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Immunomodulatory and anti-fibrotic effects of ganglioside therapy on the cardiac chronic form of experimental *Trypanosoma cruzi* infection

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ABSTRACT

Heart failure and sudden death are the most common causes of death in patients with Chagas' disease. The main drug available for Chagas treatment is benznidazole, which eradicates *Trypanosoma cruzi* parasites during the acute stage of infection. However, its effectiveness during the chronic phase remains unclear. Ganglioside GM1 administration in chronically infected patients resulted to be an effective treatment for the cardiac manifestations of Chagas' disease. However, the precise mechanisms of GM1-induced improvement during chronic *T. cruzi* infection still remain unknown. The aim of the present study was to evaluate the potential benefits of ganglioside GM1 treatment during the chronic stage of murine chagasic infection, analyzing its influence on myocardial pathology as well as its immunomodulatory effects. The results obtained showed that GM1 therapy diminished the extent of myocardial fibrosis induced by *T. cruzi* in chronically infected mice. In addition, GM1 treatment resulted in a significant reduction in the myocardial expression of the fibrogenic cytokine TGF-β as well as the proinflammatory cytokines and chemokines IFN-γ, TNF-α and CCL5/RANTES. Our experimental data indicate that GM1 could be a promising immunomodulatory agent with capacity to limit the inflammatory process leading to myocardial tissue damage in chronic chagasic patients.

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1. Introduction

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, constitutes an important public health problem in Latin America [1]. This parasitosis is characterized by an acute phase lasting for 30–60 days. The main drug available for the treatment of Chagas' disease is benznidazole, which eradicates *T. cruzi* parasites during the acute and early chronic stage of infection. However, its effectiveness during the late chronic phase remains unclear. In around 30% of infected untreated patients, a chronic stage showing heart or digestive disorders can occur [2].

Cardiac manifestations include abnormalities of the intraventricular conduction system, ventricular arrhythmias, sinus node dysfunction, aneurysm, and enlargement and dysfunction of the heart [3]. Arrhythmias can be related with cardiomyopathy by itself or with autonomic nervous system alterations [4]. Heart failure and sudden death are the most common causes of death in patients with Chagas' disease [3,5]. The hallmark of chronic Chagas' disease is the development of myocarditis with fibrosis, tissue remodeling and hypertrophy of cardiac myocytes [6,7]. The inflammatory nature of cardiac lesions and the fact that a robust cytokine and chemokine expression is observed by cells from chronic patients support the notion that these inflammatory mediators are critical for the progression of Chagas' disease. Several cytokines and chemokines such as transforming growth factor-beta (TGF-β), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and chemokine (C–C motif) ligand 5 (CCL5/RANTES) are locally produced in the myocardium of patients with Chagas' disease and may play a significant role in the pathogenesis of chronic chagasic cardiomyopathy by modulating mechanisms such as hypertrophy and fibrosis. [8,9].

Previous studies have demonstrated that ganglioside treatment of mice during their recent *T. cruzi* infection promoted long-term survival and clearance of parasites from the bloodstream and organs [10]. They also observed that ganglioside-based therapy had no significant impact on the ability of the parasite to penetrate into host cells, and therefore, a modulation of host immune response was proposed to explain the beneficial effect of this compound *in vivo*. Other authors reported that ganglioside treatment is also effective to restore normal electrocardiographic activity and enhance survival of mice acutely infected with *T. cruzi* [11]. Recently, *in vitro* assays proved that ganglioside incorporation provoked modifications on *T. cruzi* plasma membranes accompanied by ultrastructural alterations [12]. In humans, ganglioside therapy reduces arrhythmias in patients with chronic Chagas' disease [13] and increases the heart rate response to atrophine and propanolol [14].

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In this context, and given the lack of safe and effective therapeutic alternatives for indeterminate and chronic Chagas' disease, our purpose was to assess the potential benefits of ganglioside treatment during the chronic stage of murine chagasic infection, analyzing its influence on myocardial pathology as well as its ability to modulate the host immune response.

2. Materials and methods

2.1. Ganglioside

Ganglioside GM1 (Sinaxial 20 ®), was provided by TRB Pharma S.A, Buenos Aires, Argentina. Before used, GM1 was diluted in phosphate-buffered saline (PBS).

2.2. Experimental infection

Two-month-old female BALB/c mice (Comisión Nacional de Energía Atómica, CNEA, Buenos Aires, Argentina) and *T. cruzi* trypomastigotes, Tulahuén strain, were used in this study. Mice were maintained in the animal facilities at the Hospital de Niños Dr. Ricardo Gutiérrez and provided with food and water *ad libitum*. The study was performed according to the National Research Council's guide for animal care and was approved by our internal Ethics Committee.

Serum samples from each mouse were collected from the tail vein before infection. A total of 50 mice were included in this study. Thirty mice were infected by intraperitoneal injection of 20 blood trypomastigotes and 20 mice remained uninfected as controls, in 2 independent experiments. This inoculum leads to a high infection rate and low mortality during the acute phase of experimental Chagas' disease. Success of infection was confirmed both by parasitological (direct parasitemia) and serological (ELISA) methods. Parasitemia was evaluated twice a week in 5 μl of tail vein blood examined microscopically at 400× magnification.

