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Effects of *Plasmodium berghei* on thymus: High levels of apoptosis and premature egress of $CD4^+CD8^+$ thymocytes in experimentally infected mice

Carolina Francelin[a], Luciana Campos Paulino[b], Jacy Gameiro[c], Liana Verinaud[a,*]

[a] Department of Anatomy, Cell Biology and Physiology, Biology Institute, State University of Campinas - UNICAMP, Barão Geraldo, Campinas CEP: 13083-970, São Paulo, Brazil
[b] Center of Natural and Human Sciences, Federal University of ABC-UFABC, São Paulo, Brazil
[c] Department of Parasitology, Microbiology and Immunology, Federal University of Juiz de Fora - UFJF, Juiz de Fora, Minas Gerais, Brazil

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ABSTRACT

We have previously showed alterations in the thymus during experimental infection with *Plasmodium berghei*, the causative agent of Malaria. Such alterations comprised histological changes with loss of delimitation between cortical and medullar regions, a profound atrophy with depletion of $CD4^+CD8^+$ double-positive (DP) thymocytes, and severe changes in the expression of cell migration-related molecules, belonging to the extracellular matrix and chemokine protein families. Taken together, these considerations prompted us to evaluate if the acute thymic atrophy observed during *Plasmodium* infection was correlated with increased apoptotic levels of thymocytes or with their premature emigration to the periphery. Our results confirmed that the marked reduction of the thymus weight in infected animals was accompanied by histological alterations, which included a very large number of cells showing nuclear condensation and karyorrhectic changes surrounded by histiocytes suggesting increased levels of apoptosis. This was confirmed by immunohistochemistry and flow cytometry techniques. In order to verify if an accelerated emigration of thymic cells to the peripheral lymphoid organs was also occurring we analyzed the spleen and mesenteric lymph nodes from control and infected mice. No significant differences were found in the spleen, but were seen after 14 days of infection between control and infected mice in the mesenteric lymph nodes. The main alteration was the presence of double negative ($CD4^-CD8^-$) and double positive ($CD4^+CD8^+$) cells. We concluded that both apoptosis of thymocytes and premature egress of immature cells take place during infection. Additional studies will be necessary to verify how such alterations might influence the systemic immune response to the parasite.

Introduction

The thymus plays a primary role in supplying immunologically competent T cells to the periphery by providing a microenvironment for differentiation and selection of T lymphocytes (or thymocytes, while in the thymus) from lymphoid progenitors. This microenvironment is mainly composed by a three-dimensionally (3D)-oriented network of epithelial cells, macrophages, dendritic cells, fibroblasts and matrix molecules that provide mechanical support and molecular stimuli for thymocyte differentiation, selection of double-positive (DP) cells, and functional maturation of newly generated T cells (Takahama 2006; Ciofani and Zúñiga-Pflücker 2007; Petrie and Zúñiga-Pflücker 2007). The successful development of mature T cells depends on the constant migration of thymocytes through this thymic stromal compartment. So, in the absence of a regularly structured and composed *thymic microenvironment*, the essential functions of the thymus are severely altered and frequently preclude normal thymocyte development to occur (Lind et al. 2001; Petrie 2002; van Ewijk et al. 1999). Although its function declines with age, the thymus remains indispensable to T-cell-repertoire reconstitution, which ensures immune reactions in various situations until late adulthood (Shanker 2004).

The thymus has long been known to be an organ that is vulnerable to atrophy when exposed to a variety of stimuli, including many pathogens, such as *Trypanosoma cruzi* (Savino et al. 1989; Leite-de-Moraes et al. 1992; Cotta-de-Almeida et al. 2003), Human Immunodeficiency Virus (Savino et al. 1986; Sodora et al. 2002) and *Paracoccidioides brasiliensis* (Brito et al. 2003; Souto et al. 2003). All these works show that acute infection of mice is followed by strong thymic atrophy and deep disorganization of thymic architecture (Savino 2006).

Abbreviations: DP, double positive; DN, double negative; TUNEL, terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate biotin nick end labeling; ABI, apoptotic body index; PI, propidium iodide; PS, phosphatidylserine; MLN, mesenteric lymph nodes; FITC, fluorescein isothiocyanate; TRECs, T-cell receptor excision circles; IFN-γ, interferon gamma; TNF, tumor necrosis factor; LT, lymphotoxin; Tg, transgenic.

* Corresponding author. Tel.: +55 19 35216255; fax: +55 19 35216276.
E-mail address: verinaud@unicamp.br (L. Verinaud).

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Previously, using a lethal murine malaria model of *Plasmodium berghei*, we have also shown profound histological alterations and severe thymic atrophy with loss of DP cells. In addition, the presence of parasites inside the thymus was detected for the first time (Andrade et al. 2008). In another study that used the same model, we have demonstrated changes in the expression of extracellular matrix elements and chemokine protein families in *Plasmodium*-infected animals (Gameiro et al. 2009). As these molecules play a critical role in T cell maturation because they are involved in the dynamic thymocyte migration within the thymus, it is reasonable to suppose that such events may influence the process of intrathymic T cell differentiation, impairing the normal development of T cells. As a consequence, an altered peripheral immune response to the parasite can be expected due to a severe reduction of lymphocytes and/or because of the presence of cells without fully functional immune competence in blood and peripheral lymphoid tissues.

