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Therapeutic effects of vaccine derived from amastigote surface protein-2 (ASP-2) against Chagas disease in mouse liver

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ABSTRACT

This study investigated the efficacy of the vaccine in liver of mice infected with the *Trypanosoma cruzi* (*T. cruzi*) and immunized with AdASP-2. For this purpose, histopathological analysis and gene expression of COX-2, TNF-alpha, TNFR, iNOS, cytochrome *C*, caspase-3, TLR4, IL-6 and IL10 were evaluated. The following groups were used in this study: Group 1 - Control Group (CTRL) animals received AdβGal vehicle; Group 2 - Infected Group (TC) animals were infected with *T. cruzi*; Group 3 - Immunized Group (AdASP-2): animals were immunized by AdASP-2 vaccine; Group 4 - Immunized and Infected Group (AdASP-2 + TC) animals were infected with *T. cruzi* and immunized by AdSP-2 vaccine. A significant decrease of amastigote nests was noticed in the group of animals that were immunized with AdASP-2 and infected on the same day. COX-2 and TNF-alpha gene expressions increased in TC group, whereas TNF-alpha decreased in the TC + AdASP-2 group. TNFR expression was high in AdASP-2 + TC group. iNOS expression was high for all experimental groups whereas cytochrome *C* decreased for all experimental groups. Caspase 3 increased in TC and TC + AdASP-2 groups. The gene expression of TLR4 and IL-10 showed an increase in AdASP-2 + TC group. Finally, hepatic fibrosis was noticed to TC and AdASP-2 + TC groups. Taken together, our results demonstrated that vaccination with AdASP-2 was effective against the acute phase of experimental Chagas disease as a result of a more powerful and rapid immune response closely related to expression of some inflammatory genes, such as iNOS, TNF-alpha, TLR 4, and IL-10.

1. Introduction

T. cruzi has a complex life cycle undergoing several changes during the course of disease progression. The acute phase is closely associated with parasites circulating in the bloodstream followed by severe tissue parasitism [1]. This phase leads to death in children and adults with debilitating disorders [2]. It is important to stress that anti-parasitic drugs are successful in the acute phase, but they present high toxicity to cells and/or tissues. When the patient reaches the chronic phase of the disease, the therapeutic approach is very ineffective [3,4].

In general, the infection with *T. cruzi* compromises heart and/or gastrointestinal tissues, but it depends on the parasite strain, genetic susceptibility and route of infection. Inflammatory process is present in tissues and organs after *T. cruzi* infection, due to expression of several pro-inflammatory cytokines, such as TNF-alpha, COX-2 and iNOS [5,6]. This scenario is able to induce apoptosis, through cytochrome c release and subsequent caspase 3 activation [7]. Moreover, *T. cruzi* causes tissue injury in multiple organs, such as liver [8]. This organ plays a key role for controlling general metabolism, *i.e.* metabolic homeostasis of lipids, carbohydrates and proteins, in so far as the human health [9]. Herein, many researches have studied the relationship between *T. cruzi* infection and liver function so far [10]. At the first time, Plata et al. [11] showed the cross-talk between liver, *T. cruzi* infection and immune system. In recent years, some authors reported the presence of inflammatory cells, such as neutrophils and mononuclear cells after *T. cruzi* infection in liver [9]. This condition leads to the production of large amounts of inflammatory cytokines, which certainly participate in the biological mechanisms of parasite invasion in the liver [9]. Other

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researchers have investigated the immune response in the acute phase of disease in liver as a putative route of entry in the host organism [12].