2.3. Treatment protocol

Four months after infection, surviving mice were separated into the following groups: (G1) ganglioside (GM1)-treated infected mice; (G2) non-treated infected mice; (G3) uninfected, GM1-treated mice; and (G4) uninfected, non-treated mice. Animals (ten mice per group) were injected by the intramuscular route with 5 mg of GM1 per kg of body weight, 6 days per week for 4 weeks, starting upon 120 days postinfection (dpi). Untreated mice only received PBS. The dose and route of administration were chosen based on data communicated previously [10,11]. One month after treatment, mice were sacrificed under ether anesthesia and submitted to serological, parasitological, histopathological and immunological analysis.

2.4. Serological analysis

Individual blood samples were collected before and 1 month after treatment in order to determine *T. cruzi* IgG antibody profile by ELISA as described previously [15]. Briefly, *T. cruzi* antigens present in a whole homogenate (WH) of parasites were prepared from freeze-thawed epimastigote forms disrupted by sonication, further diluted to a concentration of 200 μg/ml in carbonate–bicarbonate buffer (pH 9.6) and coated onto flat-bottomed ELISA plates (Nunc, Denmark) overnight at 4 °C. The plates were then washed five times with PBS containing 0.05% Tween 20 (PBST) and saturated with 1% bovine serum albumin (BSA) in PBST for 1 h at 37 °C. Another wash step as above was followed by addition of 100 μl of mouse sera (twofold serial dilutions) in PBST/BSA. After 1 h at 37 °C and further washing, 100 μl of peroxidase-labeled goat anti-mouse IgG (Pierce, Rockford, IL) diluted 1:5000 in PBST/BSA was added to all the wells and incubated for 1 h at 37 °C. The plates were then washed five times with PBST and the color was developed by adding the ABTS [2,2′-azino-*bis* (3-ethylbenzthiazoline-6-sulphonic acid), Sigma Chemical Co., St. Louis, MO] substrate and incubating in the dark for 20 min. The optical density (OD) was measured at 405 nm using an ELISA reader (Multiskan Ex, Thermo Labsystems, Finland). End-point titers were defined as the highest serum dilution that resulted in an OD value greater than that of the mean + two standard deviations of pre-immune mouse sera.

2.5. Tissue-PCR analysis

Tissue parasitism was evaluated in myocardium by PCR detection of *T. cruzi*-specific DNA. Myocardial nucleic acid was extracted using the QiAmp tissue kit (Qiagen, CA, USA) following the manufacturer's recommendations. Parasite detection was performed using the primers #121 [5′-AAATAATGTACGG(T/G)-GAGATGCATGA-3′] and #122 [5′-GGTTCGATTGGGGTTGGTGTAATATA-3′] which amplify a *T. cruzi*-specific 330 bp fragment, corresponding to the variable regions of the *T. cruzi* kDNA minicircle [16]. Positive and negative controls were included in each run.

2.6. Histopathological examination

Fragments of myocardium were fixed in neutral formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) for inflammation assessment or Masson's trichromic for fibrosis measurement. A single-blind evaluation of the specimens was performed by light microscopy on randomized, precoded slides in a systematic fashion. Tissue sections were evaluated for the presence of inflammatory infiltrates as described previously [17,18]. Scoring of fibrosis was performed according to distribution of interstitial collagen deposits (normal = 0, focal reinforcement of connective tissue network = 1, multifocal reinforcement of connective tissue network = 2, confluent patches of connective tissue = 3).

2.7. Detection of cytokine and chemokine mRNA in myocardium

To evaluate the influence of GM1 therapy on the myocardial expression of cytokines and chemokines, we investigated by RT-PCR assay the presence of transcripts for IFN-γ, TNF-α, CCL5/RANTES and TGF-β in cardiac tissues from treated and untreated mice. Total RNA was isolated from heart using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's specifications. Five micrograms of total RNA was reverse transcribed by the addition of 1 U of Moloney murine leukemia virus RNase H-reverse transcriptase (MMLV, Promega Corporation, Madison, Wisconsin, USA), 1× MMLV buffer, 2.5 mM deoxynucleotides (dNTPs, Invitrogen), and 0.3 μM random hexamer oligonucleotides (Bio-Rad Laboratories, Hercules, CA, USA) in a total volume of 20 μl. The reaction proceeded for 45 min at 40 °C and was terminated for 5 min at 95 °C. Five microliters of cDNA was used for amplification in a 50-μl PCR reaction containing 50 μM specific primers, 200 μM dNTPs, 25 mM $MgCl_2$, 10× Taq buffer and 1.25 U Taq DNA polymerase (Invitrogen). The PCR primers (sense and antisense) and annealing temperature were as follows: IFN-γ: 5′ AGC GGC TGA CTG AAC TCA GAT TGT AG 3′, 5′ GTC ACA GTT TTC AGC TGT ATA GGG 3′, T_{ann} 55 °C ; TNF-α: 5′ ATG AGC ACT GAA AGC ATG ATC 3′, 5′ TCA CAG GGC AAT GAT CCC AAA GTA GAC CTG C 3′, T_{ann} 60 °C ; CCL5/RANTES 5′ CGC GGA TCC CCA CGT CAA GGA GTA TTT CTA CAC C 3′, 5′ CGC GGA TCC CTG GTT TCT TGG GTT TGC TGT G 3′, T_{ann} 60 °C; TGF-β: 5′ TTG CTT CAG CTC CAC AGA GA 3′, 5′ TGG TTG TAG AGG GCA AGG AC 3′, T_{ann} 58 °C. The set of primers used for amplification of the housekeeping β-actin gene was purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Negative controls for RT-PCR were omission of a target template and omission of reverse transcriptase. An optimal number of PCR cycles were determined initially by using a variable number of cycles to identify a linear range of amplification for each transcript. The

