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Thymic alterations induced by *Plasmodium berghei*: Expression of matrix metalloproteinases and their tissue inhibitors

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ABSTRACT

The thymus plays a crucial role in the generation of T-cells, and so our laboratory has been interested in the study of the intrathymic events that occur during infection diseases and may cause disruption in its functions. Previously, we showed that thymus from experimentally *Plasmodium berghei*-infected mice present histological alterations with high levels of apoptosis, changes in cell migration-related molecules, and premature egress of immature thymocytes to periphery. In addition, parasites were found inside the thymus. In this work we investigated alterations in the expression pattern and activity of matrix metalloproteinases MMP-2 and -9, and their tissue inhibitors, TIMP-1 and TIMP-2. Our results show enhanced expression and widespread distribution of these molecules in thymus from infected animals. Also, the presence of active MMP-2 was detected. These data are suggestive of MMPs and TIMPs importance in the earlier observed changes in the extracellular matrix during thymic alterations after *plasmodium* infection.

1. Introduction

The thymus, a central lymphoid organ, plays an important role in the generation of the T cell repertoire. The thymic microenvironment (composed by lymphoepithelial stroma and by the secretory products of the thymic epithelium) provides an appropriated milieu for the development and differentiation of immature T-lymphoid precursors into functionally mature T cells that will be able to carry out immune functions in the periphery. The successful development of mature T cells depends on the constant and organized migration of the immature T cell (or thymocytes while in the thymus) through different thymic niches. Such migration is essential for thymocyte receive signals (like chemokines, extracellular matrix (ECM) proteins, and their respective receptors) that lead these cells to proliferation, differentiation and generation of diversity [1–4]. So, it is plausible to suppose that alterations in the thymic microenvironment can influence the process of T cell maturation.

Since the thymus can be a target organ for several pathogens, like fungus, virus and parasites [5], our laboratory has been interested in the study of the intrathymic events that may cause a disruption of normal thymic functions [6,7]. Previously, we have reported that the thymus gland is also a target organ during the infection with *Plasmodium berghei*, the etiological agent of Malaria [8]. In addition to detecting the intrathymic presence of parasites in experimentally infected mice, we observed severe thymic alterations characterized by histological changes, high levels of apoptosis, altered expression of cytokines and ECM proteins that, conjunctively, promotes an increase of thymocyte migratory activity with consequent premature egress of immature cells to the peripheral lymphoid organs [9,10].

ECM composition and turnover are regulated by a variety of enzymes among which matrix metalloproteinases (MMPs) seem to be the most important [11]. MMPs are classified into different families [12–17] one of which is the family of gelatinases that includes MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B). Literature has shown that the expression of MMP-9, for example, is important for the remodeling of the ECM and the migration of cells [18]. As both of these functions are essential components of T-cell maturation it would be reasonable to suppose that MMPs have a crucial role during the intrathymic thymocyte migration.

Although information regarding MMPs, as well as their natural tissue inhibitors (TIMPs), during the events of thymocyte migration is quite limited, it is plausible to suppose that ECM in thymus are also controlled by these proteins. Indeed, evidences have showed that thymic microenviromental cells produce MMP-9,

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the largest and most complex member of MMPs family [19], and a potential regulatory function of MMPs on intrathymic, ECM-mediated interactions has recently been suggested [11,20]. However, roles of these MMPs and TIMPS on thymic atrophy observed during infection have not been characterized clearly.

Considering that MMPs must also play a role in the intrathymic ECM control, in this study we investigated changes in the expression pattern and activity of matrix metalloproteinases MMP-2 and MMP-9, as well as in their natural tissue inhibitors, TIMP-1 and TIMP-2, with the aim to better comprehend the events involved in the thymic alterations observed during *plasmodium* infection.

2. Material and methods

2.1. Animals

Specific pathogen free BALB/c male mice (aged 6–8 weeks), obtained from Centro Multi-Institucional de Bioterismo (CEMIB) were used. They were kept in groups of five in transparent acrylic plastic isolators under aseptic conditions on a 12 h light/dark cycle throughout the study. Sterile water and food were provided *ad libitum*. All procedures were carried out in accordance with the guidelines proposed by the Brazilian Council on Animal Care (COBEA) and approved by the animal care and use committee of the State University of Campinas (protocol no. 1789-1).

2.2. Infection of mice

Groups of five to ten animals were intraperitoneally infected with 5×10^6 *Plasmodium berghei* NK65 parasitized red blood cells obtained from a source mouse or with saline (control groups). Parasitemia and mortality were assessed every other day by the examination of Giemsa-stained thin blood smears. At days 5, 10 and 14 following infection, mice were sacrificed and their thymuses were collected for analyses.

