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RESEARCH ARTICLE

# Morphological alterations of Thymus during the early murine leishmaniasis

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**ABSTRACT**

Leishmaniasis is a neglected and endemic disease that affects poorest population mainly in developing countries. Thymus provides an essential complex environment for T cell maturation and differentiation during leishmania infection. The aim of this study was to investigate the pathological alterations of the Thymus during early *Leishmania amazonensis* murine infection. BALB/c mice were infected with $10^5$ amastigotes for 24 h, 3 days, 7 days, 15 days or 30 days. At different times of infection, the relative weight of the Thymus was obtained, and the Thymus cellularity was determined by counting total cells of one thymic lobe. The thymic lobe was, alternatively, processed for standard Haematoxylin and Eosin protocol. Our results suggest thymic alteration during the early days of BALB/c mice infection with *L. amazonensis.* The thymic hypertrophy was accompanied by histological alterations in Thymus architecture with thickening cortex at 3 days p.i. and loss of an evident delimitation between the cortex and medulla at 7 days p.i. when compared to the control mice. That is the first time that Thymus hypertrophy was observed during the early leishmaniasis. However, how it may contribute to infection susceptibility requires further investigation.

**Keywords:** Hypertrophy; thymus; leishmaniasis; histology thymic.

## INTRODUCTION

Leishmaniasis is an endemic zoonosis caused by several species of the genus *Leishmania* spp. (Protozoa, Kinetoplastida, Trypanosomatidae), a cell obligate parasite, transmitted through the bite of an infected sandfly, that can cause a range of symptoms from cutaneous to visceral lesions in humans (Oumeish, 1999; Gramiccia & Gradoni, 2005; Vijayakumar & Das, 2018). More than 1 million people are being victimized by leishmaniasis world-wide and reported fatalities is around 30,000 annually (Kamhawi, 2017). In fact, an interesting reviewing study addressed for interventions for old world leishmaniasis considers it as prominent among the global causes of death by infectious diseases (Heras-Mosteiro *et al*., 2017)

Clinical and experimental studies suggest that leishmaniasis is avaccine-preventable disease, the primary control measure is based on chemotherapy. Altough such treatments exist for leishmaniasis, they are considered inadequate due to aspects such as emergence of resistance, side effects including high toxicity, teratogenic effect, lack of efficacy, and high cost (Sundar & Jaya, 2010; Légaré & Ouellette, 2017; Zulfiqar *et al*., 2017). Thus, new studies specifically targeting the early stages of infection may improve our knowledge of the parasite-host relationship and are an attractive option for developing a novel treatment strategy for leishmaniasis.

Moreover, an effective immune response is required to support anti-leishmanial drugs as patients with immuno-deficiency may be particularly hard to cure (Monge-Maillo *et al*., 2014). In this scenario, the Thymus, a primary lymphoid organ of host may play a role by providing an essential complex environment for T cell maturation and differentiation during their migration within the cortical and medullary thymic compartments (Holländer *et al*., 2006; Kaphingst *et al*., 2010; Abramo *et al*., 2012). T cell maturation is orchestrated via interactions between T cells and mainly thymic epithelial cells, but also other stromal cells, such as dendritic cells, fibroblasts, and myeloid cells (Kondo *et al*., 2017).

Throughout leishmania human infection, T cells coordinate multiple aspects of adaptive immunity in both susceptible and resistant phenotypes exist within human populations (Sacks & Noben-Trauth, 2002). Clinical cutaneous disease ranges from a few lesions of spontaneous cure to diffuse external or internal disease, and there may be severe mucous membrane involvement. Spontaneously healing lesions are associated with positive antigen-specific T cell responsiveness, diffuse cutaneous and visceral disease with T cell non-responsiveness, and mucocutaneous disease with T cell hyperresponsiveness (Kaye & Scott, 2011). Several

studies have demonstrated that pathological changes of the Thymus are very frequently observed in patients during acute and chronic diseases. For instance, Thymic hyperplasia of lymphoproliferative origin was observed in 50–60% in the Thymus of Myasthenia Gravis patients (Cron *et al*., 2018).

