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PPARγ ligand treatment inhibits cardiac inflammatory mediators induced by infection with different lethality strains of *Trypanosoma cruzi*

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ABSTRACT

Trypanosoma cruzi (*T. cruzi*), the etiological agent of Chagas' disease, causes cardiac alterations in the host. Although the main clinical manifestations arise during the chronic stage, the mechanisms leading to heart damage develop early during infection. In fact, an intense inflammatory response is observed from acute stage of infection. Recently, peroxisome proliferator-activated receptors (PPARs) have attracted research interest due to their participation in the modulation of inflammation. In this work we addressed the role of 15-Deoxy-$\Delta^{12,14}$ ProstaglandinJ2 (15dPGJ2), a PPARγ natural ligand in the regulation of inflammatory mediators, in acute and chronic experimental mouse models of Chagas' disease with the RA and K98 *T. cruzi* strains, respectively. This work demonstrates that 15dPGJ2 treatment inhibits the expression and activity of inducible nitric oxide synthase (NOS2) as well as TNF-α and IL-6 mRNA levels. Also, expression and activity of metalloproteinases 2 (MMP-2) and 9 (MMP9) were inhibited by 15dPGJ2. Moreover GW9662, a specific PPARγ antagonist, revealed the participation of other signaling pathways since, in GW9662 presence, 15dPJG2 had a partial effect on the inhibition of inflammatory parameters in the acute model of infection. Accordingly, NF-κB activation was demonstrated, assessing p65 nuclear translocation in the hearts of infected mice with both *T. cruzi* strains. Such effect was inhibited after 15dPGJ2 treatment. Our findings support the concept that *in vivo* PPARγ and NF-κB pathways are implicated in the inhibitory effects of 15dPGJ2 on inflammatory mediators at different times depending on whether the infection is caused by the lethal or non-lethal *T. cruzi* strain.

1. Introduction

Chagas' disease is caused by infection with the protozoan kinetoplastid parasite *Trypanosoma cruzi* (*T. cruzi*). This disease is endemic throughout Central and South America, constituting an important public health problem. Serious cardiac alterations that characterize chronic symptomatic Chagas' disease are found in about 30% of infected individuals, which may develop dilated cardiomyopathy [1].

Differences in cardiac or digestive pathological manifestations might partially be attributed to particular characteristics of *T. cruzi* subpopulations, like lethality [2] tissue tropism [3] and induction of immune response [4]. Moreover, the evolution of the disease might depend on the diverse host–parasite relationships. RA and K98 *T. cruzi* strains have demonstrated contrasting characteristics [5]. While RA is highly lethal for mice and replicates actively in the reticuloendothelial system, K98 is a clone isolated from the myotropic CA-I strain with low lethality and that hardly multiplies in phagocytes [6].

There are many substantial evidence showing that cardiac tissue, an important target of *T. cruzi*, produces marked amounts of pro-inflammatory cytokines, chemokines and enzymes including inducible nitric oxide synthase (NOS2) and metalloproteinases (MMPs), resulting in inflammation and cardiac remodeling in response to parasite infection [7–9]. It has been shown that NOS2 is relevant in the pathogenesis of cardiac diseases, whereas in Chagas' disease nitric oxide (NO) has been described as a trypanocidal agent [10–12]. It has also been suggested that the high output of NO by inflammatory cells could lead to heart failure [13]. Moreover, increased MMP production, associated with a sustained inflammatory response, may lead to excessive extracellular matrix degradation impairing infarct healing and worsening early remodeling, which in turn causes cardiac rupture [14]. Peroxisome proliferator-activated receptors (PPARs), members of the steroid hormone receptor superfamily, are ligand-dependent nuclear transcription factors that have emerged as regulators of lipid metabolism as well as inflammation. There is growing evidence demonstrating the efficacy of PPAR agonists as anti-inflammatory mediators in the heart [15]. For example, the natural ligand 15-Deoxy-$\Delta^{12,14}$ ProstaglandinJ2 (15dPGJ2) has high affinity for PPARγ and is a potent anti-inflammatory molecule. It has been previously described that 15dPGJ2 can repress some genes in activated macrophages

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and cardiomyocytes including NOS2, cyclooxygenase 2 (COX2) and tumor necrosis factor α (TNF-α), being this repression partially dependent on PPARγ expression [16,17,9]. Also, it has been reported that treatment with 15dPGJ2 reduced the inflammatory infiltrate in the skeletal muscle at the site of infection and decreased the number of lymphocytes and neutrophils in the blood during acute phase of *T. cruzi* infection [18].

15dPGJ2 is normally present *in vivo* during the resolution phase of inflammation, suggesting that it may function as a feedback regulator of the inflammatory response [19]. However, little is known about the role of PPARγ ligands in *T. cruzi* infection or in Chagas' disease. It has been described that the major role of PPARγ is the trans-suppression of inflammatory gene activation by negatively interfering with the nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), signal transducers and activators of transcription 1 (STAT1) and mitogen-activated protein kinases (MAPKs) signaling pathways in a DNA-binding independent manner [20,17].

Here we investigated, in mouse models of infection with different *T. cruzi* subpopulations, the role of PPARγ as a modulator of inflammatory mediators in the heart. Treatment with PPARγ natural ligand, 15dPGJ2, inhibited the inflammatory response in terms of cytokines, NOS2 and MMP expressions in both strains regardless of the time of infection with each strain. Besides, NF-κB activation in the heart after RA and K98 infection, and its inhibition by 15dPGJ2 confirms and reveals the participation of both PPARγ and NF-κB in the modulation of pro-inflammatory mechanisms in *in vivo* models of early acute and chronic Chagas' disease.

2. Material and methods

2.1. Mice and infection

Mice used in this study were bred and maintained in the animal facility of the Department of Microbiology, Parasitology and Immunology, School of Medicine, University of Buenos Aires. For the *in vivo* experimental model of Chagas' disease, 8-week old BALB/c male mice (8–10 per group) were infected intraperitoneally with 10^5 bloodstream trypomastigotes of lethal RA (pantropic/reticulotropic) or the non-lethal K98 strains of *T. cruzi* subpopulations as previously described [5]. For experiments with RA, mice were sacrificed at 6, 12, 20, 24, 36 and 48 h or at day 10 post infections (p.i.), depending on the experimental protocol. For K98 experiments, mice were sacrificed at 1, 2, 5, 11, 14 or 22 days p.i. In addition, some mice were treated i.p. with 15dPGJ2 (1 mg/kg) (Cayman Chemical Co) every 24 h or 12 h (depending on the experimental protocol) for mice infected with RA. K98-infected mice were treated with 15dPGJ2 (1 mg/kg) every 24 h. For experiments performed with GW9662 (Sigma-Aldrich Co), mice were treated i.p. 30 min before 15dPGJ2 treatment. All animal protocols were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL), School of Medicine of the University of Buenos Aires, and are in accordance with guidelines of the Argentinean National Administration of Medicines, Food and Medical Technology (ANMAT), Argentinean National Service of Sanity and Agrifoods Quality (SENASA) and also based on the US NIH Guide for the Care and Use of Laboratory Animals.