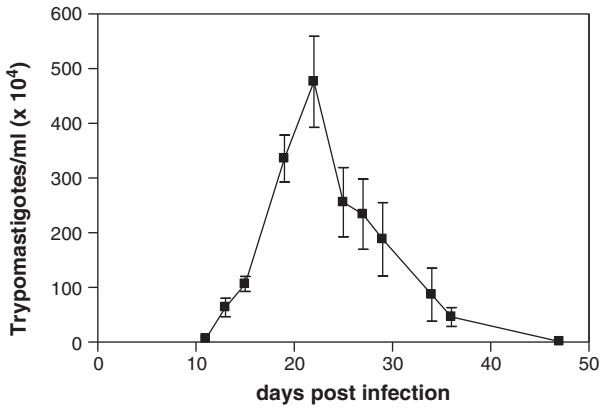

Fig. 1. Parasitemia during the acute phase of infection with Tulahuén strain of *T. cruzi* in BALB/c mice.

PCR mixture was incubated at 94 °C for 5 min followed by 35 cycles of amplification. Each cycle consisted of 1 min of denaturation at 94 °C, 1 min of annealing and 1 min of extension at 72 °C. After 35 cycles, a final extension step at 72 °C for 10 min was performed. Semiquantitative fold induction was calculated as previously described [19]. Briefly, PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. Amplified products were visualized under UV light and quantified with densitometric analysis software (SionImage version 1.54, NIH, Bethesda, Maryland, USA). The densitometry value for each gene was normalized to value for mouse β-actin.

2.8. Immunohistochemical studies

Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded cardiac specimens. Five-micrometer sections adhered onto poli-lysine-coated slides were deparaffinized using routine techniques. After blocking endogenous peroxidase with 3% hydrogen peroxide, the following primary antibodies were applied to the sections: rat monoclonal anti-mouse IFN-γ, rabbit polyclonal anti-mouse TNF-α, rabbit polyclonal anti-mouse TGF-β (Abcam, Cambridge, UK) and biotin-labeled rat monoclonal anti-mouse CCL5/RANTES (R&D Systems, Minneapolis, MN, USA). Immunodetection was performed using Ultra Tek HRP Anti-Polyvalent Lab Pack (ScyTek Laboratories, USA). The sections were then counterstained with Mayer's hematoxylin.

Controls were obtained by omitting the primary antibody and by using an isotype-matched control. All controls were negative for these inflammatory mediators.

2.9. Statistical analysis

Results were analyzed using GraphPadPrism 4.0 software (GraphPad Software Inc., San Diego, CA, USA). Student's *t* test was used for the comparative analysis of the means. The nonparametric Mann–Whitney test was used to determine the significance level for inflammation and fibrosis scores. The p values <0.05 were considered statistically significant.

3. Results

In our model, BALB/c mice infected with Tulahuén strain of *T. cruzi* exhibited the classical pattern of parasitemia during the acute phase of infection. Blood parasites were detected by day 10 pi, peaking at 21 dpi, and declining gradually to very low values by day 45 pi (Fig. 1).

3.1. Serological profile

The levels of *T. cruzi*-specific serum IgG antibodies quantified by ELISA test at 45 and 100 dpi (before the administration of GM1) and 1 month after the end of GM1 treatment (150 dpi) are depicted in Fig. 2. No significant differences between GM1-treated and untreated *T. cruzi*-infected animals were observed.

3.2. Detection of T. cruzi DNA by PCR

In order to assess post-therapeutic cure in *T. cruzi* chronic infection, we performed PCR assays to detect parasite kinetoplastid DNA (kDNA) in heart tissues from treated and untreated mice. Our data demonstrated that tissue-PCR was able to detect *T. cruzi* kDNA in all infected mice, regardless of treatment status (Fig. 3).

