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Abstract: The participation of nitric oxide (NO) in the control of blood parasitemia and parasitism during the acute phase of infection in dogs inoculated with blood trypomastigotes (BT) or metacyclic trypomastigotes (MT group) of Trypanosoma cruzi strain Berenice-78 has been evaluated. Animals of the MT group (n=4) presented increased levels of serum NO throughout the infection when compared with the BT (n=4) or control (n=4) groups, and a delay in parasitemia peak compared with the BT group. In spleen fragments, tissue parasitism was not observed but the MT group presented larger areas associated with inducible NO synthase (iNOS) in relation to BT and control groups. Heart fragments of MT-infected animals exhibited comparatively low tissue parasitism and high iNOS expression, while animals of the BT group presented high inflammatory infiltrate, high tissue parasitism and low iNOS expression. These results indicate that the source of inoculum can interfere with the development of the acute phase of Chagas disease, and may also trigger a distinct parasite-host interaction during this phase.

Belo Horizonte, april 14 2008

Editor-in-Chief
John Horton
Experimental Parasitology

Dear Dr. Horton,

I would like to submit our manuscript "***Trypanosoma cruzi:* serum levels of nitric oxide and expression of inducible nitric oxide synthase in myocardium and spleen of dogs in the acute phase of infection with metacyclic or blood trypomastigotes**" for publication in *Experimental Parasitology.*

In this manuscript, we investigated the participation of nitric oxide during canine acute infection with metacyclic or blood trypomastigotes. The rational for our study is the observation that different inoculum source conduct to differential response during acute *T. cruzi* infection (Carneiro et al., Acta Tropica, 2007). So, we decided to verify the participation of No during this moment.

The presented data are novel and unpublished. There is no overlap between the submitted manuscript and other papers under consideration elsewhere. All co-authors approved the submission of the manuscript. Animal experiments were performed in accordance with COBEA (Colégio Brasileiro de Experimentação Animal).

Sincerely yours,

Claudia Martins Carneiro

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Trypanosoma cruzi: serum levels of nitric oxide and expression of inducible nitric oxide synthase in myocardium and spleen of dogs in the acute phase of infection with metacyclic or blood trypomastigotes

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Abstract

The participation of nitric oxide (NO) in the control of blood parasitemia and parasitism during the acute phase of infection in dogs inoculated with blood trypomastigotes (BT) or metacyclic trypomastigotes (MT group) of *Trypanosoma cruzi* strain Berenice-78 has been evaluated. Animals of the MT group (*n*=4) presented increased levels of serum NO throughout the infection when compared with the BT (*n*=4) or control (*n*=4) groups, and a delay in parasitemia peak compared with the BT group. In spleen fragments, tissue parasitism was not observed but the MT group presented larger areas associated with inducible NO synthase (iNOS) in relation to BT and control groups. Heart fragments of MT-infected animals exhibited comparatively low tissue parasitism and high iNOS expression, while animals of the BT group presented high inflammatory infiltrate, high tissue parasitism and low iNOS expression. These results indicate that the source of inoculum can interfere with the development of the acute phase of Chagas disease, and may also trigger a distinct parasite-host interaction during this phase.

Index Descriptors and Abbreviations: Chagas disease; *Trypanosoma cruzi*; blood trypomastigotes (BT); metacyclic trypomastigotes (MT); nitric oxide (NO); inducible nitric oxide synthase (iNOS)

1. Introduction

Trypanosoma cruzi, the causative agent of Chagas disease, is characterised by a complex biological cycle involving an invertebrate vector and mammalian hosts. The protozoan exists in at least three morphologically distinct stages. Epimastigotes proliferate in the invertebrate host and are released as metacyclic trypomastigotes (MT) in the faeces. In the vertebrate host, amastigotes and blood trypomastigotes (BT) are the intracellular developmental and infective forms, respectively (Andrade and Andrews, 2005).

Transmission mechanisms include vectorial, transfusional and oral infection, congenital processes and laboratory accidents (Prata, 2001). Vectorial infection is the main and most frequent form of transmission and occurs when mucous membranes or abraded skin are exposed to MT-infected faeces of triatomine insects. In the domestic cycle of Chagas disease, *Triatoma infestans* and *Rhodnius prolixus* are the principal invertebrate vectors (Kollien and Schaub, 2000). Although the vertebrate host can develop a cellular and humoral immune response to the parasite, chronic infection is maintained by a reduced number of circulating BT forms. The presence of BT forms in the blood thus generates a further public health problem, and transmission of the parasite via blood transfusion has become the second most frequent route of infection (Coura et al., 2007). Transmission of *T. cruzi* resulting from congenital processes, oral infection or laboratory accidents may be mediated by either MT or BT forms (Sandler et al., 2003).