Vaccines have been recognized as a relevant therapy for treating Chagas Disease [13,14]. Herein, *T. cruzi* surface proteins have been extensively investigated as potential vaccine candidates [15]; although most studies have interest on proteins belonging to the trans-sialidase family, as for example amastigote surface proteins and trypomastigote surface antigen [16]. The amastigote surface protein 2 (ASP-2) is a promising toll so far [17,18]. It was initially described by Pan and McMahon-Pratt [19] by means of species-specific monoclonal antibody (MAb) raised against purified membrane receptors of *T. cruzi* amastigotes. An 83-kDa antigen was purified, and the N-terminal amino acid sequence was sequenced. According to Boscardin et al [20], immunization of mice with plasmids containing specific genes against *T. cruzi* activated $CD4^+$ Th1 and $CD8^+$ Tc1 cells, protecting against infection. The majority of cases for vaccination used plasmids containing antigens expressed by tripomastigotes in the non-replicative stage. In fact, it has been postulated that the use of recombinant viruses in vaccination protocols against several pathogens are efficient, mainly because they induce potent type I T cell immune responses [21,22]. Our research group has struggled with vectors to induce a protective immune response induced by vaccines from plasmid or adenoviral origin with promising results [23–25]. In particular, immunization with AdASP-2 vaccine on the same day of infection with *T. cruzi* promoted satisfactory immune response, significantly decreased parasite burdens and increased mouse survival [26]. However, there are no studies that addressed the therapeutic potential of vaccine from AdASP-2 against *T. cruzi* infection in liver cells. This justifies this work and others as well.

The aim of this study was to investigate the therapeutic potential of AdASP-2 vaccine against *T. cruzi* infection in liver. Certainly, such data will contribute to a better understanding for pathogenesis of Chagas disease and therapeutic use of vaccine AdASP-2 in liver cells.

2. Materials and methods

2.1. Animals and experimental design

The current study was approved by the Animal Committee of Federal University of Sao Paulo, UNIFESP (Protocol number 3272290115).

A total of 16 A/Sn female mice aged 5–8 weeks were obtained from Centro de Desenvolvimento de Modelos Experimentais (CEDEME), Federal University of Sao Paulo, SP, Brazil. Mice were maintained under controlled conditions of temperature (24 + / − 2 °C) and light-dark periods of 12 h and with free access to water and commercial diet (Nuvital, PR, Brazil). The mice were distributed into 4 groups, as follows (n = 4 per group): Group 1 - Control Group (CTRL) that received 50 μL of AdβGal (2×10^8 pfu/cam) by intramuscular injection (i.m.) route at day 0; Group 2 - Infected Group (TC) animals were infected with 150 bloodstream trypomastigotes of *T. cruzi* Y strain in 0.2 mL PBS by subcutaneous (s.c.) route and immunized with 50 μL of AdβGal (2×10^8 pfu/cam) at day 0; Group 3 - Immunized Group (AdASP-2): animals were immunized by i.m. route with 50 μL of AdSP-2 (2×10^8 pfu/cam) at day 0; Group 4 - Immunized and Infected Group (AdASP-2 + TC) animals that were immunized by i.m. route with 50 μL of AdSP-2 (2×10^8 pfu/cam) and infected by s.c. route with 150 bloodstream trypomastigotes of Y strain of *T. cruzi* diluted in 0.2 mL PBS on the same day (day 0). Animals from AdSP-2 + TC group were infected at 8 a.m and vaccinated at 5 p.m on the same day [26]. All animals were euthanized on 17° day after beginning the experiment.

Bloodstream tripomastigotes were obtained from mice infected 7 days earlier with parasites of the Y strain. After estimating the parasite concentration, the blood was diluted in PBS (Phosphate buffered solution). Each mouse from infected groups was inoculated with 150 trypomastigotes.

2.2. Adenoviruses used for immunization

Recombinant human type 5 replication-defective adenoviruses expressing *T. cruzi* Amastigote Surface Protein-2 (AdASP-2) was generated, characterized, grown, and purified as previously described by Machado et al. [23]. For this purpose, mice from infected groups were inoculated intramuscularly in the tibialis anterior muscle with 50 μL of viral suspension with the indicated plaque forming units (pfu).

2.3. Histopathological analysis

After 17 days following the experimental period, the livers from all animals were removed for histopathological examination. Liver tissues were fixed in 10% buffered formalin (Merck, Darm-stadt, Germany), and serial section of 3–4 um in thickness were stained with haematoxylin and eosin (H&E, Merck) and Sirius red [27]. Analyses of the livers sections were graded by the total number of amastigote nests in TC group and AdASP-2 + TC groups. For Sirius red, photomicrographs were obtained to conventional light and polarized light to differentiate type I (red and yellow) and III (green) collagen. Histopathological evaluation was performed by light microscopy. Analyses from liver sections were graded by the number of amastigote nests, inflammatory infiltrate, fibrosis, necrosis and tissue transformation.