2.2. Parasitemia and mortality

At 5, 8 and 10 days p.i., blood was obtained from RA infected or infected and 15dPGJ2 treated mice. Also, at 14, 22 and 50 days p.i. blood was obtained from K98 infected or 15dPGJ2 treated mice. Parasitemia was analyzed in infected mice through a small incision at the end of the tail. Five-fold dilutions were done in red blood cell lysis buffer (150 mM NH_4Cl, 0.1 mM EDTA, and 10 mM $KHCO_3$, pH 7.4). Parasitemia was measured in a Neubauer chamber. For survival studies, 2 independent groups (RA or K98 *T. cruzi* infected and *T. cruzi* infected and 15dPGJ2-treated mice) of 8 animals each were followed up daily until different days after infection.

2.3. Histopathology

Hearts from *T. cruzi* infected and 15dPGJ2-treated and infected mice were fixed in formalin and embedded in paraffin. Six non-contiguous sections (5 μm) were cut and stained with hematoxylin–eosin. Cellular infiltrate and the number and size of the amastigote nests were examined using a Nikon Eclipse E600 microscope (Nikon Inc.). Images were captured using a Spot RT digital camera. At least thirty random microscopic fields (400×) were analyzed in each microscopic section, using ImageJ analysis software (NIH).

2.4. Quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was obtained from heart tissue homogenates using Trizol reagent (Life Technologies, Inc.). Total RNA was reversed transcribed using Expand Reverse Transcriptase (Promega Corporation). QRT-PCR was performed using SYBR Green PCR Kit (PE Applied Biosystems Inc.) in an Applied Biosystems 7500 sequence detector. Primer sequences were: NOS2 forward: 5′-CACAGCAATATAGGCTCATCCA-3′, reverse: 5′-GGATTT CAGCCTCATGGTAAAC-3′; PPARγ forward: 5′-ATCTACACGATGCTGGC-3′, reverse: 5′-GGATGTCCTCGATGGG-3′; TNF-α forward: 5′-ATGAGCACA GAAAGCATGATC-3′, reverse: 5′-TACAGGCTTGTCACTCGAATT-3′; IL-6 forward: 5′-TGATGCACTTGCAGAAAACAA-3′, reverse: 5′-GGTCTTGGTC CTTAGCCACTC-3′. All samples were analyzed in the same run for 18S rRNA expression for normalization: forward: 5′-AACACGGGAAACCT CACCC-3′ and reverse: 5′-CCACCAACTAAGAACGGCCA-3′. PCR parameters were 50 °C for 2 min, 94 °C for 2 min, and 40 cycles of 94 °C for 30 s, 60 °C (for TNF-α, NOS2 and 18S rRNA) or 54 °C (for IL-6 and PPARγ) for 1 min. Quantification was calculated using the comparative threshold cycle (Ct) method and efficiency of the RT reaction (relative quantity, $2^{-\Delta\Delta Ct}$). The replicates were then averaged and fold induction was determined, considering the value at zero time as 1 [21].

2.5. Measurement of IL-10 production

IL-10 concentrations were measured in mice sera by ELISA method. The ELISA sets were IL-10 (BD OptEIA), and procedures were performed according to the manufacturer's instructions. The reaction was detected by peroxidase-conjugated Streptavidin followed by a substrate mixture that contained hydrogen peroxide as a substrate and ABTS (Sigma) as a chromogen. The absorbance measured at 405 nm was compared with the standard curve of IL-10.

2.6. Preparation of cytosolic, nuclear and total protein extracts for Western blot

Heart tissues (100 mg) were homogenized with 500 μl of buffer A (10 mmol/l HEPES; pH 7.9, 1 mmol/l EDTA, 1 mmol/l EGTA, 10 mmol/l KCl, 1 mmol/l DTT, 0.5 mmol/l phenylmethyl-sulfonyl fluoride, 40 μg/ml leupeptin, 2 μg/ml tosyl-lysyl-chloromethane, 5 mmol/l NaF, 1 mmol/l $NaVO_4$, 10 mmol/l Na_2MoO_4), and NP-40 was added to reach 0.5% (vol/vol). After 15 min at 4 °C, the tubes were gently vortexed for 10 s, and cytosolic extracts were collected by centrifugation at 13,000 *g* for 30 s. The supernatants were stored at −80 °C (cytosolic extracts) and heart pellets were resuspended in 300 μl buffer A supplemented with 20% (v/v) glycerol and 0.4 M KCl, and mixed for 30 min at 4 °C. Nuclear proteins were obtained by centrifugation at 13,000 *g* for 5 min, and aliquots of the supernatant (nuclear extracts) were stored at −80 °C.

Total protein extracts were obtained after washing the hearts with PBS and adding 500 μl of OGP (Sigma-Aldrich Co) lysis buffer. Then, the tubes were kept on ice for 30 min with swirling and the samples were centrifuged at 7000 *g* at 4 °C for 10 min. The supernatant was

transferred to a clean tube and stored at 20 °C. Protein concentrations of sera and heart tissue of mice were determined by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad CA., USA) reagent and bovine serum albumin (BSA) (Sigma-Aldrich Co.) as a standard [12].

2.7. Zymography

MMP activity in mice sera was measured using gelatin in-gel zymography. Mice sera were subjected to a 7.5% SDS-PAGE, in which 1 mg/ml gelatin (type A from porcine skin; Sigma Aldrich Co.) was incorporated. Following electrophoresis, gels were washed in 30% Triton X-100 for 60 min to remove SDS. Then, the gels were incubated in 50 mM Tris buffer pH 7.4, containing 0.15 mM NaCl and 30 mM $CaCl_2$, for 36 h at 37 °C. Gels were stained with Coomassie Brilliant Blue R and then distained with a mixture of 10% acetic acid and 30% methanol in water. The areas of proteolytic activity appeared as negatively stained bands in the dark background. Identification of MMPs was based on their molecular weights. A positive internal control (activated peritoneal macrophages) was run in each gel to allow the standardization of the results obtained in the different zymograms. After destaining, the clear regions representing MMP activity were quantified by densitometry using the ImageJ program developed at the NIH (USA).

2.8. Electrophoretic mobility shift assays (EMSA)

The oligonucleotide sequence 5′-TGCTAGGGGGATTTTCCCTCTCTCT GT-3′, corresponding to the consensus NF-κB binding site (nucleotides −978 to −952) of the murine NOS2 promoter, was annealed with the complementary sequence by incubation for 5 min at 85 °C in 10 mmol/l Tris–HCl, pH 8.0, 50 mmol/l NaCl, 10 mmol/l $MgCl_2$, and 1 mmol/l dithiothreitol. Aliquots (100 ng) were end-labeled with Klenow fragment of DNA polymerase I fragment in the presence of 50 mCi of [α-32P] dCTP and the other unlabeled dNTPs in a final volume of 50 ml. DNA probe (5×10^4 dpm) was used for each binding assay: 5 μg of nuclear heart proteins was incubated for 15 min at 4 °C with the probe and with 1 μg of poly (dI–dC), 5% glycerol, 1 mmol/l EDTA, 10 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, and 10 mmol/l Tris–HCl (pH 7.8) in a final volume of 20 μl. The DNA–protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris–borate–EDTA buffer. Supershift assays were carried out after the addition of the antibody against NF-κB protein (p65) (0.5 μg) to the binding reaction and incubation for 1 h at 4 °C before the addition of the probe. Analysis of competition with unlabeled oligonucleotides was performed using a 20-fold excess of double stranded DNA in the binding reaction and adding the nuclear extracts as the last step in the binding assay. Unrelated competitor oligonucleotide AP-1 (consensus) corresponding to the AP-1 motif of the albumin promoter, 5′-TTCCAAAGAGTCATCAG-3′ was used to ensure band specificity.