On the other hand, acute thymic involution is usually caused by several infectious agents such as bacteria, viruses, fungi and parasites (Watson *et al*., 1984; Morrot *et al*., 2011; Liu *et al*., 2014; Alves da Costa *et al*., 2016). In fact, the thymic athrophy is a well-studied primary event that is associated with stress hormone dysregulation in an experimental model of Chagas disease (Lepletier *et al*., 2012). In this model, activation of the receptors for glucocorticoids and prolactin was found to be mutually antagonistic. The glucocorticoids may inhibit the proliferation of intrathymic double positive (DP) cells by promoting their apoptosis, while prolactin administration leads to the opposite consequences. These results suggest possible crosstalk between these two stress hormones during *T. cruzi*-induced acute thymic involution in mice (Lepletier *et al*., 2012). Recent study has indicated that protein malnutrition is considered a primary risk factor for the development of clinical visceral leishmaniasis and leishmania infection lead to lymphoid tissue disorganization, including changes in cellularity and lymphocyte subpopulations in the Thymus (Losada-Barragán *et al*., 2017). They also suggested that protein malnutrition induced drastic thymic atrophy with a severe hypocellularity and decrease in CD4+CD8+ DP thymocytes of animals infected with *Leishmania infantum.* On the other hand, the weight of Thymus of well-nourished animals increased 31% after 14 days of viscerotropic *L. infantum* infection. In this work, we addressed whether dermotropic *Leishmania amazonensis* infection is involved in the pathological alterations of the Thymus during early development of the lesion into murine model.

## MATERIALS AND METHODS

*Leishmania amazonensis* (MHOM/BR/73/M2269) amastigotes were obtained from footpad lesions of susceptible mice as previously described (Barbieri *et al*., 1993). Sixty female BALB/c mice (6 weeks old), maintained on controlled temperature (22 ± 1 °C) and ad libitum access to food and water, were subcutaneously infected in the right hind footpad with $10^5$ amastigotes for 24 h, 3 days, 7 days, 15 days and 30 days (6 animals per group) (Arrais-Silva *et al*., 2014). Control groups (6 animals per group) were composed of animals inoculated with PBS in the same way as infected groups. At designed time, the animals were euthanized and the relative weight of Thymus was analyzed (Manente *et al*., 2017). Then the thymic lobes were then separated for histological analysis or for evaluation of cellularity. Thymus cellularity was determined by maceration of one thymic lobe in saline solution and total cells was counted in a hemocytometer. The other thymic lobe was fixed by immersion in 4% paraformaldehyde in 0.1M PBS/0.1M sucrose for 6 h and processed for standard paraffin embedding. Tissue sections (4 µm) were stained with Haematoxylin and Eosin (H&E) and five different regions of cortex or medulla were selected at random and examined under an Eclipse E200-Nikon light microscope (Nikon, Japan).

The analytical results were subjected to ANOVA using BioStat 3.0 and variable means showing significant differences were compared using the Dunnett's multiple comparisons test. All statements of significance are based on the 0.05 level of probability. All protocols were performed in accordance with the guidelines of the Federal University of Mato Grosso Committee on the Use and Care of Animals (protocol number 23108.015339/14-1).

## RESULTS

All mice infected with dermotropic *Leishmania amazonensis* amastigotes presented high levels of parasite load, which progressively increased since parasite inoculation until the end of experimental analysis. The initial number of parasites ($10^5$ amastogotes) increased with time, and by 30 days, the parasite load was approximately $10^7$ amastigotes per lesion, 100 higher than the number of inoculated parasites.

Thymic hypertrophy, with pronounced high weight of the Thymus, was observed very early during the infection process from 3 days until 7 days post-infection (p.i.). This hypertrophy was limited, but detectable, and became to the control levels after 15 days p.i., coinciding with the establishment of the leishmaniotic lesion (Figure 1A). In order to investigate whether thymic hypertrophy was associated with the higher cell density, thymocytes were counted. The results suggest that the leishmania infection exhibits a greater than 300% increase in cell density during early infection with peak on day 3 p.i. (Figure 1B). It is interesting to note that cell density became to the control levels after 15 days p.i. until the end of experimental period (30 days p.i.).

Hypertrophy was accompanied by histological alterations in Thymus architecture with thickening cortex at 3 days p.i. and loss of an evident delimitation between the cortex and medulla at 5 and 7 days p.i. when compared to the control mice. (Figure 2A-2D). Furthermore, changes in the histological