2.4. RNA extraction

The liver was homogenized in 1 mL Trizol (Invitro-gen, CA, USA). After that, chloroform, isopropanol, and 75% ethanol were added to the samples. The formed pellet was resuspended in 40 μL of diethylpyrocarbonate (DEPC)-treated water. RNA purity and integrity were assured by optical density (260/280 nm ratio between 1.8 and 2.0; Nanodrop 2000c, Thermo Scientific, Canada). Subsequently, all samples were stored at − 80 °C. Finally, the samples were treated with DNase (deoxyribonuclease I Amp Grade1, Invitrogen1, CA, USA) as recommended by the manufacturer.

2.5. RT-PCR and real-time PCR

cDNA molecule was obtained by reverse transcriptase polymerase chain reaction (RT-PCR) using the High-Capacity cDNA kit Reverser Transcription (Applied Biosystems1, Foster City, CA, EUA). The gene expression analysis was performed using primers previously designed for genes of interest in this study and endogenous control (GAPDH). The detection of amplification was made by intercalating DNA (Sybr Green1, Applied Biosystems1, Foster City, CA, EUA). The primer sequences for genes of interest are shown in Table 1.

The qPCR experiments were performed to the StepOne Plus Real Time PCR (Applied Biosystems, USA). The cycling conditions were, as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 58 °C for 1 min. Samples were normalized against the housekeeping gene using the method $2^{-\Delta\Delta Ct}$.

2.6. Statistical analysis

Histopathological analysis was performed by Chi-squared test. Real-time PCR results were evaluated by two-way analysis of variance (ANOVA) test followed by the Bonferroni's multiple comparison test using Graph Pad Prism (version 5.0). $p < 0.05$ was considered for statistical significance.

3. Results

3.1. Histopathological analysis

The histopathological analysis showed a considerable number of amastigote nests in hepatic tissue from the group of animals that were

Table 1
Primer sequences for Real Time PCR used in this study.

Gene		Sequence	Length
COX-2	Forward	AGCCCATTGAACCTGGACTG	20
	Reverse	ACCCAATCAGCGTTTCTCGT	20
TNF-alpha	Forward	ACCCTCACACTCACAAACCA	20
	Reverse	GAGGCAACCTGACCACTCTC	20
iNOS	Forward	CTGCCAGGGTCACAACTTTAC	21
	Reverse	TGTGGCCTTGTGGTGAAGAG	20
Casp-3	Forward	TCTACCGCACCCGGTTACTA	20
	Reverse	TGTCGTCATGTCCACCACTG	20
IL-6	Forward	AAGCAGGTCCAGCCACAATGTAG	23
	Reverse	CCAACTGACTTTGAGCCAACGAG	23
IL-10	Forward	CTCCAGGCTACGGTACACCAT	21
	Reverse	TCCAGGCAATTGCGTCATGCT	21
Cytoc. C	Forward	GGATGGGGCCATACACGTAG	20
	Reverse	AGAATGTTGGCTACCAGGGC	20
TNFR	Forward	GAACACCGTGTGTAACTGCC	20
	Reverse	ATTCCTTCACCCTCCACCTC	20
TLR4	Forward	GCATCATCTTCATTGTCCTTGAGA	24
	Reverse	CTACCTTTTCGGAACTTAGGTCTACT	26
GAPDH	Forward	GGAGAGTGTTTCCTCGTCCC	20
	Reverse	TGAAGTCGCAGGAGACAACC	20

infected with *T. cruzi* Y strain (TC group). In the group of animals that were immunized with the AdASP-2 vaccine and infected on the same day (AdASP-2 + TC), there was a decrease of nests, being significant statistically differences ($p < 0.05$) between groups (Fig. 1A). Fig. 1B shows replicative forms of the parasite with amastigote nest, with presence of hemorrhagic areas, inflammatory infiltrate and morphologic changes in the liver parenchyma.