2.9. Statistical analysis

For the *in vivo* experimental models of Chagas' disease, data were expressed as means ± SE. Survival curves were compared using the Kaplan–Meier's test. One-way ANOVA was used for the comparison of NOS2, MMP-2/9, IκB-α and p65 Western-blot band intensity of controls, *T. cruzi*-infected and *T. cruzi*-infected 15dPGJ2-treated mice. Data were expressed as means ± SD. Differences were considered significant at $p<0.05$. All analyses were performed with Prism Software (GraphPad version 5.01).

3. Results

3.1. Course of infection

BALB/c mice were infected with 10^5 trypomastigotes of RA or K98 *T. cruzi* strains *via* intraperitoneal route. The parasitemia levels, assessed from the 5th day p.i. revealed that the mice infected with virulent and lethal *T. cruzi* strain, RA, had a significant increase of blood parasites on days 5 and 8 post infection with maximum number of parasites on day 10 (Table 1). While, in mice infected with the non-lethal strain, K98, the parasitemia was evident and quantifiable at day 22 p.i., and on day 50 the number of parasites increased significantly (Table 1). After reaching maximum value (45–50 dpi) a plateau developed that slowly decreased over time. Consistently with parasitemia levels, mice infected with RA strain did not survive at day 15 p.i. whereas K98 non-lethal strain infected mice showed 100% survival as illustrated in Table 1.

3.2. Pro-inflammatory enzymes are inhibited by 15dPGJ2 treatment in the hearts of T. cruzi infected mice

It has been reported that PPARγ ligands are implicated in the control of inflammatory response. However, little is known about the role of PPARγ in the course of *T. cruzi* infection. One of the first aims of our work was to analyze 15dPGJ2 effects on the expression of representative inflammatory enzymes like NOS2 and MMP-2/9 in the acute and chronic experimental mouse models of *T. cruzi* infection. Therefore, mice were treated with 15dPGJ2 (1 mg/kg) every 24 h, since the first day after infection and until the day before sacrifice, with minor modifications as described previously by Kaplan et al. [22]. Fig. 1 shows the NOS2 expression in the heart at day 10 p.i. with RA strain whereas 15dPGJ2 treatment inhibits both NOS2 mRNA and protein expressions in the hearts (Fig. 1A and B). On the other hand, the hearts of mice infected with K98 strain required 22 days p.i. to express NOS2 and 15dPGJ2 treatment could also inhibit mRNA and protein expressions (Fig. 1C and D).

MMP-2/9 expressions were evaluated in the heart by Western blot and their activities were analyzed by gelatin zymography in sera of RA and K98 infected mice. Fig. 2A and B shows that MMP-2 protein expression and activity appear and increase at day 10 in mice infected with the acute strain of *T. cruzi* compared with uninfected mice. Nevertheless, MMP-9 was undetectable in this model of acute infection (Fig. 2A). However, when infected mice were treated everyday with 15dPGJ2, we observed a significant decrease in cardiac enzyme expression and in sera activity, compared to untreated infected mice. Interestingly, when MMPs were studied in mice infected with K98 *T. cruzi* strain, MMP-2 expression was not detected (Fig. 2C), showing that MMP-2 is not a typical marker in a chronic model of inflammation. However, we could determine MMP-9 expression and activity at 22 dpi as shown in Fig. 2C and D. These results are in agreement with times of increased expression of both enzymes reported by other authors [23]. Furthermore in both groups of mice treated daily with 15dPGJ2, MMP-2/9 expressions and activities were diminished (Fig. 2).

3.3. Role of 15dPGJ2 on cytokine expression in T. cruzi infected mice

Taking into account that 15dPGJ2 treatment inhibited inflammatory enzyme expression, we speculate about the possibility that it can also inhibit the expression of inflammatory cytokines.

Table 1
Parasitemia and mice survival after infection with RA and K98 *Trypanosoma cruzi* subpopulations. Evaluation of parasitemia and mortality rates in mice infected with RA and K98, at different days post infection. Results are means ± SE from at least three independent experiments. ND: not detected; +: less than 10^4 parasites/ml.

Experimental groups	*T. cruzi* (RA)				*T. cruzi* (K98)			
Day	5	8	10	15	11	14	22	50
Parasites/ml ($\times10^6$)	0.67 ± 0.017	1.5 ± 0.97	2.2 ± 0.16	−	ND	+	0.02 ± 0.08	1.21 ± 0.34
Survival (%)	100	100	60	0	100	100	100	100

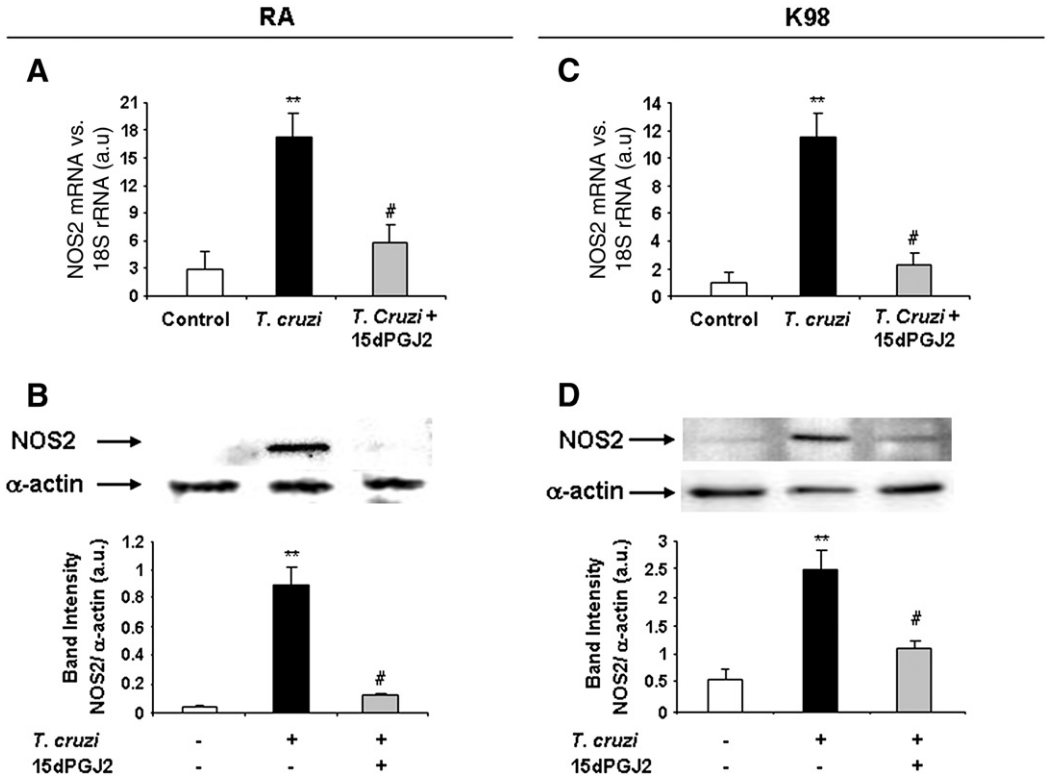

Fig. 1. 15dPGJ2 treatment regulates NOS2 expression in the hearts of mice infected with RA and K98 *Trypanosoma cruzi* subpopulations. NOS2 mRNA levels were analyzed by QRT-PCR in the heart extracts of infected mice or infected and 15dPGJ2-treated (1 mg/kg) at days 10 (RA) and 22 p.i. (K98) (A and C, respectively). The QRT-PCR results were normalized against 18S rRNA. NOS2 expression was determined in the same conditions as in A and C by Western blot with a specific antibody. Protein levels were normalized against α-actin (B and D). For A and C, results show the mean ± SD (n = 3). For B and D, results show representative experiments out of 3 performed. **p<0.05 *vs.* Control mice; #p<0.05 *vs. T. cruzi* infected mice.