During the acute phase of infection, the destruction of trypomastigotes is dependent on the production of nitric oxide (NO), a process that is catalysed by inducible NO synthase (iNOS) (Vespa et al., 1994; Aliberti et al., 1996; Holscher

et al., 1998). Studies have shown that glycosyl phosphatidylinositol-anchored mucin-like glycoproteins purified from BT forms (tGPI mucins) are potent elicitors of proinflammatory responses (i.e. cytokine and NO production) by IFN-γ primed murine macrophages. In contrast, the corresponding glycoproteins derived from MT forms (mGPI mucins) are reported to be at least 100 to 1000-fold less active than tGPI mucins in the induction of NO by murine macrophages (Camargo et al.,1997a, b; Almeida et al., 2000). Recent studies from our group have revealed that BT and MT infections are associated with distinct parasitological and serological features, together with intrinsic and inoculum source-specific changes in circulating leukocytes (Carneiro et al., 2007).

The canine model is currently considered to provide an appropriate experimental system with which to investigate various aspects of Chagas disease, since *Canis familiaris* displays high susceptibility to infection and shows considerable similarities in the manifestation of the acute and chronic phases that are representative of the human disease. Moreover, it is possible to reproduce in this model the symptomatic acute phase and the chronic phase in both the undetermined and heart forms of the disease (Lana et al*.,* 1988,1992; Bahia et al*.,* 2002).

On the basis of the above, the canine model has been employed to investigate aspects of the participation of NO during the acute phase of experimental infection by MT forms, simulating vectorial transmission, and by BT forms, simulating transfusion transmission (or, indeed, any transmission mechanism involving BT forms) of *T. cruzi* strain Berenice-78 (Be-78). The objective of the study was to obtain a better understanding of the mechanisms related to the pathogenesis of Chagas disease in the canine model.

2. Materials and methods

Details of the project were submitted to and approved by the Ethical Committee on Animal Research of the Universidade Federal de Ouro Preto. All procedures were carried out in compliance with current Brazilian regulations relating to Experimental Biology and Medicine as described in the guidelines issued by the Colégio Brasileiro de Experimentação Animal (COBEA, 2006). Study animals were maintained in the central animal facility at the Universidade Federal de Ouro Preto (UFOP), Minas Gerais, Brazil.

2.1. Parasites, animals and experimental infection

Nymphs of *Triatoma infestans* were allowed to feed on the blood of female Swiss Webster mice (weight range 20 - 24 g) that had previously been inoculated with *T. cruzi* strain Be-78. Following infection, the triatomines were maintained under starvation conditions for 15 days and then allowed to blood-feed on uninfected mice in order to induce the release of MT forms of the parasite in the faeces. BT forms of the protozoan were obtained by infecting female Swiss Webster mice with 1×10^4 blood forms of *T. cruzi* strain Be-78 per animal and collecting blood samples from the orbital veins at the parasitemia peak.

Prior to the commencement of the study, 12 mongrel dogs (6 males and 6 females), each 4 months old, were immunised against most common infectious canine pathogens and treated with anthelmintics. Study animals were maintained in quarantine for 16 weeks, during which time they received drinking water and a balanced commercial feed *ad libitum*. Two groups of 4 dogs were

inoculated intraperitoneally on study day 0 with either MT or BT forms of *T. cruzi* strain Be-78 (2000 forms per kg body weight), whilst 4 dogs were maintained uninfected and served as the control group.

2.2. Parasitological parameters

From days 1 to 35, samples (5 μL) of blood were collected daily from infected and control dogs by vein-puncture of ear veins. The numbers of parasites in the blood samples were determined under the optical microscope according to the method of Brener (1962), and parasitemia curves were plotted using the daily mean numbers of parasites per group of 4 animals.