2.3. Thymic index

The gross weight of each mouse was recorded on 5, 10 and 14 days of infection and compared with pre-infection weight. The animals were sacrificed with xylazine/ketamine (100 mg/kg hypochlorite 5 mg/kg of ketamine hydrochloride and xylazine) inoculation. After that, the thymus glands were aseptically removed and weighed. The thymic index was calculated as: (organ weight (g)/body weight (g)) $\times$ 100.

2.4. RT-qPCR/real time quantitative PCR

Total RNA was extracted from thymus using Illustra RNAspin Mini (GE Healthcare, Amersham, UK) in accordance with the manufacturer's instructions. RNA yield was evaluated spectrophotometrically (A260/A280) and RNA aliquots were stored at −80 °C until use. Generation of cDNA was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction was performed in an ABI Prism 7300 equipment and the reactions were carried out in 20 μL volume composed by Master Mix (Applied Biosystems, Foster City, CA, USA), DEPC water and specific inventoried assays of primers with TaqMan probes, as follow: 18s: HS Hs99999901_s1, MMP-2: Mm01253624_m1, MMP-9: Mm00600164_g1, TIMP-1: Mm01341361_m1 and TIMP-2 Mm00441825_m1 obtained from Applied Biosystems. Expression levels of genes in control and infected mice were represented as a relative copy numbers by using the delta threshold cycle method ($2-\Delta\Delta Ct$) [21].

2.5. Gelatin zymography

Thymus fragments (n = 5 in control group and n = 10 in infected mice) were triturated in a solution of 50 mM Tris–HCl, pH 7.4, 0.2 M NaCl, 0.1% Triton 100, 10 mM CaCl2 and 1% protease inhibitor cocktail (Sigma) for protein extraction. Total protein was quantified according to the method of Bradford [22] using bovine serum albumin (Sigma) as a standard. The zymography assays were performed on 7.5% polyacrylamide electrophoresis gels containing 0.1% gelatin and using 20 μg of protein per sample. After electrophoresis, the gels were washed with 2.5% Triton 100 at room temperature and incubated overnight in a solution of 50 mM Tris–HCl, pH 7.4, 0.1 M NaCl and 0.03% sodium azide at 37 °C. After that the gels were stained with Coomassie Brilliant Blue. The protein bands corresponding to gelatinolytic activity were observed after washing the gels with a solution containing 30% methanol and 10% acetic acid. The gel was evaluated by band densitometry using the SCION IMAGE program. Each sample was analyzed individually and the experiments were repeated three times.

2.6. Immunohistochemical analysis

In situ expression of Cytokeratin-5, Cytokeratin-8, MMP-2, MMP-9, TIMP-1, and TIMP-2 in thymus from infected and control animals were performed following protocol for immunohistochemistry stain. At sacrifice, thymuses were obtained and fixed in paraphormaldehyde in 0, 2 M Phosphate Buffer for 24 h. So, they pass through a dehydratation and parafin bath protocol composed by incubation of tissue in crescent concentration of ethanol, xilol and parafin. For last, slices of 5 um were obtained for immunohistochemistry stain procedure. Then, thymus sections were deparaffinized with xylene, hydrated in an ethanol gradient and water before stain reaction. Right after, was performed an incubation with 3% H_2O_2 in absolute methanol (v/v) to block endogenous peroxidase activity. From here all procedures were done according to ImmunoCruzTM rabbit ABC Staining System (sc-2018) or ImmunoCruzTM goat ABC Staining System (sc-2023) datasheet. Briefly, all sections were incubated with specific serum to avoid secondary antibody nonspecific binding for 1 h at 4 °C, and then they were incubated with specific primary antibodies to mouse proteins for 16 h at 4 °C. Primary antibodies were purchased from Santa Cruz (MMP-2, MMP-9, TIMP-1 and TIMP-2) or AbCam (CK5 and CK8). After washing, sections were overlaid for 1 h with a specific secondary antibody biotin-conjugated. This was followed by incubation with AB enzyme reagent to amplification of signal reaction. Bound antibodies was detected by reactivity with 3, 3'-diaminobenzidine plus H_2O_2. After tap washing, the slides were counterstained by Harris Haematoxilin and mounted with Permount. For immunohistochemistry controls primary antibodies were omitted from the staining procedure and were negative for any reactivity.

2.7. Statistical analysis

Statistical analyses were determined using unpaired t test and Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as mean ± SD, and p values equal or lower than 0.05 were considered to be statistically significant.