As shown in Fig. 3, the hearts of RA and K98 infected mice expressed considerable amounts of TNF-α mRNA and IL-6 mRNA measured by QRT-PCR, with a significant peak 24 h after infection with RA and 22 days after K98 infection. Moreover, we found that these cytokines were significantly diminished in the groups of mice pre-treated with 15dPGJ2, as we have seen in the study of inflammatory enzyme expressions (Figs. 1 and 2). As a consequence, we also evaluated the capacity of 15dPGJ2 to induce a modulatory immune response by measuring IL-10 levels in sera of mice infected with both *T. cruzi* strains. Fig. 4A and B shows a significant increase of IL-10 levels in infected mice pre-treated with 15dPGJ2 in comparison with untreated infected mice.

On the other hand, 15dPGJ2 treatment slightly increased parasitemia levels in RA infected mice as shown in Fig. 5A. However, the mortality rates remained unchanged (Fig. 5B). 15dPGJ2 treatment in K98 infected mice did not modify the parasitemia at days 22 and 50 p.i. As expected, treatment with PPARγ ligand did not modify the survival rates of K98-infected mice. Moreover, PPARγ ligand-treated mice showed significantly higher amastigote nest sizes than untreated mice both for RA (Fig. 5C) and K98 (Fig. 5D), respectively.

3.4. 15dPGJ2 inhibits NF-κB activation in the hearts of mice infected with acute and chronic T. cruzi strains

15dPGJ2 is a high-affinity ligand for PPARγ. We have previously demonstrated that it inhibits the induction of inflammatory genes in both lipopolysaccharide stimulated and in *in vitro T. cruzi* infected cardiomyocytes through PPARγ dependent or independent pathways, like NF-κB [9,17]. To further assess the role of PPARγ in 15dPGJ2 effects in our *in vivo* model, we determined PPARγ mRNA expression in the hearts of RA and K98 infected mice, 10 and 22 days after infection, respectively. Fig. 6A and B shows a significant increase in PPARγ mRNA expression in the hearts of infected mice in comparison with those of uninfected ones. Additionally, mice infected with RA strain were pre-treated with the specific and irreversible PPARγ antagonist GW9662, which partially prevented 15dPGJ2 inhibitory effect on cardiac NOS2 expression, MMP-2 serum activity and the increase in parasitemia of infected mice (Fig. 7).

Therefore, we proposed to analyze the mechanism by which 15dPGJ2 inhibited the expression of inflammatory mediators in the acute and chronic *in vivo* experimental models of Chagas' disease, since GW9662 partially prevented 15dPGJ2 inhibitory effects. Then, our next aim was to determine the possible activation of NF-κB in the hearts of *T. cruzi*-infected mice. First, we studied the cytosolic expression and disappearance of IκBα, an NF-κB inhibitor, in the hearts of mice infected with both *T. cruzi* strains. Fig. 8A shows the NF-κB activation since IκBα disappeared in cytosolic heart extracts at 24 h and 36 h after infection with RA *T. cruzi* strain. These results were confirmed by EMSA assays, showing activation of p65–NF-κB subunit 48 h after infection in nuclear extracts of heart tissue (Fig. 8B). Anti-p65 caused a supershift of nuclear heart extracts after 48 h of infection and excess of unlabeled oligonucleotide as well as an unrelated competitor oligonucleotide (AP-1) was used to ensure band specificity (data not shown).

Secondly, we analyzed NF-κB activation in the heart extracts of K98 infected mice. We observed, contrasting with mice infected with the RA strain, that those infected with K98 require longer time for NF-κB activation. Fig. 8C shows IκBα disappearance only after 11 days post-infection. EMSA assays are consistent with these results, since NF-κB activation is demonstrated by p65–NF-κB subunit appearance in nuclear extracts 11 days after K98 infection (Fig. 8D).

In view of the NF-κB activation evidenced by the disappearance of IκBα, we asked whether the effects of 15dPGJ2 were exerted through NF-κB pathway in addition to PPARγ. Therefore, RA and K98 infected mice were treated with 15dPGJ2. p65–NF-κB could not translocate to the nucleus as shown by EMSA assay (Fig. 9), confirming PPARγ independent pathway on 15dPGJ2 effects in *T. cruzi* infected mice.

4. Discussion

In this study we investigated the ability of 15dPGJ2 to modulate *in vivo* pro-inflammatory mediators in the hearts of *T. cruzi* infected mice as well as the contribution of PPARγ-independent mechanisms to this regulation. An acute lethal and a chronic non-lethal model of infection were employed. Both infection models show the expected behavior with peaks of parasitemia at 10 dpi (RA) and reaching the maximum level of a plateau at 45–50 dpi (K98), as described by Pino Martínez 2011 [MSC thesis] for BALB/c mice and Celentano et al. for inbred C3H/HeN mice and outbred Rockland mice [24]. Although the ability of 15dPGJ2 to modulate the inflammatory response has been described in a number of pathological situations, the details of its whole mechanism remain largely unidentified. This work focuses for the first time on the role of the natural PPARγ ligand in *in vivo* experimental models of *T. cruzi* infection. Our data show that 15dPGJ2 inhibits the expression and activity of pro-inflammatory enzymes such as NOS2 and MMP-2 and it also modulates cytokine expression. Moreover, increased parasitemia associated with 15dPGJ2 treatment in the hearts of *T. cruzi* infected mice probably reflects NOS2 inhibition and the consequent diminished NO levels. Regarding the role of NO in the control of parasite multiplication *in vivo* Cummings and Tarleton [25] showed that NOS2-KO mice may compensate for the absence of NO production with the upregulation of cytokines important in immune control of *T. cruzi* infection. In the same line Marinho et al. [26] using a non-lethal *T. cruzi* strain showed that NOS2-KO mice are also able to control parasitemia and tissue parasitism. The explanation for these controversial results may lie in the genetic differences in the parasite strains and mice used.

We have previously shown that 15dPGJ2 is a potent modulator of the inflammatory process, through PPARγ dependent and independent pathways in cultures of *T. cruzi* infected neonatal cardiomyocytes [9]. In the present study we also determined the capacity of PPARγ natural ligand to exert anti-inflammatory effects by inhibiting the expression of inflammatory cytokines like TNF-α and IL-6 or enzymes like NOS2 and MMP-2/9 in RA and K98 infected mice. Of note, NO regulation has an important role, since it is required for intracellular killing of *T. cruzi*, but its synthesis in excess may damage the myocardium [7,8]. Different studies underline the importance of NO in the control of intracellular parasite multiplication during