2.3. Serum levels of NO

Peripheral blood (5 mL) was collected from the brachial cephalic veins of experimental animals prior to inoculation and weekly thereafter (on days 7, 14, 21, 28 and 35), and serum was separated and stored frozen (-20°C) until required for analysis. Levels of endogenously synthesised NO in the serum were assessed by reducing nitrate to nitrite with nitrate reductase (1 U/mL) followed by the Griess reaction (Green et al., 1982). Nitrite concentrations were determined by extrapolation from standard curves obtained using various concentrations of sodium nitrite, and the results were expressed in μM.

2.4. Histopathological examinations and immunohistochemical analyses

Experimental animals were submitted to necropsy during the acute phase of infection, and fragments of the heart and spleen were resectioned, fixed in 10% buffered formalin (pH 7.2) and embedded in paraffin. Sections (5 μm thick) were mounted on poly-L-lysine-coated glass slides and were either stained with

Hematoxylin-Eosin (HE) for standard histological procedures, or submitted to immunohistochemical analysis.

2.4.1. Expression of iNOS

The immunohistochemical detection of iNOS expression was performed using streptavidin-biotin-peroxidase. Endogenous peroxide was blocked by incubating embedded tissue sections with 3% hydrogen peroxide (H_2O_2) in methanol for 30 min. Sections were then heated in a microwave oven (700 W) for 10 min to retrieve the antigens and cooled to room temperature. After washing with phosphate-buffered saline (PBS), the sections were further blocked with normal horse serum (Vector Laboratories Burlingame, CA, USA) to reduce non-specific antibody binding, and then incubated overnight at 4°C with the primary antibody against iNOS diluted 1:200 (Cat. No. sc-651; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). After washing with PBS for 3 x 5 min, the sections were incubated with the secondary antibody conjugated with biotin (Elite ABC Kit, Vector Laboratories) for 30 min at 37°C, washed again with PBS, and incubated with streptavidin-peroxidase complex for 30 min at 37°C. The reaction products of peroxidase were visualised by incubation with PBS (250mL) containing 50 mg of 3.3'-diaminobenzidine (DAB) and 500 μL of H_2O_2. Finally the sections were stained for nuclei with Harris's hematoxylin solution. Negative control slides were prepared in the same manner but in the absence of the primary antibody.

2.4.2. Determination of tissue parasitism

Embedded tissue sections were incubated overnight at 4°C with polyclonal anti-*T. cruzi* serum (obtained from a rabbit that had been immunised with *T.*

cruzi Y strain) diluted 1:1000. Subsequently, sections were incubated with secondary antibody anti-rabbit IgG and peroxidase-anti-peroxidase complex, and the label was detected by incubations DAB. Sections obtained from murine acute myocarditis, that were rich in amastigote nests, were used as positive controls. Finally the sections were stained for nuclei with diluted Harris's hematoxylin solution. Negative control slides were prepared in which the primary antiserum was substituted by PBS.

2.5. Morphometric studies

Morphometric studies of iNOS expression and tissue parasitism were performed by analysing images of 20 randomly-selected fields (total area 1.5 x 10^6 μm^2) of tissue fragment sections on a single slide per animal. Inflammatory infiltration in the heart was quantified by counting the cell nuclei present in sections of heart fragments, while iNOS and *T. cruzi* immunoreactive areas were measured in sections of heart and spleen fragments. All analyses were performed using a 40 X objective, and images were analysed with the aid of Leica QWin software (Leica Microsystems, Wetzlar, Germany).

2.6. Statistical analyses

Since the area beneath the parasitemia curve was non-parametric in character, comparative analyses between groups were performed using the Kolmogorov-Smirnov test (Conover, 1980). Serum levels of NO, iNOS expression and inflammatory infiltration were analysed among the different groups using one-way analysis of variance (ANOVA) and Tukey post tests. Student's *t*-test was employed to determine the significance of differences in

tissue parasitism. In all cases, differences were considered statistically significant when *P* values were < 0.05.

3. Results

3.1. Production of NO and levels of parasitemia

The dynamics of the levels of NO in serum collected from groups of experimental animals within the period commencing immediately before innoculation and for the following 35 days are presented in Fig. 1. Animals of the MT group showed significantly higher levels of NO when compared with those of the BT and control groups at days 14 and 28 after infection. In the MT group, enhanced production of NO began at day 7 but diminished after day 28 day, while in the BT and control groups the levels of NO were similar throughout the 35 day period. No significant differences in serum NO levels were observed in the longitudinal analyses. Although no significant differences were observed between groups MT and BT with respect to the areas under the parasitemia curves (Fig. 1), the early production of NO in MT-infected animals may be related to the later peak of parasitemia observed in this group.