3. Results

Parasitemia and mortality rates in the experimental group infected with *Plasmodium berghei* NK65 parasitized erythrocytes

followed a regular time course, showing a peak parasitemia at 15 days of infection and 100% mortality at 16 days post-infection (Fig. 1).

A severe decrease of the thymus relative weight in infected group was observed when compared to the control group (Fig. 2A). This atrophy was also accompanied by changes in the organ histological pattern that shows constriction of the cortical layer, more diffuse medullar region, and decreased cellularity of cortex and medulla regions as depicted by immunolabeling for cytokeratin-8 and cytokeratin-5, respectively (Fig. 2B).

Fig. 3 shows the mRNA expression levels of metalloproteinases as well as their natural tissue inhibitors during thymic atrophy induced by *Plasmodium berghei* NK65. An increased gene expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 can be observed in infected animals at 10 days post-infection. At 14 days after infection, however, the gene expression of these molecules is similar to that observed in the beginning of infection (at 5 days of infection).

Immunohistochemical localization and expression intensity of the matrix metalloproteinases MMP-2 and MMP-9 and their tissue inhibitors, TIMP-1 and TIMP-2, within the thymus during *plasmodium* infection was performed.

It can be noted that at 5 days of infection MMP-2 and MMP-9 are more prevalent in the medullar region of the organ, but they acquired a widespread distribution throughout the thymic tissue

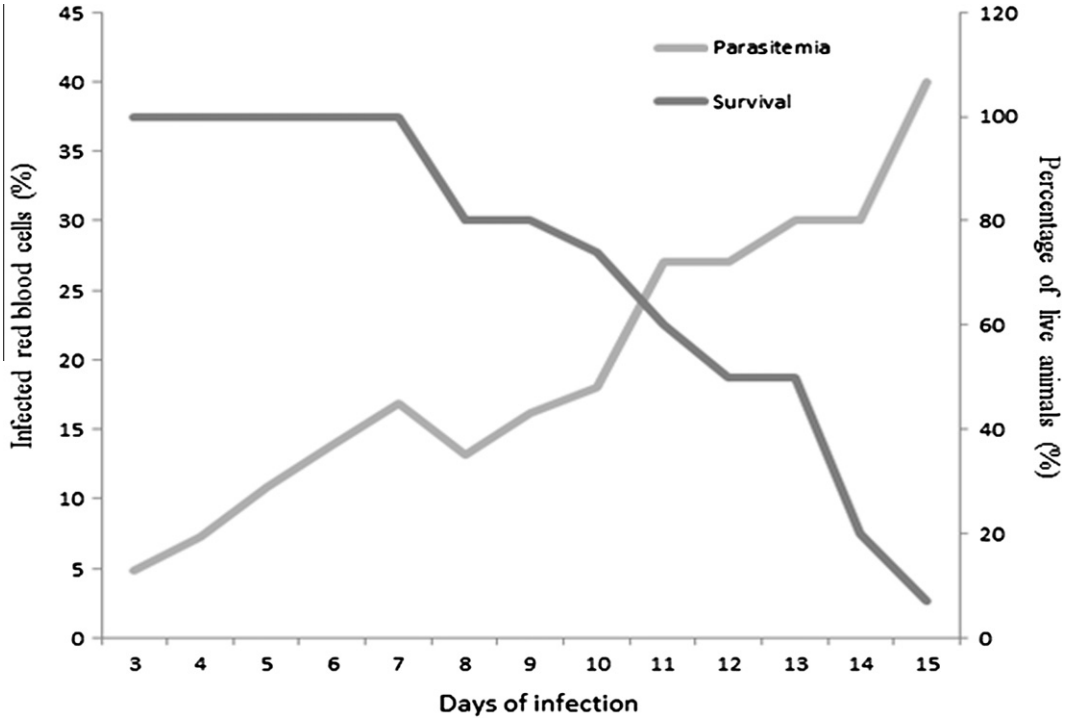

Fig. 1. Parasitemia and mortality rates in mice infected with 5×10^6 parasitized RBCs. At 15 days of infection, animals presented the peak of parasitemia and 100% were dead.