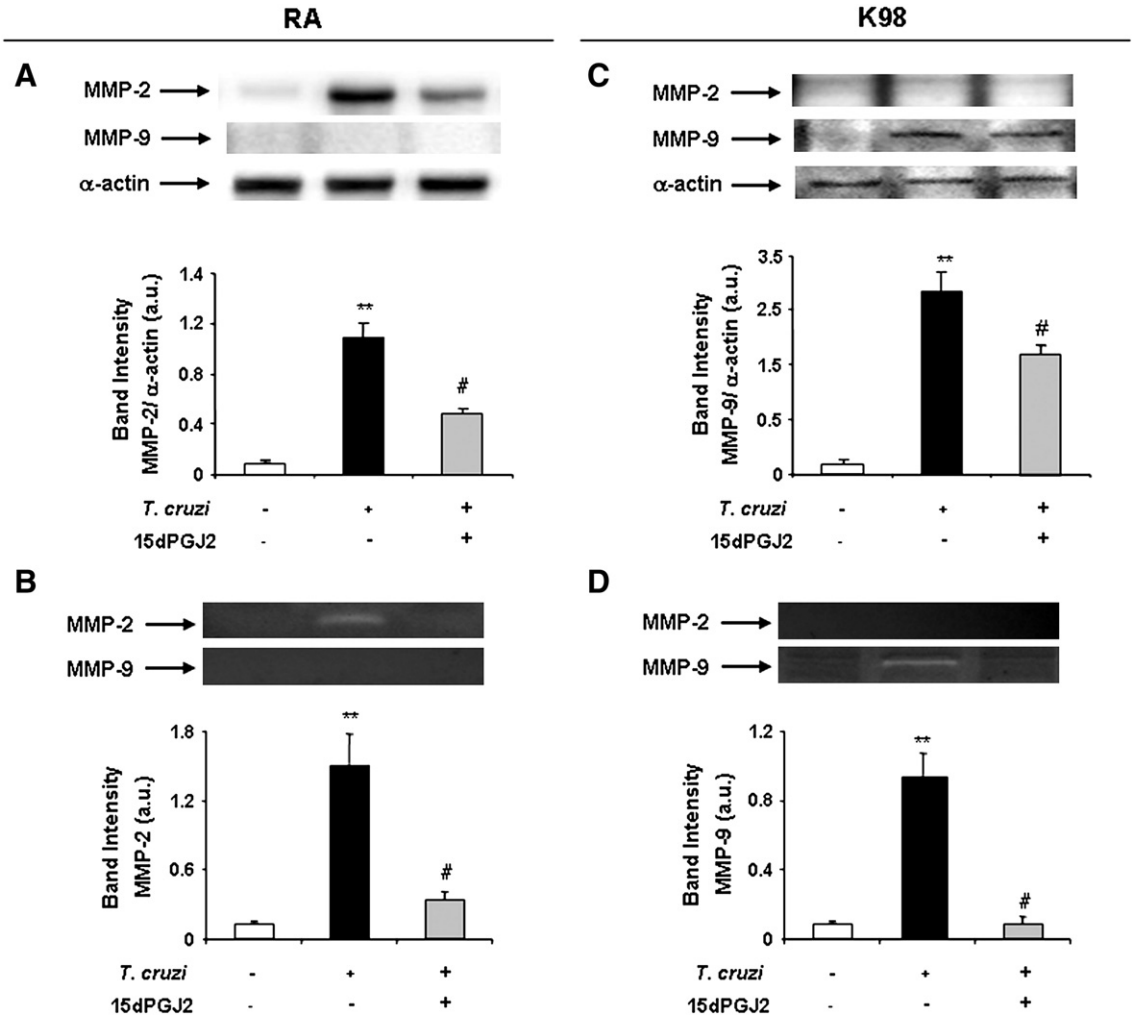

Fig. 2. Inhibition of MMP-2 and MMP-9 expressions and activity by 15dPGJ2 treatment in mice infected with RA or K98 *T. cruzi* subpopulations. MMP expression was determined by Western blot in the heart extracts at day 10 post infection with RA and at day 22 for K98 infection, or in 15dPGJ2-treated (1 mg/kg) mice. MMP-2 and MMP-9 specific antibodies were used and protein levels were normalized against α-actin (A and C, respectively). Sera from RA- or K98-infected or *T. cruzi*-infected and 15dPGJ2-treated mice were evaluated by zymography for MMP-2 and MMP-9 activities in the same conditions as in A and C (B and D). For A, B, C and D results show representative experiments out of three performed. **$p<0.05$ *vs.* Control mice; #$p<0.05$ *vs. T. cruzi* infected mice.

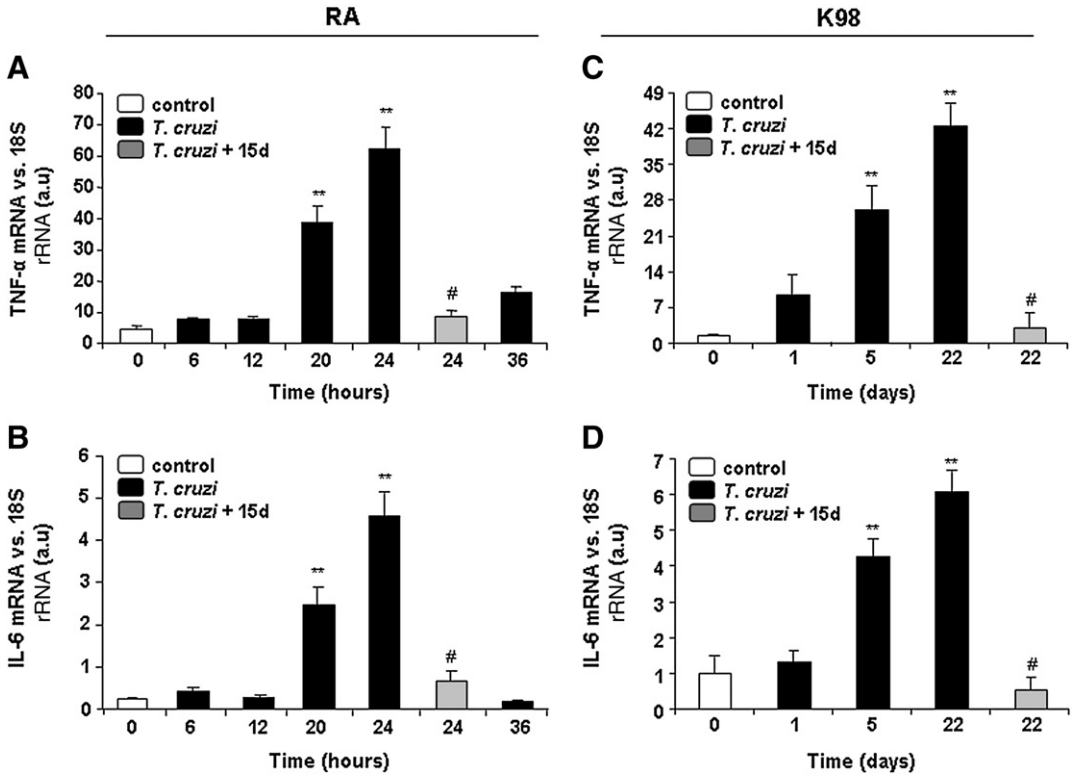

Fig. 3. Regulation of inflammatory cytokine expression by 15dPGJ2 treatment in the hearts of *T. cruzi*-infected mice. Mice were infected with RA *T. cruzi* subpopulation for 36 h or with K98 subpopulation for 22 days. QRT-PCR assays were performed in infected or infected and 15dPGJ2-treated (1 mg/kg) mice and TNF-α (A and C) and IL-6 (B and D) mRNA expressions were analyzed. The results were normalized against 18S rRNA. Results show representative experiments out of three performed. **$p<0.05$, *vs.* Control mice; #$p<0.05$ *vs. T. cruzi* infected mice.