3.2. Heart inflammatory infiltration

The results of the morphometric analysis of inflammatory infiltration in the heart are presented in Fig. 2A. Statistically significant increases were observed in the numbers of cell nuclei present in the heart fragments of MT- and BT-infected animals in comparison with those of the control group. Moreover, the numbers of inflammatory cells were greater in animals of the BT group compared with the MT group ($P < 0.05$). In infected animals, the inflammatory

infiltrate comprised predominantly mononuclear cells (Fig. 3B and C), the majority of which exhibited the morphology of lymphocytes. However, animals of the MT group exhibited focal inflammatory infiltrates while in those of the BT group the inflammation was diffuse.

3.3. Parasitological parameters

The results of the quantitative evaluation of tissue parasitism in heart fragments derived from MT- and BT-infected animals are shown in Fig. 2B. Tissue parasitism was observed to be discreet in all infected animals, and morphometric analysis of different areas of heart tissue revealed no significant differences in parasitism between the two groups (Fig. 2B and insert Figs. 3B and C). No parasitism in spleen tissue was observed in animals of either group.

3.4. iNOS expression in heart and spleen

Immunohistochemical staining for iNOS was carried out after necropsy during the acute phase of infection. Results of the quantification of areas of sections of heart and spleen exhibiting iNOS immunoreactivity are shown, respectively, in Figs. 2C and D, while the histopathological aspects are displayed in Fig. 3. Heart fragments from animals of the MT group presented significantly larger iNOS positive areas compared with the control group. With respect to the BT group, however, the iNOS positive areas were not statistically different in size compared with either the MT or the control groups. In comparison with control animals, significant staining was detected in the arteries and arterioles in the heart fragments of infected animals (inserts to Figs. 3D, E and F). Moreover, a blush of iNOS immunoreactivity throughout the

myocardium was observed in all infected animals, and was most intense in animals of the MT group (Figs. 3D, E and F).

With respect to spleen fragments, the pattern of iNOS expression was very similar to that established for levels of serum NO, i.e. MT-infected animals presented a significantly larger area of iNOS immunoreactivity compared with both the BT and the control groups (Figure 2D).

4. Discussion

The aim of the present study was to evaluate serum NO levels, blood parasitemia, expression of iNOS and tissue parasitism in spleen and heart tissues of dogs infected with MT or BT forms of *T. cruzi* strain Be-78. The two infective forms of the parasite are known to exhibit distinct characteristics that induce different immune responses *in vitro* and *in vivo* (Brener and Gazzinelli, 1997; Carneiro et al., 2007). Infection mediated by metacyclic forms *in vitro* can, in part, mimic that caused by vectors *in vivo*. On the other hand, *in vitro* infection with blood forms imitates blood transmission or even laboratory accidents. It is worth mentioning that the majority of experimental infections are conducted using blood trypomastigotes because these forms are easier to obtain and maintain.

Although iNOS is not considered essential for the control of *T. cruzi* infection (Cummings and Tarleton, 2004), the elimination of BT forms in the acute phase is dependent on a number of factors (Pascutti et al., 2003; Cardillo et al., 2007), one of which is the production of NO catalysed by iNOS (Vespa et al., 1994; Petray et al., 1995; Aliberti et al., 1996; Holscher et al., 1998). Thus macrophages infected with *T. cruzi* produce IL-12, a powerful cytokine that

induces INF-γ synthesis by NK cells. INF-γ plays an important role in the activation of macrophages that produce high levels of NO and effectively control parasite replication (Cardillo et al., 1996; Sardinha et al., 2006).

The results obtained in the present study showed that dogs infected with MT forms of the parasite exhibited elevated expression of iNOS and higher serum levels of NO in comparison with BT-infected animals ($P < 0.05$). Camargo et al (1997a,b) have demonstrated that, in a murine model, tGPI-mucins of BT forms are potent elicitors of the immune system and produce large amounts of NO. The disparity between these two sets of findings could be due to the experimental model used (i.e. dogs versus mice) or to the type of experiment (i.e. *in vitro* versus *in vivo*) carried out. Additionally, the earlier work focused on a single component of the parasite form whereas in the present investigation all components were involved.