3.3. Histopathologic findings

To investigate whether GM1 treatment during the chronic phase would affect the influx of inflammatory cells to the heart and the occurrence of myocardial fibrosis, we analyzed the extent of inflammation as well as the presence of fibrous tissue 1 month after completion of chemotherapy. Both GM1-treated and untreated mice infected with *T. cruzi* exhibited mild to moderate myocarditis characterized by a focal or diffuse inflammatory cell infiltrate, consisting mainly of mononuclear cells. Nevertheless, no significant differences between groups were found. No inflammatory cell infiltrates were detected in the myocardium of uninfected animals, regardless of treatment status.(Fig. 4). In contrast,

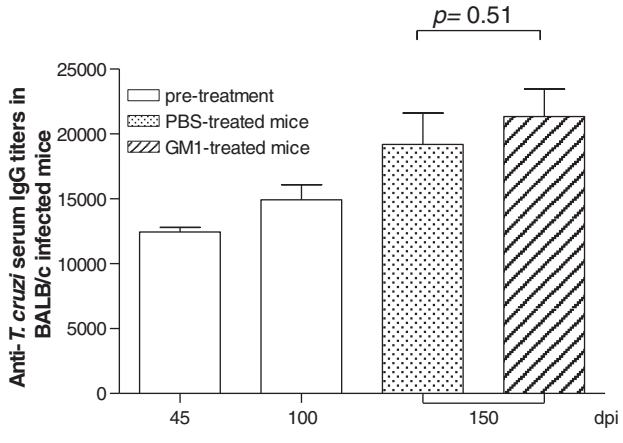

Fig. 2. Anti *T. cruzi* serum IgG titers in BALB/c infected mice before (45 and 100 dpi) and 1 month after GM1 treatment (150 dpi).

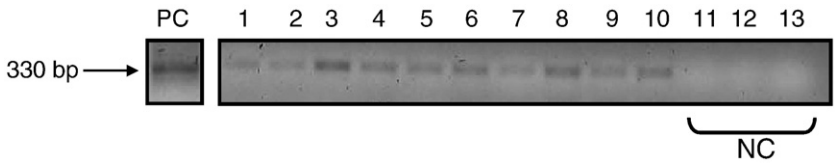

Fig. 3. Representative 2% agarose gel showing *T. cruzi* amplified kDNA by PCR. PC: positive control (*T. cruzi* kDNA). Lanes 1–5: myocardium from untreated *T. cruzi*-infected mice. Lanes 6–10: myocardium from GM1-treated *T. cruzi*-infected mice. Lane 11: control of reagents used in the DNA extraction. Lane 12: control of reagents used in the PCR. Lane 13: myocardium from uninfected control.

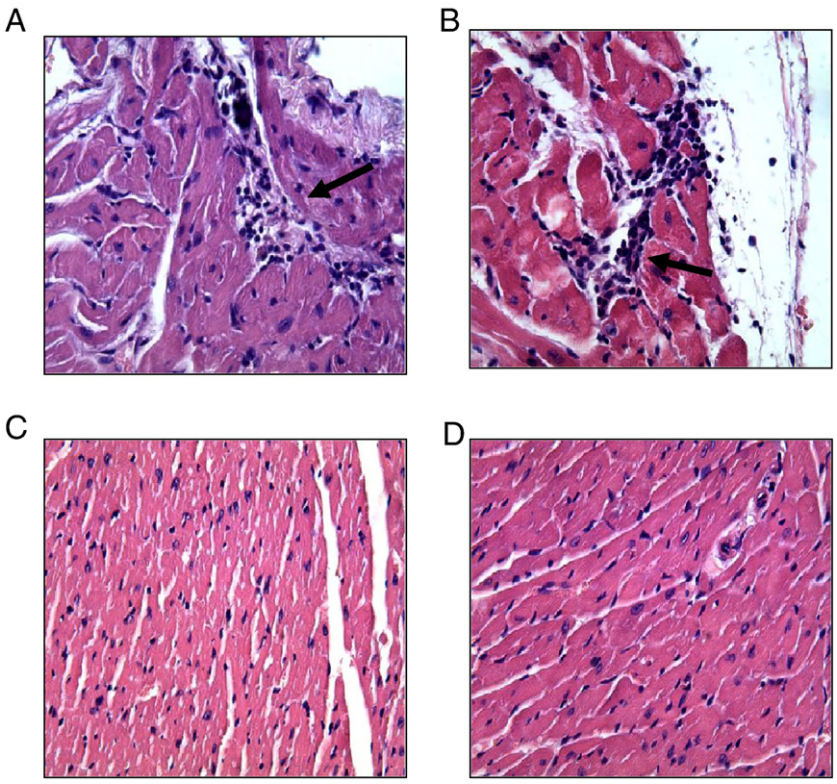

Fig. 4. Representative photomicrographs of H&E-stained histological sections of myocardium showing inflammatory infiltrates. (A) Untreated *T. cruzi*-infected mouse. (B) GM1-treated *T. cruzi*-infected mouse. (C) Uninfected GM1-treated mouse. (D) Uninfected mouse. Arrows indicate the presence of inflammatory infiltrates. Original magnification, 400×.