T. cruzi infection. NOS2 knock-out mice showed higher parasitemia in acute infection in comparison with infected wild type mice [27].

Several factors contribute to chagasic cardiomyopathy and tissue damage, mainly including parasite persistence in the heart tissue [28]. The MMP family may also contribute to the development of chagasic cardiomyopathy, facilitating parasitic infection. In this regard, mice treated with an MMP inhibitor showed significantly decreased heart inflammation [29]. We have previously studied MMP cardiac expression by means of two different experimental models, lipopolysaccharide challenge and *T. cruzi* infection *in vitro*, confirming that cardiomyocytes were able to express MMP-9 and MMP-2 [9,17,21]. The fact that MMP9 but not MMP2 is detected in the heart extracts of K98-infected mice suggests that the last one may play a role at stages when heart damage is more severe. Of note, histological examination showed amastigote nests but scarce inflammatory reaction and slight necrosis. Moreover, our results of acute and chronic *in vivo* models confirm different reports showing that MMP-2 has rapid effects in regulating diverse early cellular functions independently of its action on the extracellular matrix [30] and also causes acute myocardial dysfunction after ischemia–reperfusion injury [31]. Furthermore, we found that pre-treatment of isolated cardiomyocytes with 15dPGJ2 inhibited MMP-9 and MMP-2 mRNA expressions as well as their gelatinolytic activity, being the regulation by 15dPGJ2 mediated through transcriptional mechanisms [21,17,9]. In the present work we confirm that exogenous 15dPGJ2 regulates MMP-2/9 expressions and activity in *in vivo* mouse models of *T. cruzi* infection.

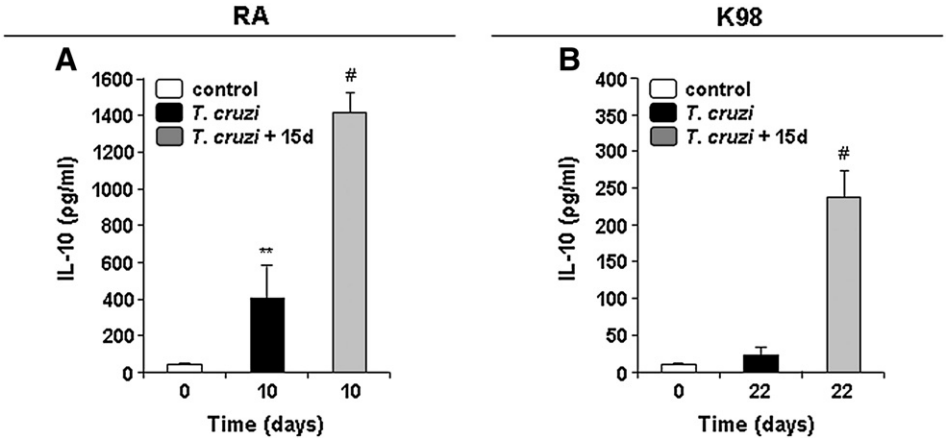

Fig. 4. Effect of 15dPGJ2 treatment on IL-10 expression in sera of RA and K98 infected mice. IL-10 production was measured by ELISA in sera from RA infected or infected and 15dPGJ2-treated (1 mg/kg) mice at day 10 post infection (A). Sera from K98 infected or infected and 15dPGJ2 treated mice were collected at day 22 post infection and IL-10 production was detected (B). Results are the means ± SD from three independent experiments. **$p<0.05$, *vs.* Control mice; #$p<0.05$ *vs. T. cruzi* infected mice.

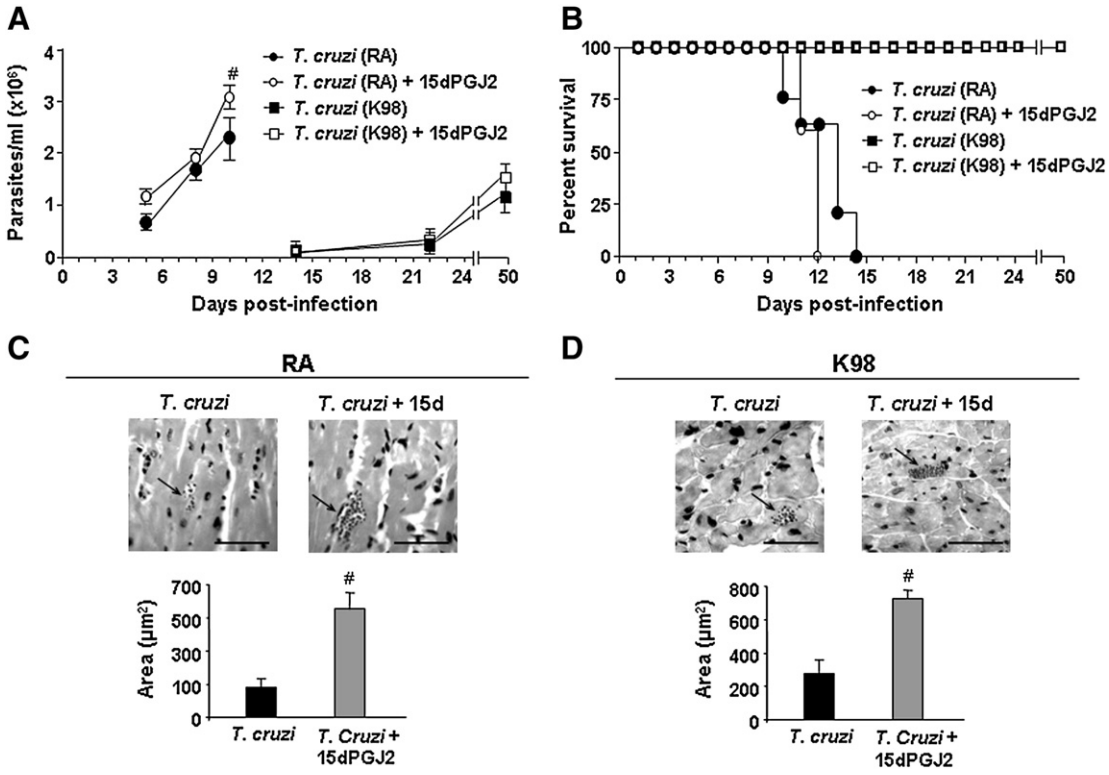

Fig. 5. Mice parasitemia, survival and heart parasitism after RA and K98 *Trypanosoma cruzi* subpopulation infection. Parasitemia was analyzed until day 10 of infection with RA and until day 50 of infection with K98 in 15dPGJ2 (1 mg/kg) treated (open symbols) or untreated (filled symbols) mice (A). Mice were infected and treated as in A and survival rates were analyzed by Kaplan–Meier's test (B). Cardiac parasitism was analyzed in histological sections of the hearts of infected and 15dPGJ2-treated or untreated mice stained with hematoxylin–eosin. Bar graph: area of amastigote nests ± SD (µm²), #$p<0.02$. Arrows show amastigote nests. Bar: 50 µm. Micrographs (400×) are representative of 3 experiments out of 8 mice per group (C and D). For A, results are the means ± SE ($n=6$) and for B results are survival percentage from at least three independent experiments.