The increases in serum levels of NO observed in the MT group could be related to the high expression of iNOS detected in different tissues (i.e. heart and spleen) that have direct contact with blood. In this manner, such compartments could contribute to the systemic increase in NO in the MT group. On the other hand, the differences in profile of serum NO observed between the MT and BT groups could arise from the parasite-host interaction initially established by the two infective forms, since towards the end of the acute phase the differential effect had disappeared.

Considering previous results of Carneiro et al. (2007) concerning the phenotyping of peripheral blood, a positive correlation between serum levels of NO and the frequency of $CD5^+$ T lymphocytes and subpopulation $CD4^+$ was observed only in animals of the MT group (data not shown). This suggests that

these cell types could be participating directly or indirectly in the production of NO. $CD4^+$ T cells could generate NO directly through the action of iNOS, as has been observed by Taylor-Robinson et al. (1994). Conversely, $CD4^+$ T lymphocytes may produce INF-γ, which in turn activates macrophages to produce NO by the action of iNOS (Russo et al., 1988; Araujo, 1989; Rottenberg et al., 1993, 1995).

Additionally, Carneiro et al. (2007) observed that, in the experimental dogs employed in the present study, there was a significant decrease of $CD4^+$ cells in the peripheral blood of MT-infected animals on day 28 in comparison with those of the BT group. Such a finding may indicate a greater migration of $CD4^+$ cells to the sites of inflammation in the MT group, and this could be responsible for the increased production of NO in the tissues of MT-infected animals. Numerous attempts have been made by our research group to characterise the T cells ($CD4^+$ and $CD8^+$) in heart and spleen samples, both frozen and embedded in paraffin, but currently without success.

From the 28^{th} day after infection, the serum levels of NO in animals of the MT group showed a decline, and at day 35 were similar to those of the BT and control groups. These findings are consistent with those reported by Saeftel et al. (2001), who demonstrated that in the murine model NO was important for the control of infection in the first 3 weeks following infection, but declined in level thereafter.

Park et al. (2000) detected the presence of iNOS mRNA in cells from normal human heart, implying that heart muscles express iNOS constitutively under baseline conditions. This possibility would explain the expression of iNOS in different areas of the heart of control animals as observed in the present work.

Immunohistochemical studies by Chandrasekar et al. (1998) revealed high expression of iNOS in the arteries and arterioles of rats commencing 36 h after infection but reducing by the 15th day. In contrast, iNOS expression was intense in cardiomyocytes on the 15th day after infection. The same pattern was observed in the present study, with strong expression in the arteries and especially in cardiomyocytes.

Animals of the BT group showed greater inflammatory process compared with the control and MT groups, but the areas associated with iNOS were, in general, smaller. This fact could be related to the presence of anti-inflammatory cytokines such as IL-10 and TGF-β (Oswald et al., 1992; Sher et al., 1992), which can disable the macrophages and thus prevent expression of iNOS.

No parasitism was detected in spleen tissue fragments derived from any of the experimental dogs, a finding that could be explained by the myotropism observed in *T. cruzi* strain Be-78.

The results presented herein reinforce those obtained by Carneiro et al, (2007), who found that the source of inoculum could interfere with the development of the acute phase of Chagas disease in respect of parasitology, serology and phenotype of the peripheral blood cells. Apparently, inoculation of animals with MT forms induces an effective initial response that limits parasitemia and subsequent tissue parasitism, resulting in a lower inflammatory response and only minor tissue damage. In contrast, infection with BT forms appears not to induce an early immune response that is sufficiently elaborate to achieve the rapid control of the infection, and this permits the development of intensive histological changes, especially in the heart. Moreover, our findings

suggest that the inoculum source may trigger distinct parasite-host interactions during acute Chagas disease.