3.2. Analysis of collagen fibers

To evaluate the presence of collagen fibers in the liver, it was performed Picro Sirius staining in this setting. The results showed that it was possible to identify the connective tissue between liver cells. In particular, it was observed increased connective tissue as depicted by the presence of collagen I and III types nearly blood vessels, being characteristic of fibrosis in groups TC and AdASP-2 + TC as a result of inflammatory process (Fig. 2).

3.3. Real time polymerase chain reaction (qPCR)

The expression of some inflammatory cytokines, such as, Cox-2, TNF-alpha, its receptor TNFR and iNOS, TLR4, IL-6 and IL-10 and apoptosis, cytochrome c and caspase-3 were evaluated as well. The gene expression of Cox-2 showed a significant increase in the TC and AdASP-2 groups when compared to the control group ($p < 0.05$). There was a significant increase in TNF-alpha gene expression from TC and AdASP-2 + TC groups when compared to the control group ($p < 0.05$), whereas the expression of its receptor (TNFR) increased in the AdASP2 + TC group when compared to the respective controls. ($p < 0.05$). There was also a significant increase in the expression of this gene in the AdASP-2 group when compared to the control group ($p < 0.05$) (Fig. 3). Regarding iNOS expression, the findings indicated an increased expression in the TC, AdASP-2 and AdASP-2 + TC groups when compared to the control group, being significant statistically differences ($p < 0.05$) between AdASP-2 + TC and TC groups. These findings are presented in Fig. 4.

Regarding genes related to apoptosis process, cytochrome c, and caspase-3, the responses were different when compared to inflammatory host response induced by *T. cruzi* infection and vaccine. Cytochrome c gene expression showed that TC, AdASP-2 and AdASP-2 + TC groups decreased when compared to the control group ($p < 0.05$). For the caspase-3, TC and AdASP-2 + TC groups presented higher expression ($p < 0.05$) when compared to the control group. However, no significant differences ($p > 0.05$) in the expression of this gene were noticed between the control group and those animals only immunized with AdASP-2 (Fig. 4).

The expression of Toll like receptor 4 (**TLR4**) was higher in the AdASP-2 and AdASP-2 + TC groups, with significant statistically differences ($p < 0.05$) in relation to the control and TC groups, respectively (Fig. 5).

With respect to IL-6, the results revealed a decrease in the gene expression from TC and AdASP-2 + TC groups when compared to control group ($p < 0.05$). IL-10 expression was higher ($p < 0.05$) in the TC, AdASP-2 and AdASP-2 + TC groups when compared to the control group. The gene expression of the anti-inflammatory biomarker in the group of animals that were immunized and infected on the same day was also higher in the group of animals that were infected (TC). Such findings are summarized in Fig. 4.