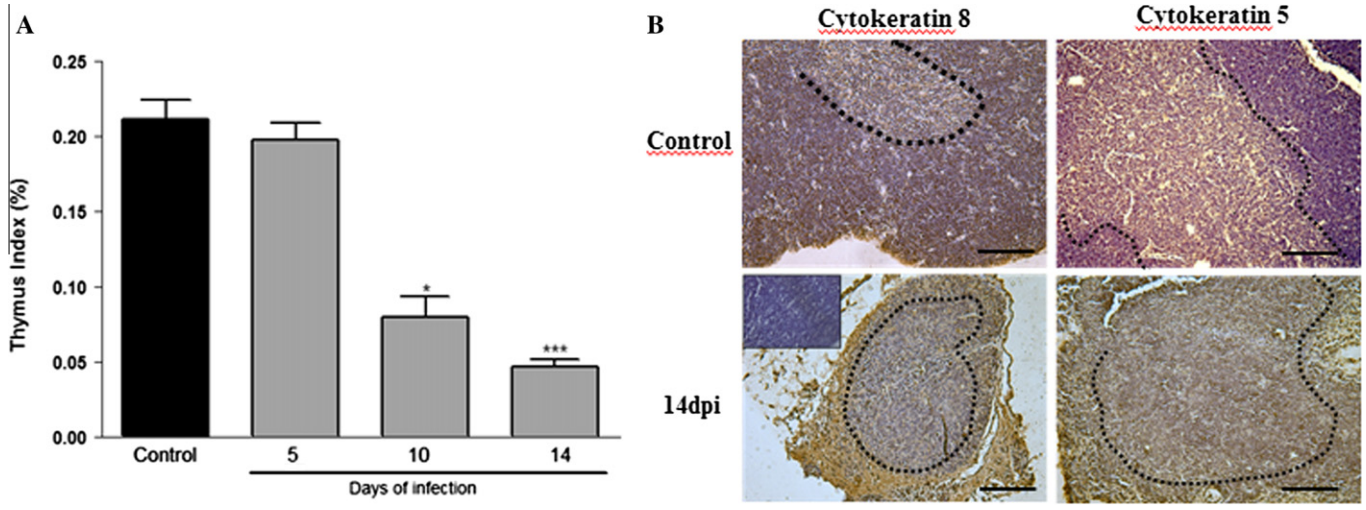

Fig. 2. Thymic alterations in *Plasmodium berghei*-infected mice. (A): a severe decrease of the thymus relative weight is observed in infected group. Results are expressed as mean ± standard error (SE) for at least five animals; $^{*}p \leqslant 0.01$; $^{***}p \leqslant 0.001$. The control bar is representative since the data were similar at each time point. (B): constriction of the cortical layer, more diffuse medullar region, and intense reduction of cellularity in both cortical (cytokeratin-8) and medullar (cytokeratin-5) thymic regions was observed at 14 days of infection. The figures are representative of at least three experiments performed on different experimental days. Insert: negative control. Bar: 10 μm.

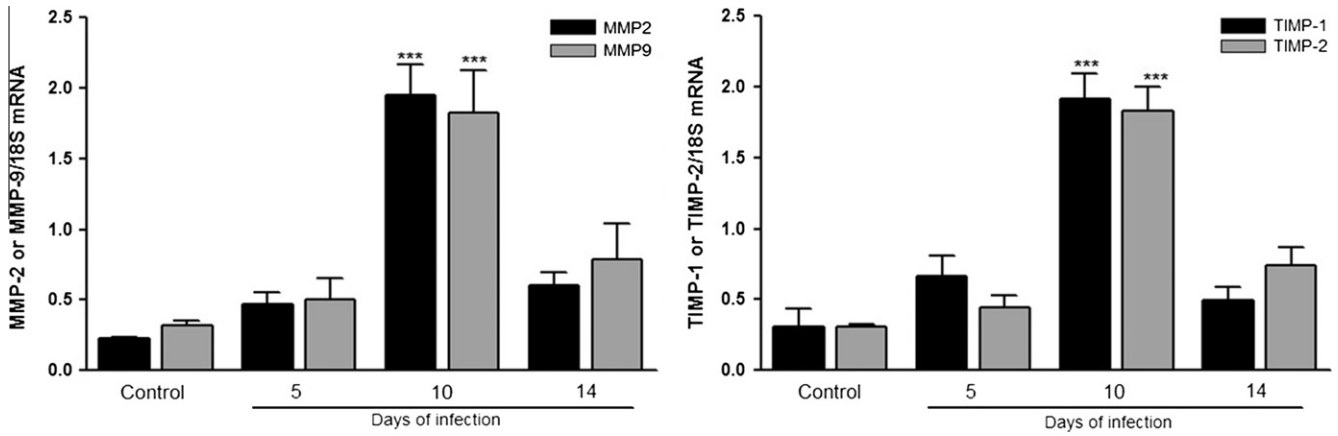

Fig. 3. Relative mRNA expression levels of MMPs and TIMPs during thymic atrophy induced by *Plasmodium berghei* NK65. At 10 days after infection, an intense increase of specific messenger RNA for MMP2 and MMP9, as well as for TIMP1 and TIMP2, was observed in the thymus from infected mice. Results are expressed as mean ± SE for at least five animals. ($^{***}p \leqslant 0.0001$). The control bars are representative since the data were similar at each time point.

as the disease progresses (Fig. 4, upper panel). The expression intensity of MMP-2 and MMP-9 is markedly increased at 14 days after infection when compared to the control group (Fig. 4, bottom panel).