Our results confirm that 15dPGJ2 modulates the parasitism in *T. cruzi* infected mice. The number of bloodstream trypomastigotes was higher in treated than in untreated infected mice. Besides, 15dPGJ2 treatment increased heart parasitism in both models. While mobilization of inflammatory cells was observed as perivascular infiltrates as well as some interstitial infiltrates (data not shown), infected cells (amastigote nests) were devoid of surrounding inflammatory reaction in both models (Fig. 5C and D). This might suggest that 15dPGJ somehow increases intracellular parasitism. In fact, *in vitro* cultured neonatal cardiomyocytes infected with the RA strain of *T. cruzi* show increased parasitism upon treatment with 15dPGJ2 in a model where inflammatory cells are absent [9]. Moreover, no differences in the mortality between experimental groups were observed. The balance between downregulation of expression of pro-inflammatory cytokines and upregulation of IL-10 could allow mouse survival. Downregulation in the levels of systemic cytokines by 15dPGJ2 has been described in models of inflammatory response [32]. Gallardo-Soler et al. suggest in *Leishmania major* infection model, that arginase induction by PPAR ligands changes macrophage functions to a more permissive stage that is suitable for parasite growth. This activation status closely resembles the one triggered by Th2 response, since it is comparable to that achieved in the presence of IL-4. Inhibition of parasite growth by PPAR antagonists demonstrates that this nuclear receptor is a key factor in macrophage alternative activation [33] consistent with previous works by Kopf and colleagues in which dyslipidemia inhibited protective Th1-type immunity and increased host susceptibility to *L. major* infection [34]. Moreover, Cuervo and colleagues have demonstrated that Th2 cytokines induce arginase expression, which may influence host and parasite cell survival but which might also downregulate the deleterious effects triggered by NOS2, in the heart tissue during *in vivo* acute *T. cruzi* infection of BALB/c and C57BL/6 mice [35].

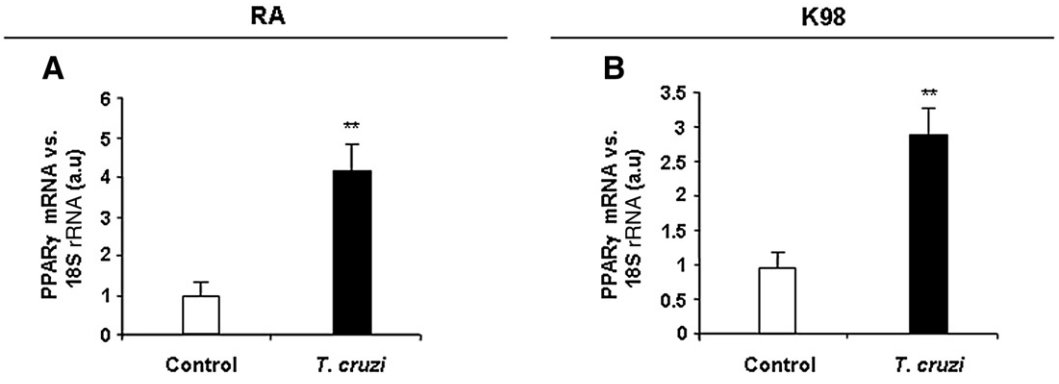

Fig. 6. PPARγ expression after *Trypanosoma cruzi* infection. PPARγ mRNA expression was analyzed by QRT-PCR in the hearts of control, RA- or K98-infected mice at day 10 post infection (A) and at day 22 post infection (B), respectively. The results were normalized against 18S rRNA. Results show the mean ± SD ($n=4$). **$p<0.05$, *vs.* Control mice.

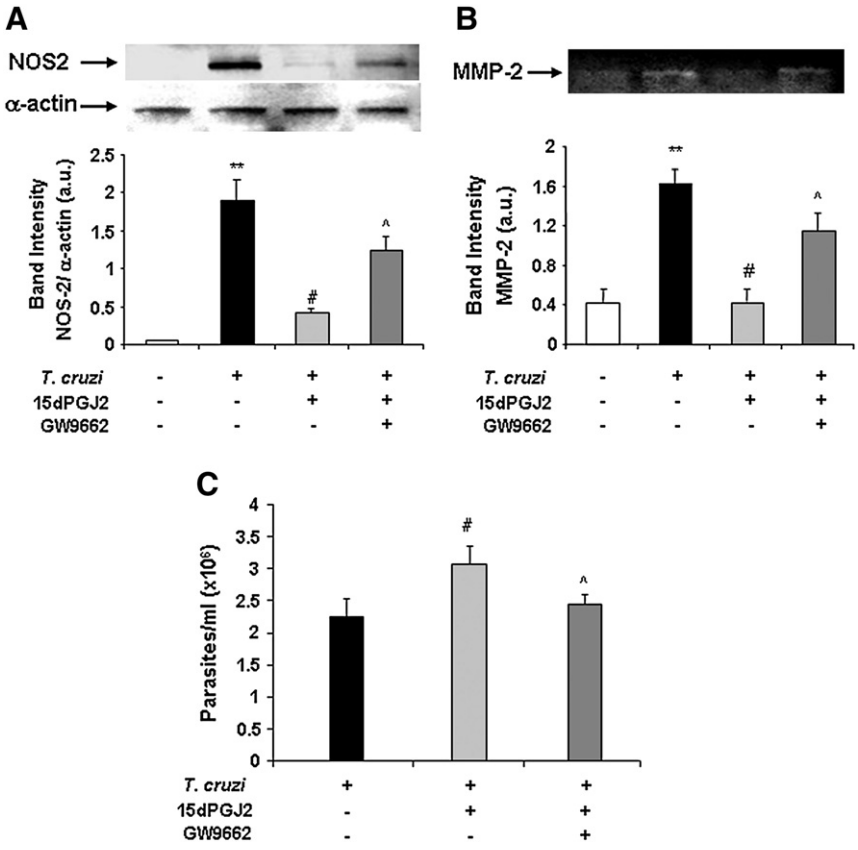

Fig. 7. PPARγ participation in 15dPGJ2 effects on NOS2 expression, MMP-2 activity and parasitemia. Mice were infected and treated daily with the PPARγ antagonist GW9662 (1 mg/kg) and 15dPGJ2 (1 mg/kg), 30 min later. At day 10 post-infection the heart extracts were obtained and Western blot analyses were done for NOS2 expression. Protein levels were normalized against α-actin (A). Mice were treated as in (A) and sera were subjected to gelatin zymography to detect MMP-2 activity (B). Mice were treated as in (A) and the parasitemia values were evaluated at 10 day post-infection (C). For A and B, results show representative experiments out of 3 performed. For C, results are the means ± SE (n = 6). **p<0.05 *vs.* Control mice; #p<0.05 *vs. T. cruzi* infected mice. ^p<0.05 *vs. T. cruzi* + 15dPGJ2 infected mice.

In our work, the increase in parasitemia levels after PPARγ ligand treatment could be explained by the upregulation of IL-10 levels in 15dPGJ2-treated RA and K98 infected mice, a cytokine mainly secreted by Treg, B cells and some Th2 cells. The levels of serum IL-10 are several times lower in mice infected with non-lethal strain, K98, with respect to those infected with RA. However, it must be noted that the levels of IL-10 in 15dPGJ2-treated to untreated mice ratio remain approximately the same (4.5–5 times) for both parasite strains. Besides, Carrera-Silva et al. [36] showed that pro-inflammatory cytokines were effectively countered by IL10 as well as TGF-β regulatory cytokines. Therefore, TGF-β might be playing a compensatory role for regulation in the case of K98, which produces significantly lower absolute levels of IL-10 than RA.