4. Discussion

The aim of this study was to evaluate the efficacy of vaccine Ad-

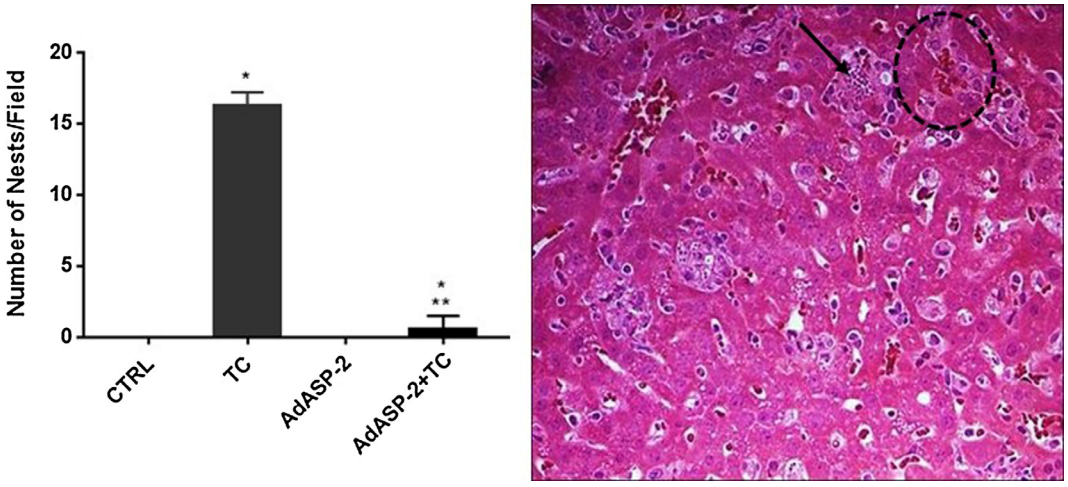

Fig. 1. (A). Number of amastigote nests in hepatic tissue of mice A/ Sn. Infected with *T. cruzi* and vaccinated with AdASP-2. Values expressed as mean ± standard deviation with $^{*}p < 0.05$ when compared to the control group; $^{**}p < 0.05$ when compared to the group to the TC group. **(B)** Photomicrography of hepatic tissue of the TC group. **Arrow**: replicative forms of the parasite; **Circle:** hemorrhagic area. H.E. Stain. Magnification 400×.

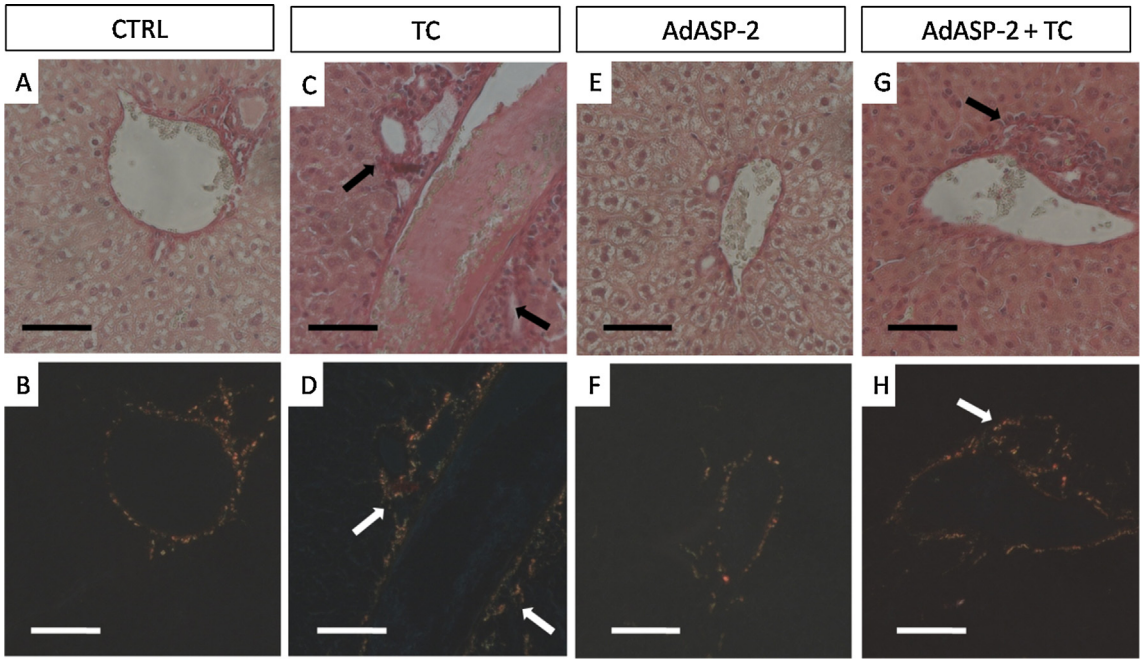

Fig. 2. Photomicrographs of hepatic vessels for all groups stained with Sirius Red with normal (**A**, **C**, **E**, **G**) and polarized light (**B**, **D**, **F**, **H**). Polarized light revealed type I collagen (red and yellow) and type III collagen (green) for all groups evaluated. Note the presence of connective tissue around the vessels, characterizing fibrosis in TC and AdASP-2 + TC (**D** and **H,** white arrows) because of inflammatory process (**C** and **G**, black arrows). Magnification 400×. Scale Barr = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