Immunohistochemical results show that in infected groups the expression of TIMP1 and TIMP2 is mild and restrict to the endothelia at the beginning of infection, but intense and widespread at 14 days of infection (Fig. 5, upper panel). As observed for metallo-

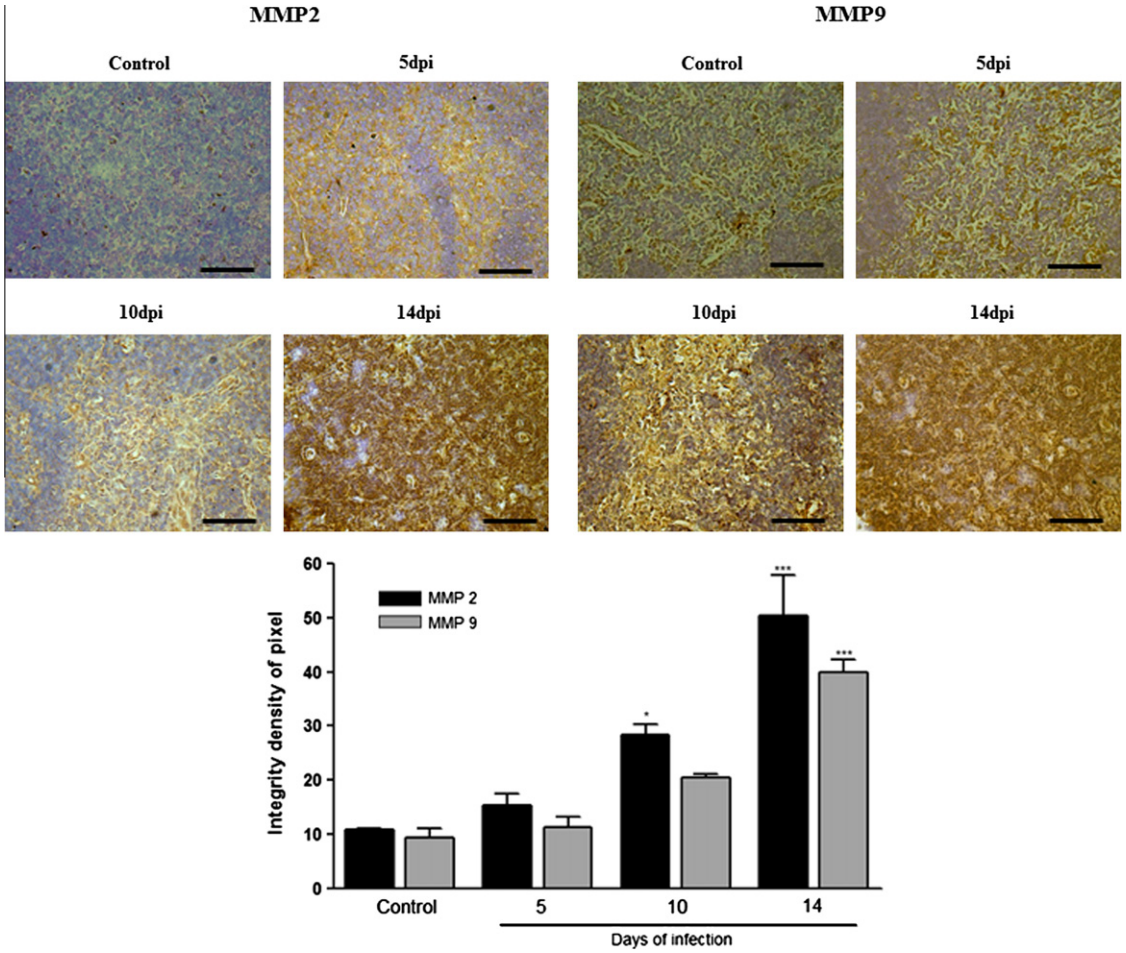

Fig. 4. Immunohistochemical localization (upper panel) and expression intensity (bottom panel) of metalloproteinases, MMP-2 and MMP-9, during thymic atrophy induced by *Plasmodium berghei* NK65. Upper panel: Note that MMP-2 and MMP-9 are more prevalent in the medullar region of the organ, but they acquired a widespread distribution throughout the thymic tissue as the disease progresses. Bar: 5 μm. Bottom panel: expression intensity of MMP-2 and MMP-9 was higher at 14 days after infection when compared to the control group. ($^{*}p \leqslant 0.01$; $p^{***} \leqslant 0.001$). The control bars are representative since the data were similar at each time.

