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Different Activation of Lymphocytes in Reaction to *Babesia microti* Infection in iNOS-/- and Wildtype Mice

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ABSTRACT

The reaction of lymphocyte populations, in iNOS-/- mice and wildtype control mice, to *Babesia microti* infection was investigated in this study. During the course of infection, in both groups of mice there was gross enlargement of the spleens but the spleen cell population decreased by 50%. At peak parasitemia iNOS-/- mice had a low lymphocyte count and on overall, a slight increase in leukocyte population in peripheral blood. On the other hand control mice showed a significant increase in leukocyte population but lymphocyte numbers remained unchanged. During infection the iNOS-/- mice had a higher percentage of B lymphocytes and $CD4^+$ T cells in the spleen, compared to the control mice. In response to *B. microti* infection iNOS-/- mice produce less IL-4, but more IFN-γ compared to control mice. Parallel to the higher percentage of B cells, iNOS-/- mice also produced higher amounts of *B. microti*-specific antibodies. These results suggest that in the early stages of infection NO protects the lymphocytes against *B. microti* invasion and that the immune defense in iNOS-/- mice involves to a large extent humoral immune response.

INTRODUCTION

Babesia microti is a rodent pathogen, species of the hemoprotozoan parasite *Babesia. Babesiae* invade erythrocytes where they multiply and cause hemolysis and anemia in the host (Callow and Dalgliesh 1982). *Babesia microti* and *B. equi* are the only *Babesia* species which invade the host's lymphocytes and multiply

there initially before they invade the erythrocytes. This characteristic places them close to the genus *Theileria* (Mehlhorn and Schein 1984). Cell-mediated immunity by $CD4^+$ T cells and IFN-γ plays an important role in the protection against primary infection with *B. microti* (Igarashi et al. 1994; Shimada et al. 1996). Our earlier experiments with iNOS-/- mice unable to produce nitric oxide in response to *B. microti* infection demonstrated that NO is involved in early immune defense against the parasite. Differences in the expression patterns of IFN-γ and TNF-α during the course of infection proved that iNOS-/- mice use different immune defense mechanisms against the invading parasites as compared to those employed by the wildtype control mice with the ability to produce NO (Remer et al. 1998, in print). The objective of the present study is to identify the lymphocyte subsets involved in immune defense against *B. microti* in iNOS-/- and wildtype mice.

MATERIALS AND METHODS

Mice and parasites

Breeding pairs of mice with disrupted iNOS gene (iNOS-/-) were generously provided by Dr. J. S. Mudgett (Merck Research Laboratories). The C57BL/6 mice used as controls for this study were purchased from CLEA (Tokyo, Japan). All mice were between 6-9 weeks of age at the beginning of the experiments and all experiment groups were sex and age matched. *B. microti* (Munich strain) was kindly provided by Prof. A.O. Heydorn from the Institute of Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin. The mice were inoculated intraperitoneally (i.p.) with 1×10^7 parasitized erythrocytes (PRBC) and the course of parasitemia was monitored every 2 days by counting the percentage of PRBC on Giemsa-stained blood smears.

Spleen size and cell numbers in the spleen

Groups of uninfected mice and infected mice at the peak and at the end of the parasitemia were sacrificed. The spleens were removed and their weight measured with an electronic balance. The spleens were homogenized between two frosted glass slides with Turk's solution (KANTO Chemical Co. Inc., Tokyo, Japan) and the cells in the suspension quantified using a modified Neubauer chamber. The cell-numbers were calculated as cells/mg spleen weight.

Leukocyte-numbers in the blood

Blood from infected and uninfected mice was collected by cardiac puncture. For leukocyte-numbers the blood was diluted with Turk's solution in a haemocytometer and the cells counted with a modified Neubauer chamber. For the proportion of lymphocytes in the total leukocyte population Giemsa-stained blood

smears were examined under light microscope.

Flow cytometrical analysis of lymphocyte subsets

Infected and uninfected mice were sacrificed and the spleen removed. The spleens were then homogenized between two frosted glass slides with phosphate buffered saline (PBS) + heparin (100 U/ml) and the suspension filtered through a nylon mesh to remove tissue fragments. Erythrocytes in the suspension were lysed with 0.83 % ammonium chloride, the cells were washed 2 times with PBS and resuspended with PBS to a concentration of 2×10^7 cells/ml. Cell suspensions were incubated with FITC-conjugated anti-T cell and anti-$CD8^+$ mAb or PE-conjugated anti-B cell and anti-$CD4^+$ mAb for 30 min on ice, washed 3 times and analyzed by flow-cytometer (COULTER® Epics®XL, Coulter TM, Miami, USA).

Measurement of IFN-γ and IL-4

Blood samples of infected and uninfected mice were collected by cardiac puncture. The serum was separated and stored in -80°C until use. The concentrations of IFN-γ and IL-4 in the serum were measured by ELISA test kits (ENDOGEN Inc., Cambridge, USA).