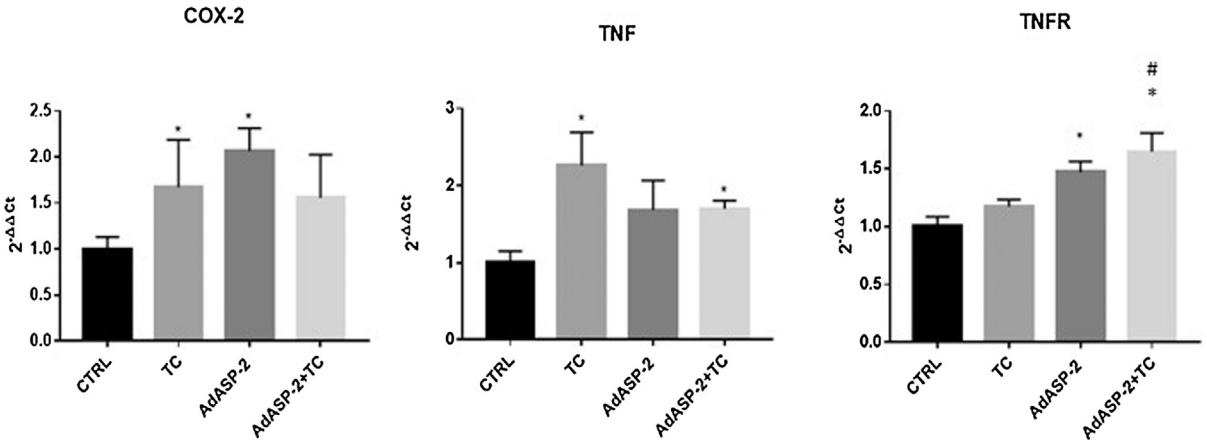

Fig. 3. Gene expression (mRNA) **of Cox-2, TNF-alpha and TNFR** in hepatic tissue of mice A/Sn. Values are expressed as mean ± standard deviation with $^{*}p < 0.05$ when compared to the control group; $\#\ p < 0.05$ when compared to the TC group.

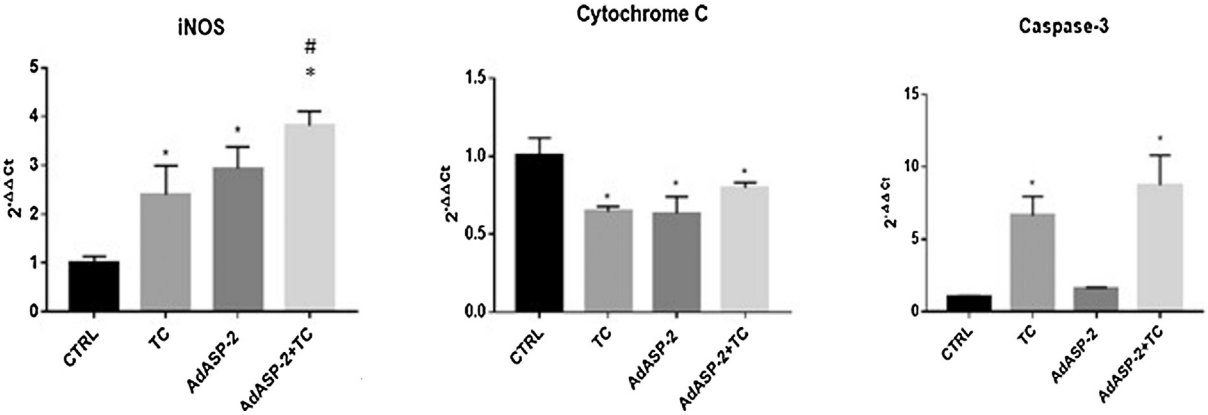

Fig. 4. Gene expression (mRNA) of **iNOS, Cytochrome *C* and caspase-3** in hepatic tissue of mice A/Sn. Values are expressed as mean ± standard deviation with $^{*}p < 0.05$ when compared to the control group; $\#p < 0.05$ when compared to the TC group.