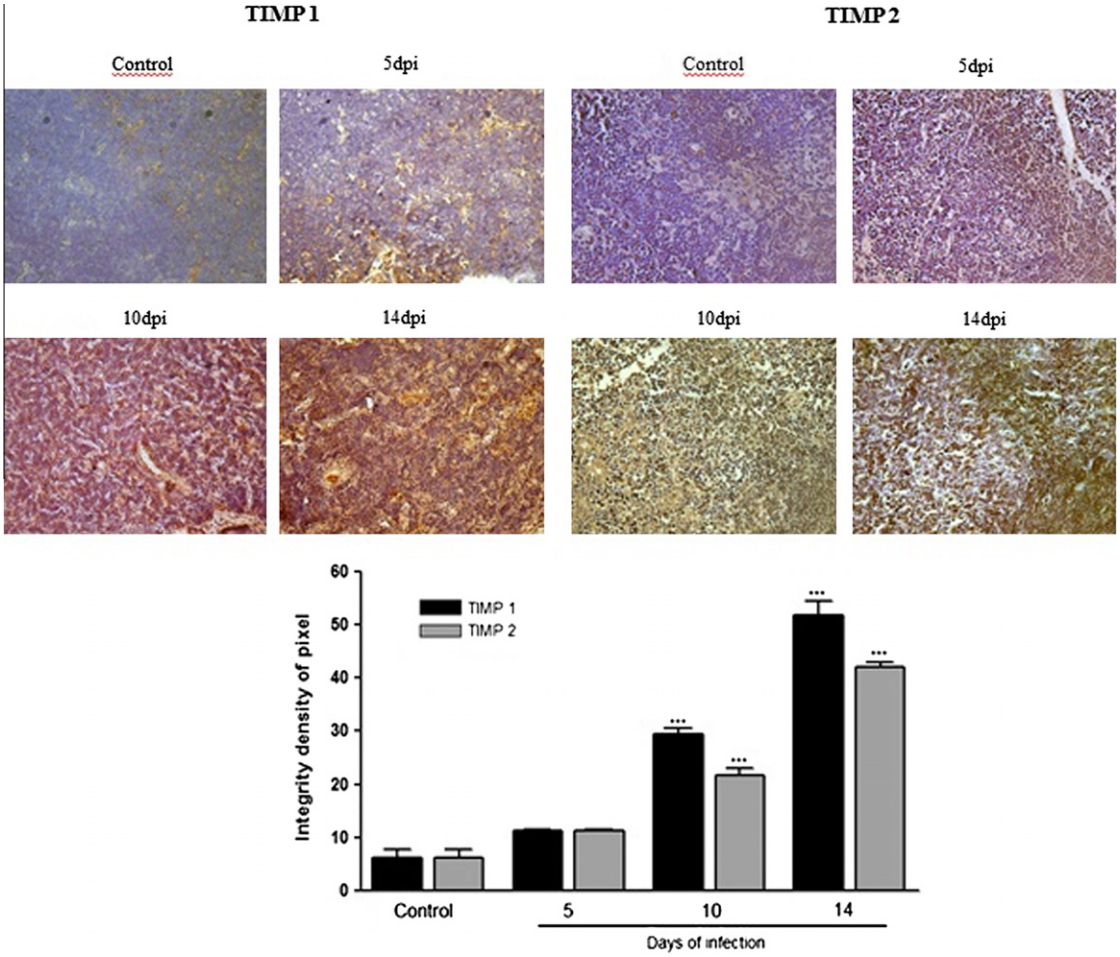

Fig. 5. Immunohistochemical localization (upper panel) and expression intensity (bottom panel) of natural tissue inhibitors of metalloproteinases, TIMP-1 and TIMP-2, during thymic atrophy induced by *Plasmodium berghei* NK65. Upper panel: Note that the expression of TIMP-1 and TIMP-2 is mild and restrict to the endothelia at the beginning of infection, but intense and widespread at 14 days of infection. Bar: 5 μm. Bottom panel: expression intensity of TIMP-1 and TIMP-2 was higher at 14 days after infection when compared to the control group. ($^{***}p \leqslant 0.001$). The control bars are representative since the data were similar at each time.

proteinases, expression intensity of TIMP-1 and TIMP-2 is higher at 14 days post-infection (Fig. 5, bottom panel).