In this work we found increased levels of PPARγ mRNA expression in the hearts of infected mice compared to uninfected ones. It has also been demonstrated that PPARγ expression is increased in the airway mucosa of asthmatic patients when compared with healthy subjects [37]. Recent evidence in a mouse model of leishmaniasis suggested that PPARγ expression was not modified in the liver but it was increased in the spleen upon infection [38]. In fact, PPARγ levels in inflammatory models are controversial since during the cardiovascular hypodynamic phase of septic shock PPARγ expression decreases in endothelial and aortic vascular smooth muscle cells [39]. In order to evaluate the implication of increased PPARγ in 15dPGJ2 effects, RA infected mice were pre-treated with a specific PPARγ antagonist, GW9662, revealing partial reversion of 15dPGJ2 inhibitory effects on NOS2 heart expression and NO production. Also, GW9662 prevented the inhibitory effect of PPARγ natural ligand on MMP-2 activity in serum of infected mice.

Herein we have demonstrated NF-κB activation in the heart after mice infection and, also, the inhibition of this pathway by 15dPGJ2 pre-treatment. In previous studies we observed that 15dPGJ2 exerts anti-inflammatory effects in LPS or *T. cruzi*-stimulated cardiac cells by means of PPARγ-dependent and -independent mechanisms involving transcription factors like NF-κB [17,9]. Moreover, several authors have demonstrated that 15dPGJ2 exerts a strong anti-inflammatory effect by attenuating the expression of pro-inflammatory mediators in activated monocytes/macrophages. This effect is mainly exerted through the inhibition of NF-κB-dependent transcription of inflammatory genes [16,40].

Taken together, our data suggest that 15dPGJ2 downregulates inflammatory enzymes and cytokines after *T. cruzi* infection. Despite the increased parasitism, probably due to decrease in NO levels, its effect may be beneficial, reducing the detrimental effects of the inflammatory response. The fact that GW9662 partially restored NOS2 and MMP-2 levels and activity after 15dPGJ2 treatment, strongly suggests that the participation of PPARγ-independent signaling pathways is operative *in vivo*. In summary, the regulation of the heart's inflammatory response by the PPARγ natural ligand in acute and chronic Chagas' heart disease through PPARγ-dependent and NF-κB pathways opens new avenues to putative pharmacological treatments.

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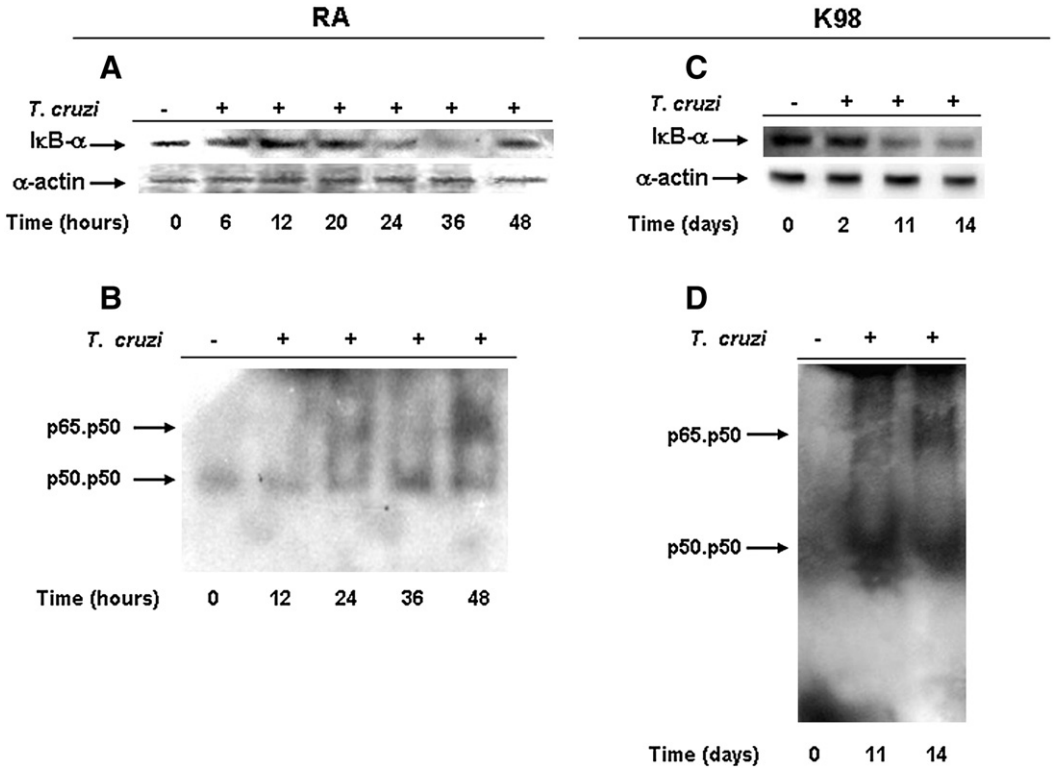

Fig. 8. Activation of NF-κB pathway in RA- and K98-infected mice. Control and infected mice were sacrificed at different times. Western blot analyses were carried out in cytosolic heart extracts of RA (A) or K98 infected mice (C). IκB-α expression was determined with a specific antibody and protein levels were normalized against α-actin. Electrophoretic mobility shift assays (EMSA) were carried out for determination of NF-κB activation in RA (C) and K98 infected mice (D). For A, B, C and D results show representative experiments out of three performed.

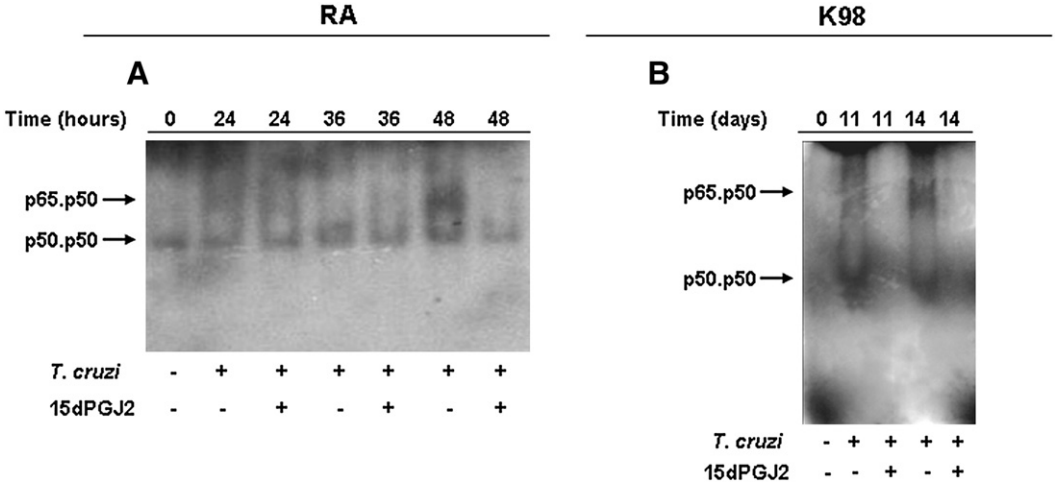

Fig. 9. 15dPGJ2 treatment inhibits NF-κB activation induced by *T. cruzi* infection. Infected mice were treated or not with 15dPGJ2 (1 mg/kg) and at the indicated times. Electrophoretic mobility shift assays (EMSA) were performed for determination of NF-κB activation in nuclear extracts from RA (A) or K98 (B) infected mice. For A and B, results are representative of three assays performed.

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