In the present study, we evaluate whether the acute thymic atrophy observed during *Plasmodium* infection correlates with increased apoptotic levels of thymocytes or with their premature egress from the thymus to the periphery.

Materials and methods

Animals

Specific pathogen-free BALB/c male mice, 6–8 week-old, were purchased from CEMIB/UNICAMP (Campinas, São Paulo, Brazil) and housed in microisolator cages with free access to water and food. All protocols were conducted in conformity with the guidelines of the State University of Campinas' Committee on the Use and Care of Animals.

Infection of mice

Groups of five animals were intraperitoneally infected with 10^6 parasitized red blood cells obtained from a source mouse or with saline (control groups). Parasitemia was assessed daily by counting the number of infected erythrocytes in Giemsa-stained thin blood films. At days 7 and 14 following infection, mice were sacrificed and their thymuses and mesenteric lymph nodes collected and analyzed by histological methods, cytofluorometry and/or immunohistochemistry.

Thymus and mesenteric lymph node histology

For microscopic histological evaluation, organs were collected and fixed in a solution 4% paraformaldehyde for 12 h at 4 °C. The specimens were submitted to diafanization with xylene, dehydrated by graded ethanol, embedded in paraffin and cut in 5-μm-thick sections. Histologic changes were evaluated on sections stained with hematoxylin and eosin (H&E).

Quantification of relative thymus weight

For the analysis of relative thymus weight, body weight of the respective mice was measured before thymi of control and infected mice were isolated by microsurgery. The thymus gland was weighted and the thymic index (%) was calculated as: 100 × organ weight (g)/body weight (g).

TUNEL staining of apoptotic cells

The distribution of apoptotic thymocytes in tissue sections of thymi from control and infected mice was determined by the terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) method. In brief, 5 μm sections were de-waxed, and rehydrated specimens were incubated in proteinase K (40 μg/ml) for 1 h at 37 °C, and were then treated with 3% H_2O_2 in methanol for 30 min at room temperature. After, the specimens were incubated with TdT and digoxigenin (DIG)-dUTP for 1 h at 37 °C. Anti-DIG peroxidase was added to the slides, followed by incubation for 30 min at 37 °C. Slides were stained with diaminobenzine for 10 min and counterstained with hematoxylin. Controls for the TUNEL procedure were handled in the same manner as the test samples except that the TdT enzyme was omitted in the nucleotide mixture and was replaced with dH_2O. Apoptotic cells were identified by TUNEL in conjunction with characteristic morphological changes, such as cell shrinkage, membrane blebbing, and chromatin condensation, to distinguish apoptotic cells and apoptotic bodies from necrotic cells. The latter were not considered apoptotic cells. In total, 500 cells were counted for each specimen by using an ocular grid. The apoptotic body index (ABI) was performed by a single observer (C.F.A.) blinded to the animal status, and defined as follows: apoptotic body index (%) = 100 × apoptotic cells/total cells.

Annexin V and antibody staining

The analysis of surviving, dead and apoptotic cells was based on gating of cells by size (side and forward scatter), plasma membrane integrity [propidium iodide (PI) staining] and redistribution of plasma membrane phosphatidylserine (annexin V binding). Briefly, 1×10^6 thymocytes were resuspended in 100 μl of buffer containing 10 mM HEPES, pH 7.3, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$, and incubated with 0.3 μg/ml of APC-conjugated annexin V and 5 μg/ml propidium iodide (PI) for 15 min. After incubation, samples were diluted 4 times with buffer containing 1.8 mM $CaCl_2$ and analyzed by flow cytometry. As a negative control for all annexin V staining procedures, cells were washed extensively in Ca^{2+}-free PBS and stained with annexin V in the absence of Ca^{2+}. To assess the phenotypic characteristics of the thymi, cells were stained with anti-CD3mAb coupled to PeCy7, anti-CD8mAb coupled to PE, and anti-CD4mAb coupled to FITC. All reagents were purchased from Pharmingen/Becton Dickinson (San Diego, CA, USA). Fluorochrome-labeled isotype-matched negative controls for the specific mAbs were also Pharmingen products. All samples labeled with antibody were washed in staining buffer after 25 min on ice and cells resuspended in 500 μl of staining buffer for flow cytometric analysis.

Flow cytometry

Thymus and mesenteric lymph nodes were dissected and mechanically disaggregated. Single cell suspensions were obtained and red blood cells were removed by NH_4Cl lysis. Cells were diluted in 10% BSA-phosphate-buffered saline and incubated with anti-FcγR antibody (BD Biosciences) to block the binding of conjugated antibodies to FcγR. Cells then were incubated with the proper antibodies as described above and samples were acquired on a FACSAria® cytometer (BD Biosciences, San Jose, CA, USA). Postacquisition analysis was performed using FlowJo software (Tree Star, San Carlos, CA, USA). As presented in the 'Results' section PI-positive cells were gated out of all profiles for thymocytes. $CD4^+$ and $CD8^+$ cell populations were analyzed by gating on $CD3^+$ cells.

Statistical evaluation

Statistical evaluation of the results was made by unpaired Student's *t* and Kruskal–Wallis tests. The GraphPad Instat software