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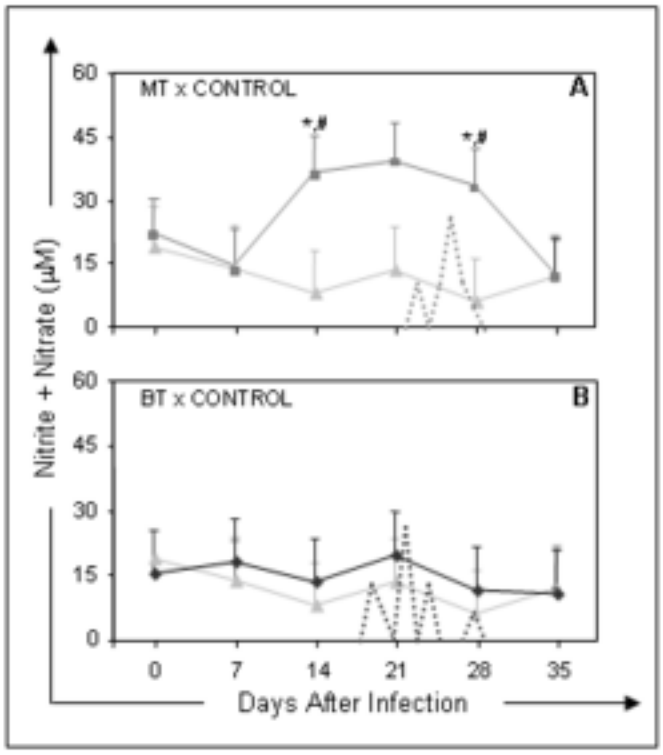

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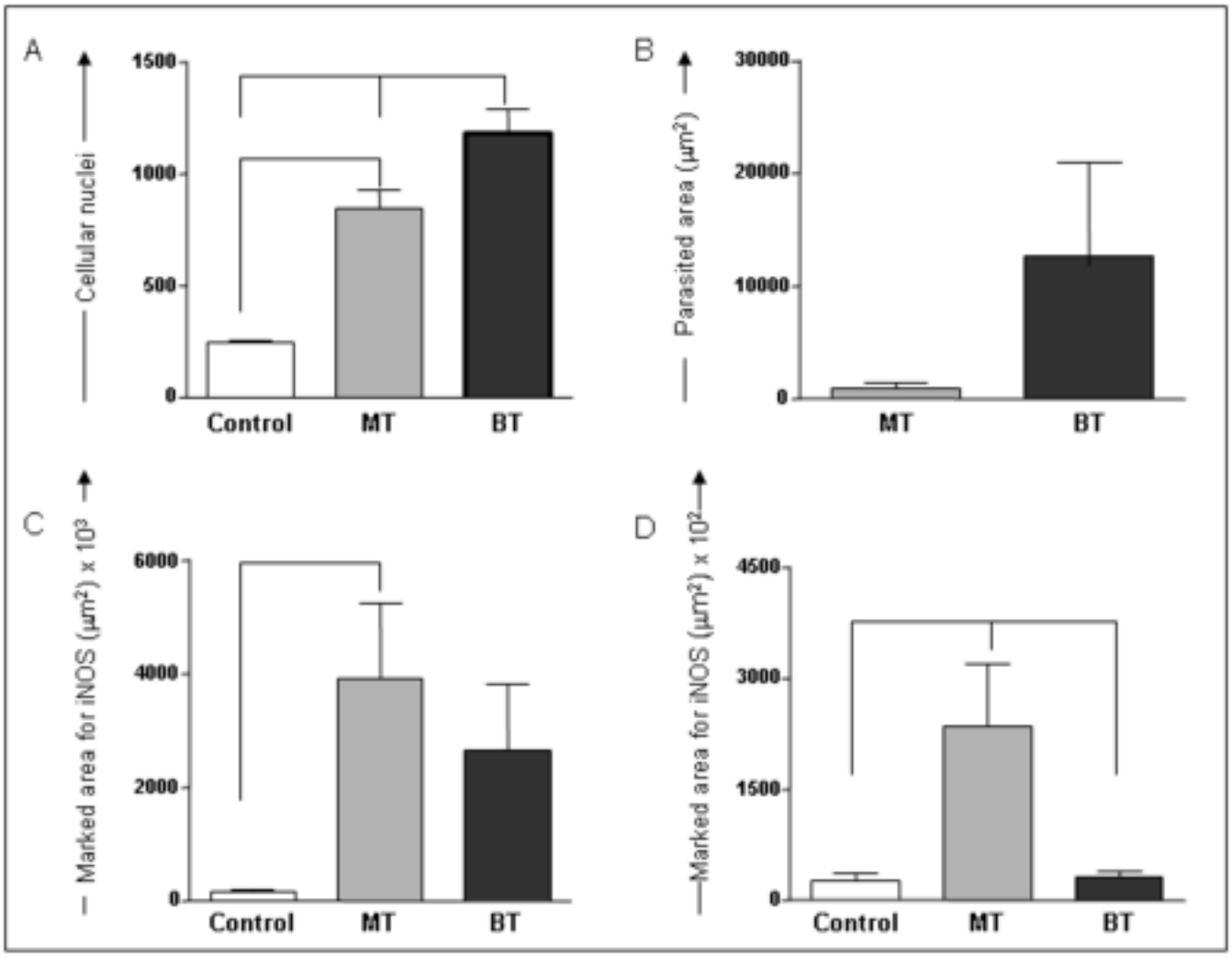