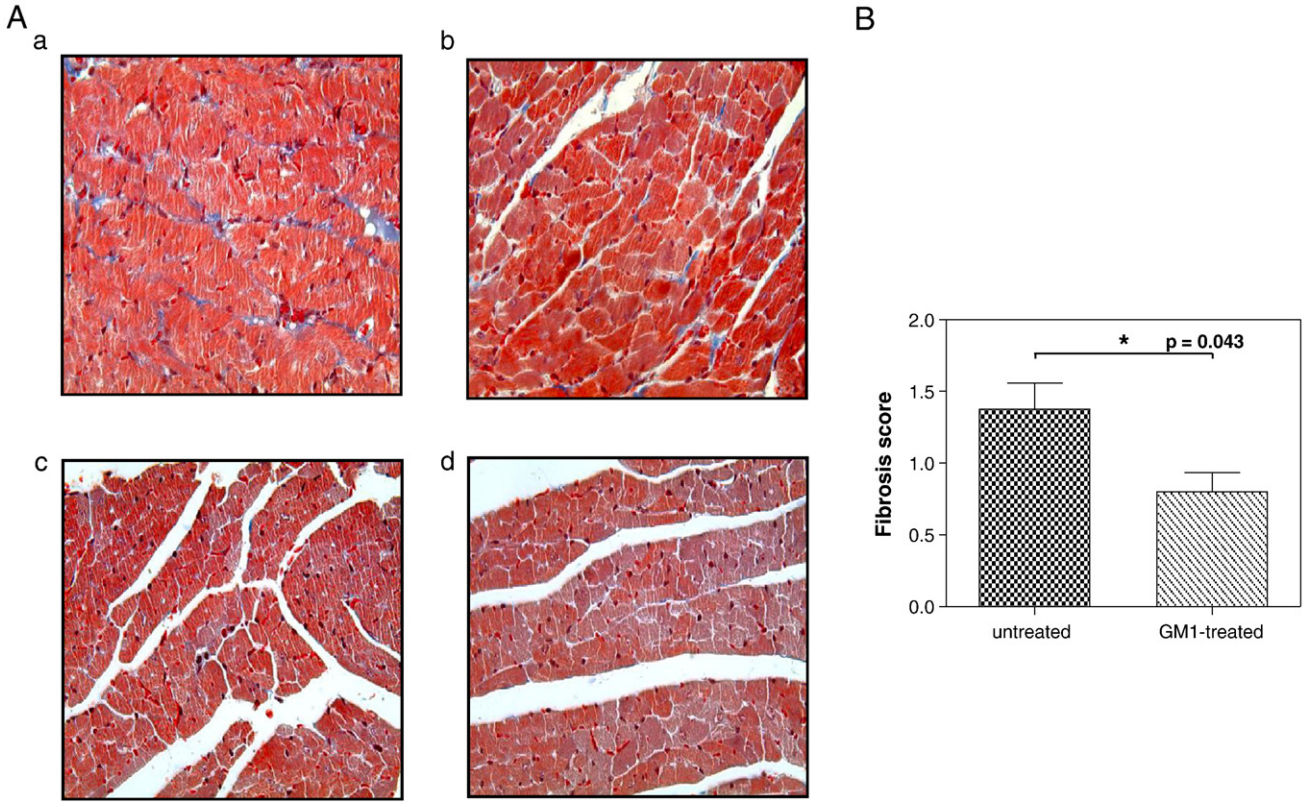

Fig. 5. (A) Representative photomicrographs of Masson's trichrome-stained histological sections of myocardium. Note the connective tissue (blue) surrounding myocardial fibers. (a) Confluent patches of connective tissue in myocardium of untreated *T. cruzi*-infected mouse. (b) Slight fibrosis in myocardium of GM1-treated *T. cruzi*-infected mouse. (c) Uninfected GM1-treated mouse. (d) Uninfected mouse. Original magnification, 400×. (B) Fibrosis intensity in myocardium of *T. cruzi*-infected mice.

the quantification of interstitial fibrous tissue in myocardium showed lower ($p = 0.043$) levels of collagen deposits in GM1-treated infected mice when compared with untreated infected animals (Fig. 5A and B).

3.4. Detection of cytokine/chemokine mRNA in myocardium

When mRNA expression of IFN-γ and TNF-α and the chemokine CCL5/RANTES was investigated, a strong reduction in transcript levels of these inflammatory mediators was found in the hearts of infected mice after GM1 treatment ($p = 0.0053$, $p = 0.042$ and $p = 0.049$, respectively). Moreover, gene expression analysis of TGF-β revealed that this fibrogenic cytokine was drastically ($p = 0.0018$) down-modulated in myocardium from GM-1 treated, *T. cruzi*-infected animals (Fig. 6). Expression of IFN-γ, TNF-α , CCL5/RANTES and TGF-β was not observed in specimens from uninfected mice.