Antibody titer

Babesia microti-specific antibody-titers were determined by indirect immuno-fluorescence antibody test (IFAT). *Babesia microti*-antigen covered slides were kindly provided by Dr. Avarzed. The antigen-spots were covered with the PBS-diluted serum from infected and uninfected mice for 30 min, incubated with FITC-conjugated anti-mouse IgG for 30 min, washed in PBS and examined with a fluorescence microscope (Microphot-FX, NIKON, Tokyo, Japan).

RESULTS AND DISCUSSION

The spleens of uninfected iNOS-/- mice were slightly smaller in size and weight but contained relatively more cells than the spleens of normal C57BL mice. During the course of infection with *B. microti* the spleens increased in size in both iNOS-/- and wildtype mice. The increase in iNOS-/- mice at the end of the parasitemia was only slightly more than in normal mice (Fig. 1). In the same period the relative numbers of cells in the spleen decreased in both groups, however the decrease was greater in iNOS-/- mice as compared to wildtype mice (Fig. 2). These findings correspond with histology results of earlier experiment's which showed that during *B. microti* infection there is little follicle activation in the spleen of iNOS-/- mice as compared to that observed in C57BL controls (Remer et al. 1998, in print). Therefore, the increase in iNOS-/- mice spleen

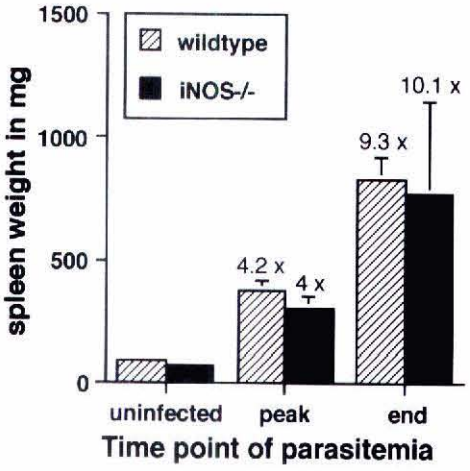

Fig. 1 Increase in spleen weight in iNOS-/- mice and wildtype C57BL mice during *B. microti* infection. The numbers over the columns show the relative increase in weight compared to the spleen of uninfected animals.

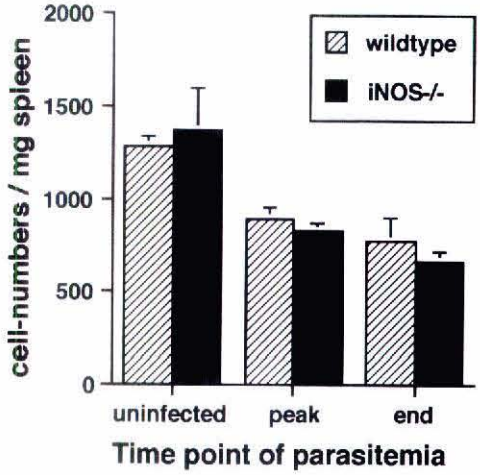

Fig. 2 Relative cell-numbers in the spleen of iNOS-/- and wildtype C57BL mice during *B. microti* infection. Cell-numbers calculated in cells/mg spleen weight.

weights, whilst relative cell numbers remain relatively lower, is caused by an increase in red pulp size rather than proliferation of the white pulp in reaction to the parasites (Hildebrandt 1981).

Uninfected iNOS-/- mice had lower leukocyte-numbers in the blood than their wildtype controls. During the course of infection leukocyte-numbers in the controls increased markedly but returned to normal before the end of parasitemia. In contrast, there was only a modest rise in leukocyte-numbers of iNOS-/- mice, which was maintained until the end of parasitemia (Fig. 3). The percentage of lymphocytes in iNOS-/- mice decreased during the course of parasitemia, while in C57BL control mice it remained unchanged (Fig. 4).

Taking into consideration that *B. microti* is one of the *Babesiae* that has an early phase of multiplication in the host's lymphocytes (Mehlhorn and Schein 1984), the higher percentage of lymphocytes in wildtype mice suggests that NO has the ability to protect the lymphocytes against destruction by the parasite. How these findings are connected with the differences in spleen activation (Remer et al. 1998, in print) and blood leukocyte-numbers is yet not well known and deserves closer examination.

To differentiate the subsets of the lymphocytes flowcytometry was performed. Here it turned out that uninfected iNOS-/- and wildtype control mice had almost the same proportions of T to B lymphocytes (1 : 1.2 and 1 : 0.81,

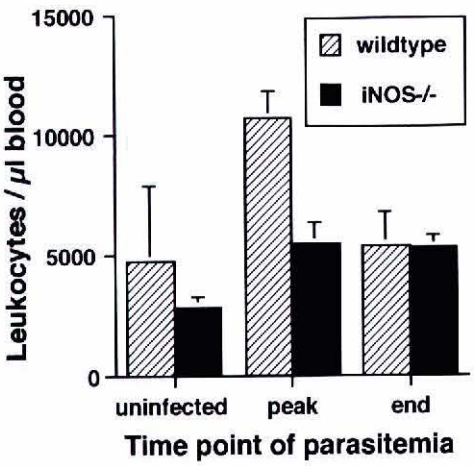

Fig. 3 Leukocyte numbers in the blood of iNOS-/- mice and wildtype C57Bl mice during *B. microti* infection.