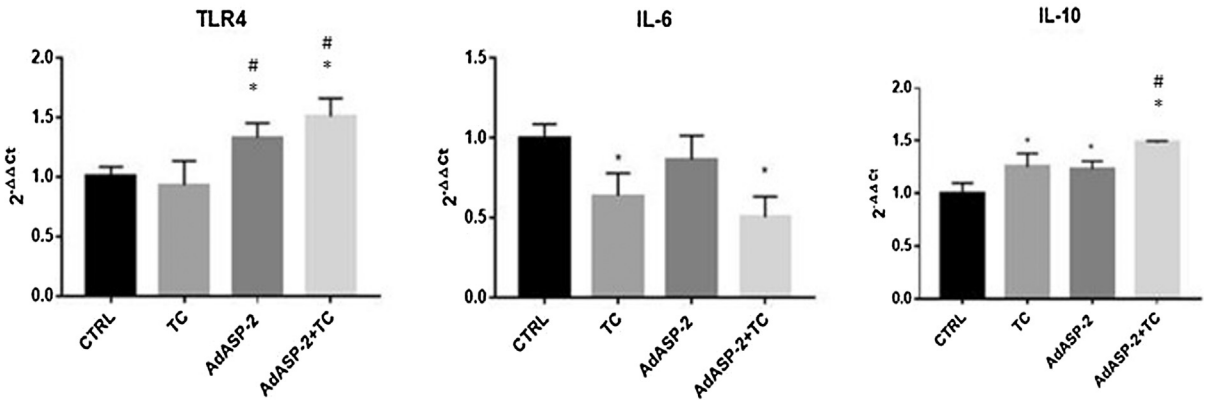

Fig. 5. Gene expression (mRNA) of **TLR4, Il-6 and IL-10** in hepatic tissue of mice A/Sn. Values are expressed as mean ± standard deviation with $^{*}p < 0.05$ when compared to the control group; $\#p < 0.05$ when compared to the TC group.

ASP-2 against *T. cruzi* infection in liver. For this purpose, histopathological analysis and gene expression of biological markers closely associated with inflammatory process and cellular death (apoptosis) were evaluated. Our results demonstrated that some inflammatory cytokines play important role for controlling the infectious disease, such as TNFR, iNOS, TLR 4 and IL-10.

Histopathological changes showed that vaccine AdASP2 was able to reduce the infection even when administered on the same day. The analysis of collagen fibers demonstrated that there was an evident increase of connective tissue around the vessels in the group of mice that was infected with *T. cruzi*. The same was observed in the AdASP-2 + TC group. These data reflect the great capacity of parasite to promote an intense inflammatory process even with a dose of sub-lethal infection. Moreover, the vaccine AdASP-2 promoted tissue regeneration as a result of eliminating *T. cruzi* in liver.

Regarding Cox-2, the expression in TC and AdASP-2 groups increased when compared to control group. According to Moraes et al. [28], Cox-2 expression was highly expressed in the early stages of *T. cruzi* infection in liver cells declining to baseline levels after 48 h of infection. Our results are in line with the published results. Nevertheless, it seems that time of exposure plays a critical role following *T. cruzi* infection in liver when considering COX-2 expression. Therefore, we assume that the involvement of COX-2 is present to *T. cruzi* infection in the acute phase of disease, but it did not participate in animals infected and vaccinated with AdASP-2, probably because the stimulatory combination of vaccine and infection was not able to modulate the biological response of COX-2 in liver cells. Further studies are necessary to clarify the issue.