3.5. Cytokine/Chemokine detection by immunohistochemistry

To determine if the differences observed in gene expression also occur at the biologically relevant protein level, we evaluated the production of IFN-γ, CCL5/RANTES, TNF-α and TGF-β in heart tissues by immunohistochemistry. The results of this analysis showed a marked downregulation of these molecules in the myocardium of GM1-treated, *T. cruzi*-infected mice compared to untreated infected animals (Fig. 7). IFN-γ, CCL5/RANTES and TGF-β staining was associated predominantly with cardiac myocytes, and in the case of TNF-α with inflammatory cells infiltrating the interstitium. No production of these inflammatory mediators was detected in myocardial sections of uninfected GM1-treated and untreated controls.

4. Discussion

Our present study demonstrates that treatment with GM1 diminishes the extent of myocardial fibrosis induced by *T. cruzi* in chronically infected mice in concert with a significant reduction in the myocardial expression of TGF-β. TGF-β is a key molecule promoting collagen synthesis and fibrosis development, a phenomenon observed in the chronic phase of this parasitic illness and associated with heart failure [20,21]. Interestingly, this cytokine was reported to be increased in plasma of patients with Chagas' disease cardiomyopathy [22]. Pharmacological inhibition of TGF-β has been demonstrated to prevent heart damage in a preclinical mouse model of Chagas' disease, suggesting that

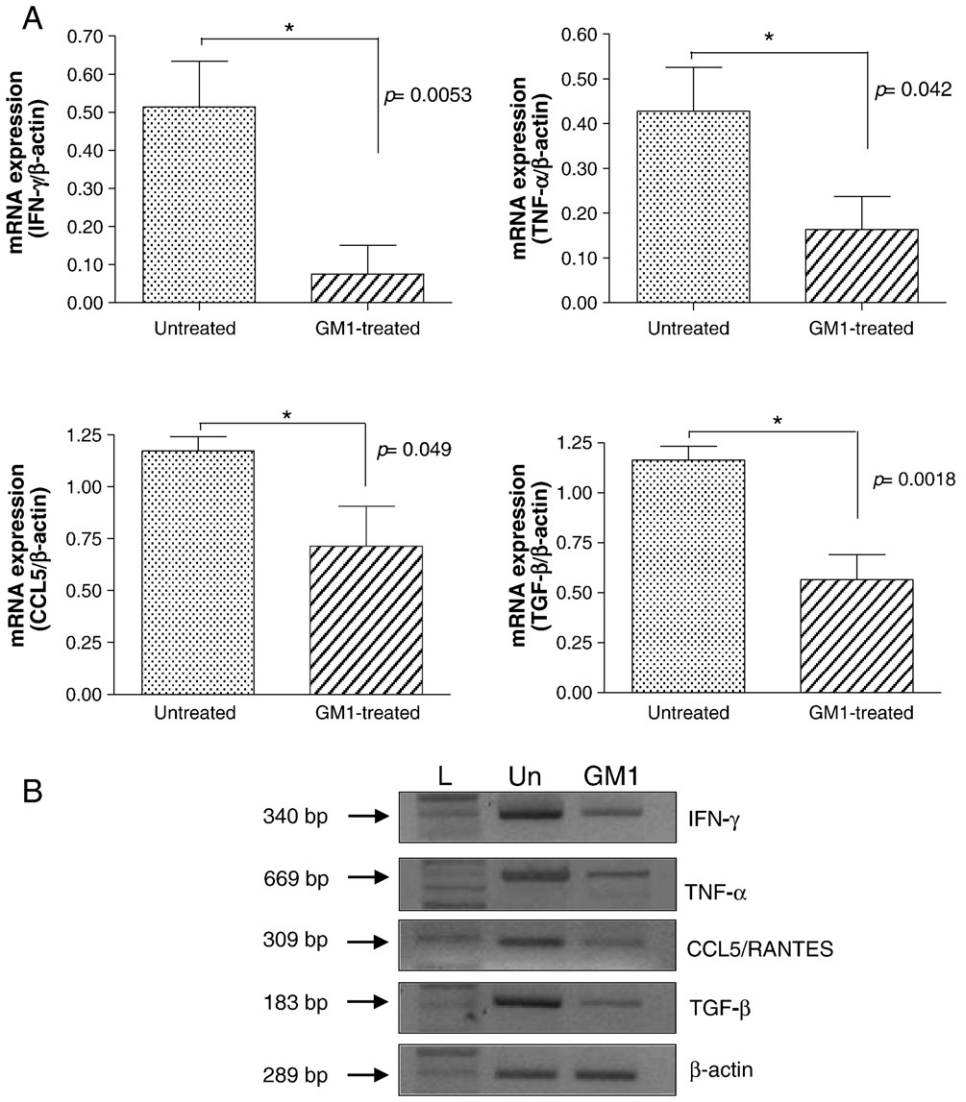

Fig. 6. (A) RT-PCR analysis of IFN-γ, TNF-α, CCL5/RANTES and TGF-β mRNA expression in myocardium from untreated and GM1-treated BALB/c mice infected with *T. cruzi*. Values represent the mean ± SEM. (B) Representative agarose gel photograph showing amplification of cytokine/chemokine mRNA. β-Actin was included as an internal standard. Each lane of the gel corresponds to the result obtained from a single animal, representative of all mice from each group. L: 100 bp ladder; Un: untreated mice; GM1: GM1-treated mice.