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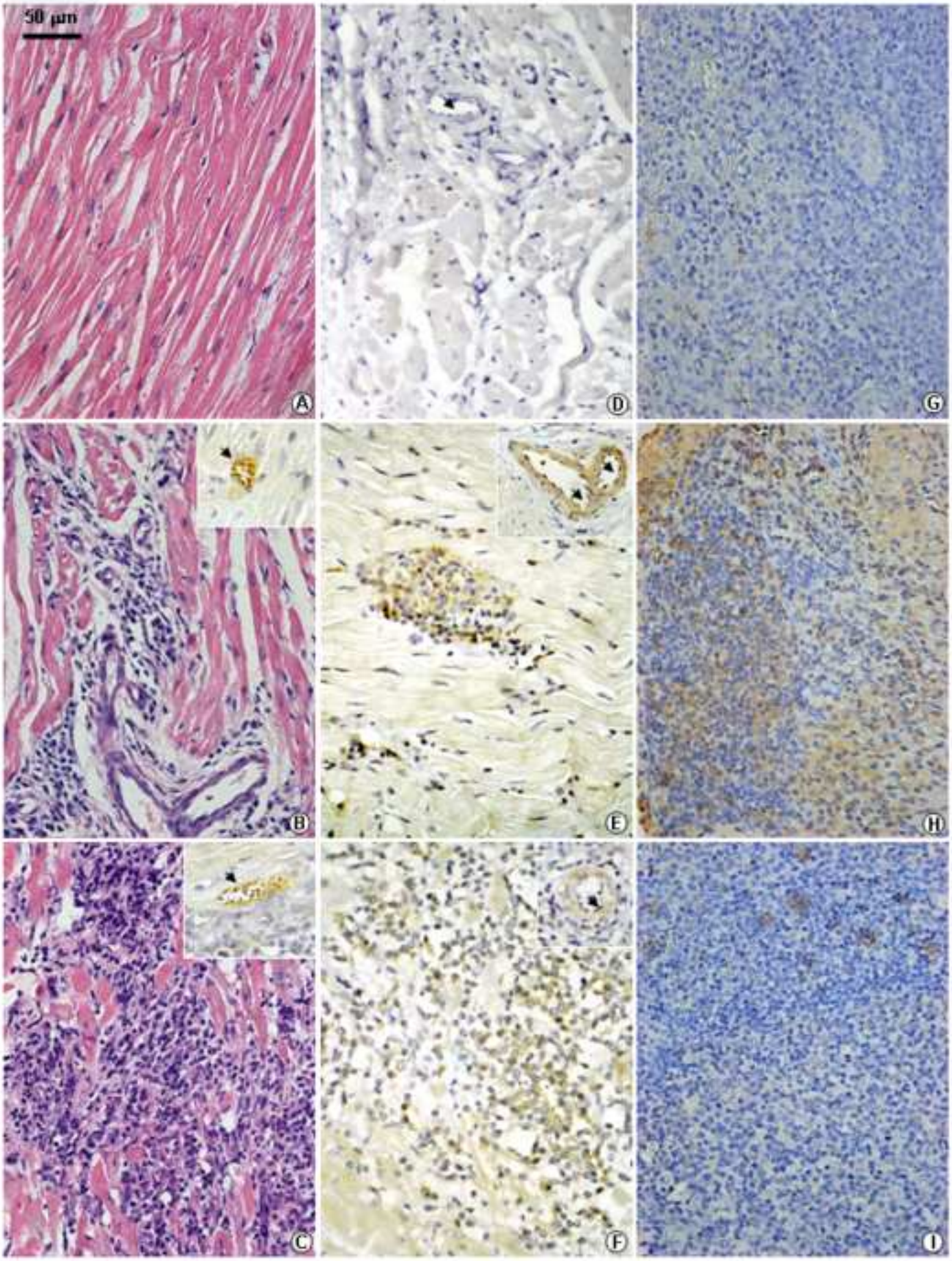

*** Reviewer Suggestions**

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