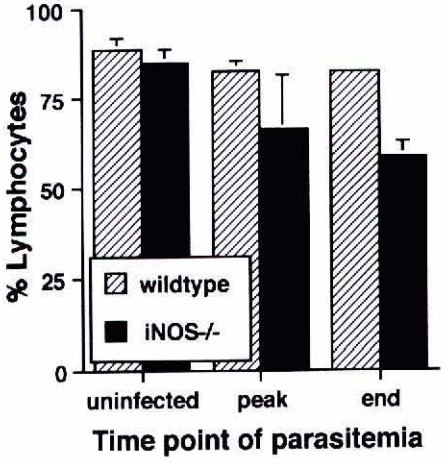

Fig. 4 Percentage of lymphocytes in the differential-blood smear of iNOS-/- mice and wildtype mice during B. *microti*-infection.

respectively), but there were significant differences in ratios between the two groups during infection (Table 1). There was also a distinct difference in the proportions of $CD4^+$ to $CD8^+$ cells: iNOS-/- mice had initially a lower percentage of $CD4^+$ cells, but developed a higher $CD4^+$: $CD8^+$ ratio during infection.

Table 1. Ratio of lymphocyte subsets in iNOS-/- mice and their C57BL controls during the course of infection with *B. microti*.

	T / B ratio		$CD4^+$ / $CD8^+$ ratio	
	wild type	iNOS-/-	wild type	iNOS-/-
uninfected	1 : 0.81	1 : 1.20	3.27 : 1	2.57 : 1
peak	1 : 2.47	1 : 3.90	4.83 : 1	6.30 : 1
end	1 : 1.53	1 : 2.27	3.63 : 1	4.57 : 1

To find out the relative dominance of Th1 or Th2 subsets among the $CD4^+$ cells, the concentrations of IFN-γ, IL-4 and *B. microti*-specific antibody-titer in the serum were determined. iNOS-/- mice had higher amounts of IFN-γ, lower amounts of IL-4 and a higher titer of specific antibodies, compared to wildtype C57BL mice (Figs. 5 and 6, and Table 2).

The importance of the $CD4^+$ T cells in resolving *B. microti* primary infection in normal mice has already been reported (Igarashi et al. 1994; Shimada

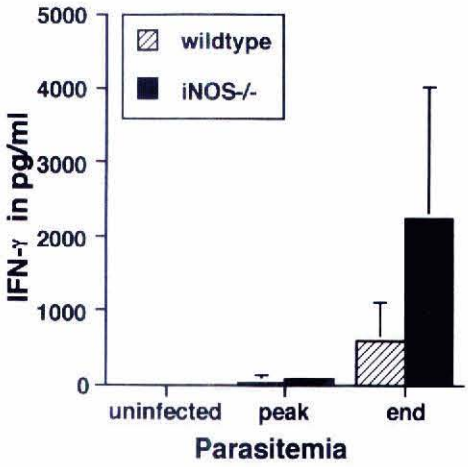

Fig. 5 IFN-γ in the serum of iNOS-/- and wildtype C57BL mice during *B. microti* - infection.

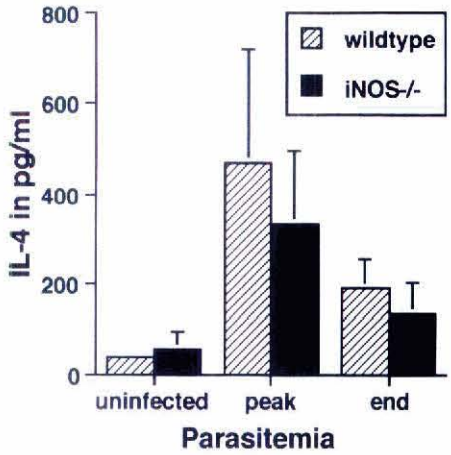

Fig. 6 IL-4 in the serum of iNOS-/- and wildtype C57BL mice during *B. microti*- infection.

Table 2. *Babesia microti*-specific antibody-titer in iNOS-/- and wildtype C57BL mice during the course of infection.

mouse	uninfected	peak	end
wildtype	-	1 : 1,024	1 : 4,096
iNOS-/-	-	1 : 1,024	1 : 16,384

et al. 1996). The results of our experiments demonstrate that $CD4^+$ T cells also play an important role in the immune defense in iNOS-/- mice. Wei et al. (1995) reported that a high concentration of NO prevents the overexpansion of the Th1 subset following a strong antigenic challenge. This may explain the high concentrations of IFN-γ found in iNOS-/- mice with *B. microti*-infection (Remer et al. 1998, in print; Wei et al. 1995). The higher proportions of B lymphocytes and the high *B. microti*-specific antibody-titer despite the general lower numbers of total lymphocytes in iNOS-/- mice indicate a greater importance of humoral immune response to primary infection in iNOS-/- mice.

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