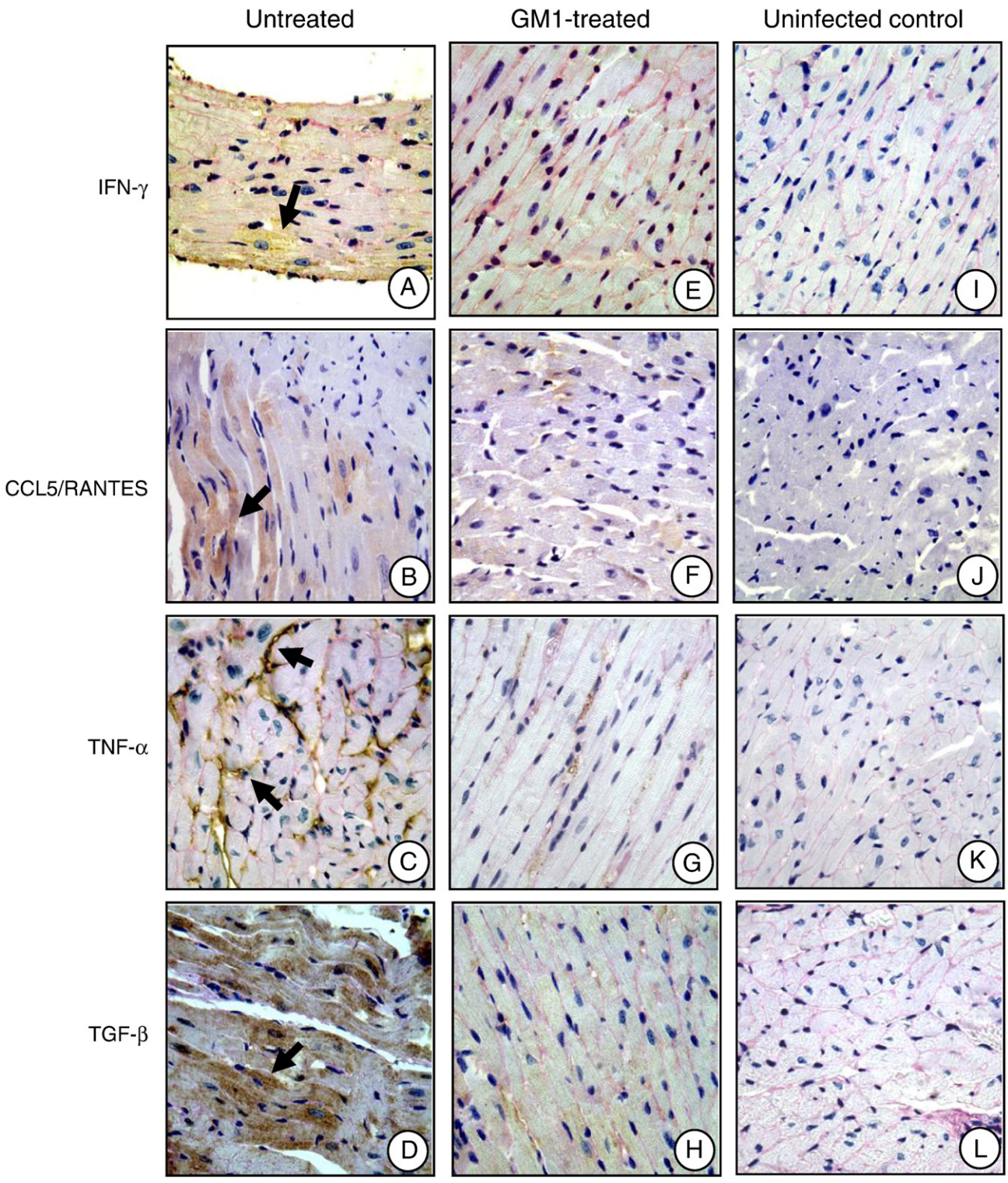

Fig. 7. Immunohistochemical labeling of IFN-γ, CCL5/RANTES, TNF-α and TGF-β in heart sections from BALB/c mice infected with *T. cruzi*. A–D, Untreated infected mice; E–H, GM1-treated infected mice; I-L, GM1-treated uninfected mice. Note the attenuation of staining in sections from *T. cruzi*-infected mice treated with GM1 compared to untreated animals. IFN-γ, CCL5/RANTES and TGF-β are expressed predominantly by cardiac myocytes, whereas TNF-α is produced by inflammatory cells infiltrating the interstitium (arrows). Original magnification, 400×.

this class of molecules may represent a new therapeutic target for acute and chronic stages of the infection [23]. In canine models of heart failure, the expression of atrial TGF-β was demonstrated to increase, and inhibition of this expression prevents the development of atrial fibrillation and fibrosis [24].