T. cruzi infection has been closely associated with the production of pro-inflammatory cytokines, such as interleukins and interferon-gamma (IFN-ɣ). This picture induces the synthesis of nitric oxide by macrophages, which is responsible for the parasite elimination [29,30]. TNF-alpha has also been implicated in the activation of macrophages as a secondary signal to the production of nitric oxide [31,32]. Studies with mice genetically modified for iNOS and infected with *T. cruzi* demonstrated high levels of parasitemia in the acute phase leading to rapid mortality to the animals [33]. We observed increased levels of TNF-alpha in the *T. cruzi* infected animals, but those infected with *T. cruzi* and vaccinated did not increase the levels of TNF-alpha when compared to *T. cruzi*. Interestingly, the levels of TNFR were increased in animals from these groups. These findings emphasize the role of host immune response induced by vaccine against the *T. cruzi,* especially by activating TNFR in liver cells. Regarding the analysis of iNOS expression, it was detected an increased expression for all experimental groups evaluated in this study. It is important to highlight that animals infected and vaccinated on the same day showed high levels of iNOS expression, when compared to *T. cruzi* group. In light of these results, we assume that vaccine was able to potentiate iNOS expression as an additional effort for eliminating *T. cruzi* in liver. As a secondary biological evidence, it is demonstrated that *T. cruzi* was able to increase iNOS expression in liver cells. Taken as a whole, iNOS expression is closely involved in the etiopathogenesis of Chagas disease in liver.

To further elucidate the cytotoxicity induced by *T. cruzi* and/or vaccine, cytochrome c and caspase 3 expressions were evaluated in this setting. Cytochrome c decreased for all experimental groups. Nevertheless, caspase 3 increased in TC and AdASP-2 + TC groups when compared to the respective controls. Animals exposed to vaccine AdASP-2 did not show remarkable changes. Apart from these findings, some possible explanations should be considered. First, *T. cruzi* is able to activate apoptosis in liver cells. Since the levels of cytochrome c decreased for all infected groups, regardless of immunization, it is assumed that apoptosis is activated by the extrinsic signaling pathway in mouse liver cells infected with *T. cruzi*. Second, the vaccine was not able to trigger apoptosis in mouse hepatocytes.

Some authors have demonstrated the biological relevance of Toll-like receptors (TLR) for understanding the pathogenesis of *T. cruzi* infection [34]. For this reason, we decide to investigate TLR4 expression in this study. Some authors used genetically modified mice for one or more recipients; the results revealed the role of TLR2, TLR4, TLR7 and TLR9 for understanding the mechanisms of resistance to *T. cruzi* [35]. Our results demonstrated that there was an increased TLR4 expression in both AdASP-2 group and immunized and infected group (AdASP-2 + TC). Comparatively, da Silva et al. [36] have postulated that increased expression and subsequent activation of TLR4 in patients with the asymptomatic form of the Chagas disease would be related to the predominance of anti-inflammatory cytokines present in serum. In this context, IL-6 is known to be a cytokine synthesized at the site of lesion in the early stages of infection being its pleiotropic activity able to activate some vasoactive amines from acute phase, such as C- reactive protein and fibrinogen [37]. IL-10 is also recognized as a potent anti-inflammatory cytokine able to modulate the host immune response, and preventing from uncontrolled inflammatory process to autoimmune diseases [38,39]. According to Fioranelli and Grazia [40], IL-10 exerts modulatory effects on both resident and circulating immune cells being its inhibitory action induced by classical inflammatory markers, such as IL-1, IL-6, TNF-alpha and IFN-gamma. Regarding IL-6 and IL-10 gene expression in liver tissue, our results show that there was no differences in IL-6 expression in the *T. cruzi* infected groups, but there was a decrease in these groups when compared to control. Conversely, we observed that IL-10 expression was higher in the AdASP-2 + TC group when compared to the infected and negative control groups. These findings lead to a clear indication that vaccine is responsible for maintaining tissue homeostasis by means of IL-10 upregulation in order to stabilize the immune response in liver following *T. cruzi* infection.

In summary, our results suggest that vaccination with AdASP-2 was effective against the acute phase of experimental Chagas disease as a

result of a more powerful and rapid immune response depicted by the expression of TNF-alpha, iNOS, TLR4, and IL-10. AdASP-2 vaccine was not able to alter apoptosis by means of cytochrome c and caspase 3 expressions. Therefore, it seems that some inflammatory cytokines play an important role when testing therapeutic action of vaccines. Since millions of people are already infected with *T. cruzi* around the world, associated with available drugs that are not able to eradicate the parasite, this study suggests that the use of Ad-ASP2 vaccine for therapeutic purposes is suitable for these cases.

Conflict of interest

None declared.

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