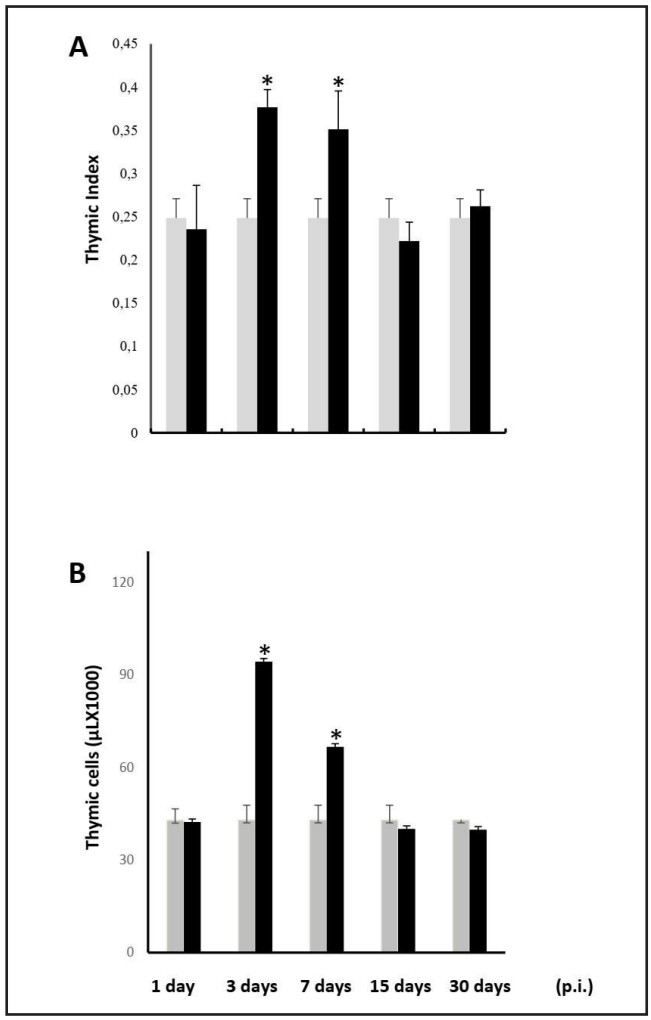


**Figure 1.** Thymic weight index (A) and thymic cell number (B) in *Leishmania* amazonensis-infected (■) and control (■) BALB/c mice. Data are expressed as mean and standard deviation of the mean (SD) (n = 8). * p<0.01.