Previous reports from other authors have stated that ganglioside treatment decreases parasitemia and heart inflammation during the acute phase of murine *T. cruzi* infection [10]. In our experimental model of chronic chagasic cardiomyopathy, we were able to detect parasite DNA in both GM1-treated and untreated infected animals. Moreover, the intensity of *T. cruzi*-induced heart inflammation was not modified by GM1 treatment. However, GM1 therapy did diminish the expression of inflammatory mediators (IFN-γ, TNF-α and CCL5/RANTES) in the myocardium. This finding is very interesting since

chronic proinflammatory cytokine production, such as IFN-γ and TNF-α, plays a pathogenic role in *T. cruzi*-induced cardiomyopathy [9]. A deleterious effect of these factors has already been also in other experimental and human heart diseases [25–27]. On the other hand, it has been suggested that chronic production of TNF-α prior to heart failure may play a role in chronic Chagas' disease cardiomyopathy progression [28], probably altering myocytes contractility [29]. A positive correlation between levels of TNF-α and the degree of heart dysfunction was also found [30]. Furthermore, IFN-γ- and TNF-α-producing cells are detected in inflamed hearts of chronic cardiomyophatic chagasic patients [22]. However, the high concentrations of both cytokines in heart tissue are not related to the intensity of the chronic inflammation but with its persistence [31]. These results agree with our observations showing that GM1 treatment did not modify the intensity of installed inflammatory process although did reduced the expression of IFN-γ and TNF-α in the myocardium. We cannot rule out the possibility that the lack of effect of GM1 on the intensity of myocardial inflammation in our experimental model of chronic *T. cruzi* infection could be due to the dose employed, as it was observed with other compounds. For example, administration of a low dose of Captopril® reduced cardiac fibrosis in mice acutely infected with *T. cruzi* without affecting inflammation; higher doses led to a decrease intensity of this phenomenon [32].

IFN-γ-inducible chemokines have also been linked to inflammatory cardiomyopathy [33]. Persistent expression of chemokines results in prolonged inflammatory injury and their inhibition can limit inflammatory activity and fibrosis. A predominance of mRNA for CCL5/RANTES, along with IFN-γ and TNF-α in cardiac tissue during the chronic phase of *T. cruzi* infection was reported in experimental models [34] and Chagas' disease patients [9]. Moreover, the administration of a CCL5/RANTES antagonist was demonstrated to diminish deposition of fibronectin in chronically *T. cruzi*-infected mice [35]. Accordingly, we observed that the reduced myocardial CCL5/RANTES expression in GM1-treated infected animals was associated with a decrease of fibrosis.

Gangliosides, glycolipids containing sialic acid, are ubiquitous constituents of cell membranes that can exert a variety of cellular functions, including the triggering and modulation of transmembrane signalling. These compounds have multiple immunomodulatory activities, decreasing the lymphoproliferative responses and modulating cytokine production [36]. In experimental autoimmune encephalomyelitis, exogenous administration of gangliosides provoked suppression of clinical signs by modulating the Th1/Th2 cytokines balance [37]. Ganglioside GM1 has been described as a major ligand for galectin-1 (Gal-1) [38,39], a new class of bioactive molecules with potent anti-inflammatory and immunoregulatory properties. It is produced by many cell types, such as fibroblasts, T cells and B cells, and is abundantly expressed by different tissues, such as heart, muscle, lymph nodes, spleen, thymus, and lung. Interestingly, during *T. cruzi* infection Gal-1 expression is up-regulated *in vitro* and *in vivo* [40]. Cross-linking of GM1 ganglioside expressed by T effector cells and Gal-1 expressed by regulatory T cells has shown to be a crucial event in immunosuppression [41]. Therefore, although we cannot identify the precise mechanism responsible for the beneficial effect of GM1 treatment observed in our experimental model, one possible explanation may be that exogenous GM1 modulates the proinflammatory cytokine and chemokine synthesis, as well as the TGF-β production, by enriching the content of ganglioside in cellular membranes, followed by cross-linking of these molecules to target cells expressing Gal-1. This, in turn, could result in reduced expression of proinflammatoty mediators and fibrogenic factors. In fact, it has been reported that membrane enrichment of human monocytes with exogenous GM1 inhibited the proinflammatory cytokine production [41]. Nevertheless, to assess precisely the mechanisms of GM1-induced immunomodulation during chronic *T. cruzi* infection, more experimental work needs to be performed.

In conclusion, the results of the present study suggest that the down-modulation of proinflammatory cytokines/chemokines such as IFN-γ, TNF-α and CCL5/RANTES, as well as the pro-fibrogenic cytokine TGF-β in myocardium may account, at least in part, for the beneficial effect of GM1 treatment during the chronic stage of *T. cruzi* infection. An important feature of chagasic cardiomyopathy is the intense extracellular matrix deposition in the heart damaged areas [28], a phenomenon associated with the development of heart failure. A reduction or delay of this process might contribute to limit cardiac damage in chronic chagasic patients.

Acknowledgments

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