Gelatinolytic activities with various molecular masses were detected in the thymus extracts (Fig. 6A). The 62, 68, and 72 kDa bands correspond, respectively, to the active, intermediate, and precursor states of MMP-2. The bands for the latent and intermediate states are present in both infected and non-infected groups, while the 62 kDa band is observed only in the infected group at 14 days of infection. The presence of 92 kDa band corresponds to the precursor state of MMP-9, and is present in both infected and non-infected groups. It may be also noted a 130 kDa band that is suggestive of a complex of metalloproteinases, which is seen only at 14 days after infection. Also, the densitometric analysis of the bands shows that latent MMP-2 increases from the 5th day of infection and peaks at 14 days after infection, and its active form can be observed only at 14 days after infection. On the other hand, the MMP-9 could be observed only in its precursor form with significant expression at 14 days of infection (Fig. 6B).

4. Discussion

The thymus is the central lymphoid organ where bone marrow-derived lymphocyte precursors mature into functional and immunocompetent T-cells. In this context, a functionally competent thymic microenvironment is essential for stromal cells to provide the appropriated signals to T-cell precursors (or thymocytes) that lead to proliferation, differentiation and generation of diversity [3,23].

There is now accumulating evidence that thymus can be severely affected by many different pathogens and that thymic involution is frequently found during infectious diseases [5]. Our laboratory has also reported that the thymus is a target organ during *Plasmodium berghei* infection. Besides the intrathymic presence of parasites [6], it was observed profound thymic atrophy accompanied by severe structural disorganization, characterized by loss of cortico-medullary delimitation, depletion of double-positive thymocytes, and alterations in the expression of ECM elements and chemokines, in such a way that thymocyte migration inside the thymus is severely compromised [10].

Since MMPs are collectively implicated in the degradation and remodeling of ECM and vascularization, we decided to investigate if MMP-2 and MMP-9, as well as their natural tissue inhibitors TIMP1 and TIMP2, could also play a role in the thymic atrophy observed during *Plasmodium berghei* infection.

Data presented here show enhanced MMP-2, MMP-9, TIMP-1 and TIMP-2 gene expression in the thymus from *Plasmodium berghei*-infected animals, at 10 days after infection. At 14 days after infection, by immunohistochemical technique, we detected the presence of these proteases in thymic tissue. It is interesting to note that as the disease progresses the distribution of these molecules is extended to the whole organ. Furthermore, gelatin zymography test performed in thymic tissue extracts has demonstrated