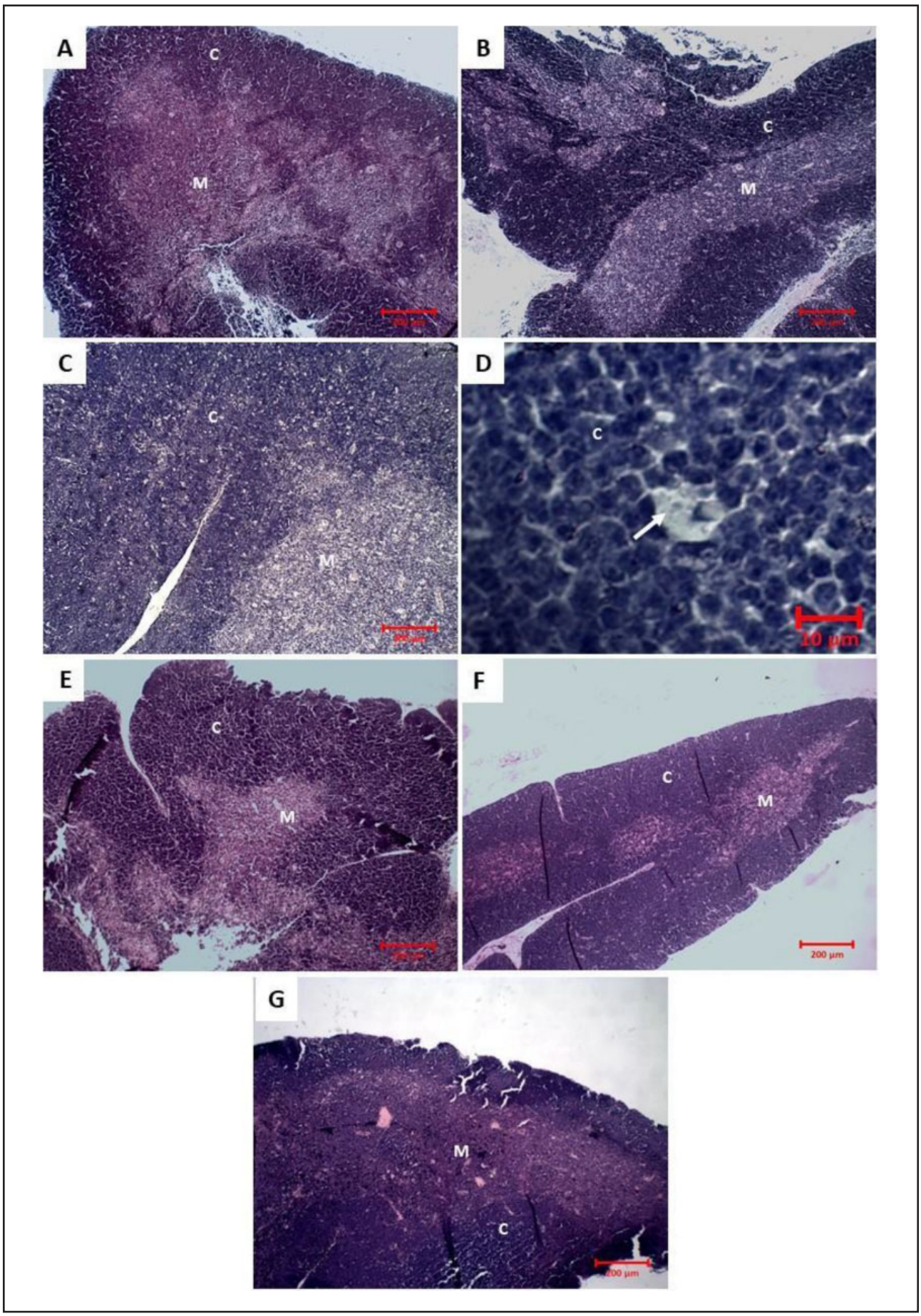


**Figure 2.** Photomicrograph of thymus sections from *Leishmania amazonensis*-infected and control mice. A) Control – Thymus with clear corticomedullary delimitation; B) 1 day p.i.; C) 3 days p.i. – Note loss of clear corticomedullary delimitation; D) 3 days p.i. (1000X); E) 7 days p.i.; F) 15 days p.i.; G) 30 days p.i.. Note C (cortical region) and M (medullar region).

pattern of the Thymus were detected at 30 days p.i. when no further alterations hypertrophy were detected (Figure 2E).

## DISCUSSION

We described for the first time thepathological alteration of Thymus during *Leishmania amazonensis* early infection, a dermotropic species. Our results have shown thymic hypertrophy that was accompanied by histological alterations in Thymus architecture with thickening cortex at 3 days p.i. and loss of an evident delimitation between the cortex and medulla at 7 days p.i. when compared to the control mice.

Two interesting studies had already addressed about pathological alteration of Thymus during viscerotropic *Leishmania infantum* experimental infection (Savino *et al*., 1989; Alves da Costa *et al*., 2016). These works have suggested that Thymus has been atrophied in infected malnourished animals and, on the other hand, its weight has been increased in well-nourished mice infected with *L. infantum*, in both cases the thymic alterations have been analyzed after 14 days of infection (Cuervo-Escobar *et al*., 2014; Losada-Barragán *et al*., 2017). In fact, thymic involution is broadly accepted and it is usually caused by several infectious agents such as bacteria, viruses, fungi and parasites (Savino, 2006). These previous studies have broken two important misconceptions that the Thymus is an immune-privileged site protected from infection and thymic function is only important during early life and dispensable after puberty (Arrais-Silva *et al*., 2014). Therefore, our results corroborate with the understanding that the Thymus is both a target for infection and a place where immune responses are recruited and may widely respond to different infection condition (Panesar, 2011; Reiley *et al*., 2012; Manley, 2013). Interestingly, infection-induced thymic atrophy often correlates with strain virulence and specific microbial factors directly promote thymocyte death. Hence, thymic atrophy during these infections process seems to be caused by decreasing cell number in Thymus tissue and it is detectable, for this reason, after several days of infection. Specifically, our result has shown an unusual hypertrophy occurring in the beginning (3 to 7 days post infection) of well-nourished BALB/c mice infection with *L. amazonensis* parasite. Losada-Barragan (2017) have already suggested thymic hypertrophy in malnourished mice after 14 days of infection with *L. infantum* parasites. However, they have not investigated the initial days after infection (Losada-Barragán *et al*., 2017).

Furthermore, it is well stablished that the Thymus is required for T cell maturation. This process (T cell maturation) may be altered by Thymus local infection and by the inflammatory mediators during systemic infection, which may disrupt the immune response in general (Chaudhry *et al*., 2016). Thus, resolving infection in the Thymus is important because chronic persistence of microbes impairs the differentiation of pathogen-specific T cells and decreases resistance to infection (Josefowicz *et al*., 2012). However, to clarify to relevance of our results, future issues should be addressed, as the parasite determination into Thymus, and the morphological pattern analysis.

## CONCLUSION

Our result corroborates the understanding that invading parasites disrupted the sterile tissue of the Thymus in the very earlier stages of infection, differently of previous studies had indicated. Moreover, the early thymic hypertrophy was associated with the higher cell density.

**Conflict of interest**

The authors declare that there is no conflict of interest.

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