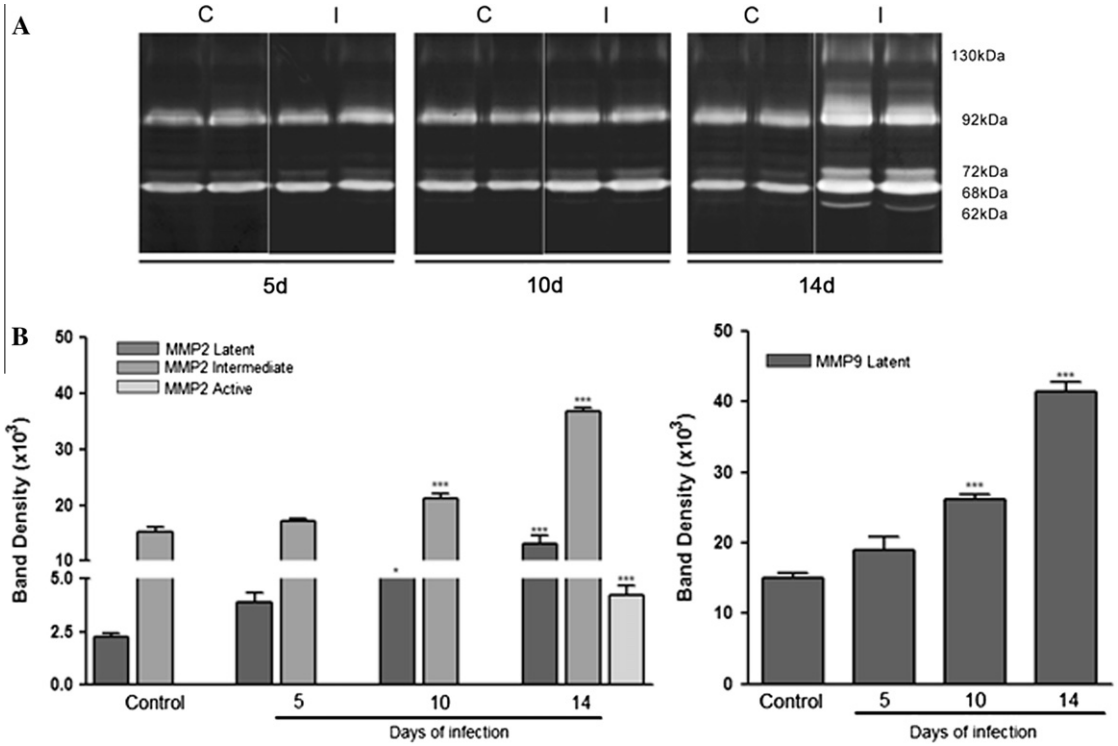

Fig. 6. (A): Representative gelatin zymography of MMP-2 and MMP-9 in thymus from non-infected (C) and *P. berghei* NK65-infected mice (I) after 5, 10 and 14 days of inoculation. Gelatinase activities at 62, 68, and 72 kDa bands correspond, respectively, to the molecular weights of the active, intermediate, and precursor states of MMP-2, and gelatinase activity at 92 kDa corresponds to the molecular weight of the precursor MMP-9. (B): Densitometric analysis of (A) shows that precursor MMP-2 increases from the 5th day of infection and peaks at 14 days after infection, and its active form can be observed only at 14 days after infection. MMP-9 could be observed only in its precursor form with significant expression at 14 days of infection. ($^{*}p \leqslant 0.05$; $^{***}p \leqslant 0.0001$). The control bar is representative since the data were similar at each time point.

that MMP-2 (gelatinase A) is present in its precursor (72 kDa) and active forms (68 and 62 kDa). These results are suggestive of MMP-2 importance in the earlier observed changes in the ECM during thymic atrophy after *plasmodium* infection. On the other hand, the predominance of the band at 92 rather than 82 kDa indicated that MMP-9 (gelatinase B) is primarily inactive. However, activation of MMP-9 requires a complex network of mechanisms involving other matrix metalloproteinases [24,25].The most likely activator of MMP-9 is thought to be MMP-3 [26] but this protein was not evaluated in this study. So, more studies are necessary to investigate the importance of MMP-9 expression changes during the course of this infection.

Although little data exist on the MMPs and TIMPs activities in the thymus, the presence of MMPs in different pathophysiology has already been reported [12,19,27], Kivelä-Rajamäki1 et al. [28] and Pereira et al. [29] observed an increased expression of MMP-9 by thymic stromal cells during the development of thymic lymphoma.

The presence of high levels of TIMPs in the atrophic thymic tissue is intriguing since they are kinetic inhibitors of MMP enzymatic activity and thus should contain the extensive tissue degradation observed. However, literature has shown that in, some cases, they are involved in the activation of MMPs from their inactive zymogen form to their active form [30,31]. On the other hand, it has also been related that proinflammatory cytokines, such as Tumor Necrosis Factor-alpha (TNF-α), are able of inhibiting the activity of TIMPs [32,33]. In fact, previous studies from our group have shown that TNF-α is increased at 10 days after infection, and this could explain the presence of TIMPs and their lack of action to inhibit MMPs.

A possible limitation of our study could be due to the death of animals at 15 days after infection. In order to verify MMPs and TIMPs activities after the parasitemia peak, experiments, using mice treated with cloroquine, are being conducted now in our laboratory. On the other hand, it is also important to note that literature shows MMPs as essential molecules for cell migration into the tissues [34–37]. In this sense, it would be very interesting to investigate MMPs and TIMPs activities at the beginning of the infection to examine their possible involvement during intrathymic thymocyte migration.

5. Conclusions

The results of this work shown a progressive increase of MMPs and TIMPs produced in the thymus. At present, the activation of MMP-2 appeared to be linked to the intense degradation of thymic extracellular matrix (ECM) observed during plasmodium infection. We speculate that such alterations can result in loss of thymocytes by apoptosis and/or in a premature migration of immature T cells to the periphery, and both of these events could influence the host immune response to the infection. It is therefore clear that the proteolytic process occurring in thymic atrophy during plasmodium infection require further study to clarify the significance of MMPs and TIMPs in both physiological and pathological conditions.

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