HISTOPATHOLOGICAL CHARACTERIZATION OF *L. DONOVANI* INFECTION IN RAG2$^{-/-}$ MICE

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ABSTRACT

A recent study demonstrated lower incidence of fever and hepatosplenomegaly in HIV-positive VL patients than HIV-negative VL patients, indicating that acquired immunity have important roles in development of hepatosplenomegaly also in human VL. To explore the roles of acquired immunity in pathology of VL, pathological characterization of L. donovani infection in RAG2$^{-/-}$ mice was performed. One hundred million promastigotes were inoculated intraperitoneally to RAG2$^{-/-}$ mice as well as BALB/c mice, and organs were harvested from the animals at 2, 4, 8 and 12 weeks after infection and examined for parasite burdens and pathological changes. L. donovani infection induced splenomegaly in BALB/c mice; the length of the spleen after 12 weeks of infected mice was as twice as that of naive mice. In contrast, such the enlargement was not observed in RAG2$^{-/-}$ mice. Accumulations of mononuclear cells were found in the liver of infected BALB/c mice, whereas such the formations were less found in RAG2$^{-/-}$ mice. These results suggest that development of hepatosplenomegaly during experimental VL is dependent on the T cell or B cell rather than the presence of parasites.

KEY WORDS: leishmania, spleen, liver, immunohistochemistry, granuloma

INTRODUCTION

Leishmaniasis is endemic in areas of the tropics, subtropics, and southern Europe, in settings ranging from rain forests in the Americas to deserts in western Asia, and from rural to peri urban areas (6). Annual incidence of VL is estimated at 500,000 cases in the world and mortality is officially estimated at 59,000 but it is clearly a severe underestimation (2).

The amastigote forms of *L. infantum* and *L. donovani* are found inside neutrophils and mononuclear phagocytes, or freely, mostly in the spleen, which may harbor the largest parasite burden. Bone marrow also is highly parasitized. The liver and lymph nodes are also important sites of infection (18).

Protracted fever, anemia, wasting, hepatosplenomegaly, hemorrhages, and bacterial co-infections are typical features. Patients who lost more weight had a higher parasite burden, and patients with epistaxis, abdominal pain, edema, and jaundice. This study suggests that higher parasite load is influenced by wasting, which may lead to

more severe disease (18). Disseminated intravascular coagulation and thrombocytopenia are the main causes of bleeding.However, profound immunosuppression is demonstrable in acutely ill patients, which is manifested in the form of lack of T cell responsiveness to leishmanial antigen or even to mitogens (7, 15, 16). Recently, some comparative studies about the clinical presentation and outcome of VL in HIV-infected and immunocompetent patients have been reported. Gastrointestinal leishmaniasis representing diarrhea, dysphagia, odynophagia, abdominal pain, epigastric pain, gastrointestinal hemorrhage, and rectal discomfort (10). HIV-infected patients had a greater frequency and degree of leukopenia, lymphocytopenia, and thrombocytopenia (14). A recent study demonstrated lower incidence of fever and hepatosplenomegaly in HIV-positive VL patients than HIV-negative VL (1). This indicates the influence of $CD4^+$ T cells to pathology of VL. However, immunological mechanisms underlying for pathology of VL remain largely unclear.

Some studies have focused on the roles of acquired immunity in pathological changes during leishmaniasis. Previous studies in our laboratory have demonstrated that $CD4^+$ cells are indispensable for ulcer development in murine CL (20-22). In contrast, development of skin lesion associated with macrophage accumulation during murine CL does not require T cell or B cell (5,20, 21). These works suggest that the roles of acquired immunity in pathology vary for each manifestation during CL.

Taken together, this paper was designed to explore the roles of acquired immunity in the development of pathology during VL. $RAG2^{-/-}$ mice were used in this study as a model of mice defective in acquired immunity. $RAG2^{-/-}$ mutants are viable that fail to produce mature B or T lymphocytes. Very immature lymphoid cells were present in primary lymphoid organs of mutant animals as defined (17). Here, $RAG2^{-/-}$mice were infected with *L. donovani*, and pathological changes were examined by comparing with those in infected BALB/c mice.

MATERIALS AND METHODS

Parasites

Leishmania donovani (MHOM/NP/03/D10) was obtained from National BioResource Project at Nagasaki University. The parasites isolated from the Nepalese VL patient were used in this study (13). Promastigotes of *L. donovani* D10 were cultured in TC 199 medium at pH 7.2 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), supplemented with 10% heat-activated fetal bovine serum (Thermo Electron Corporation, Australia) and 25 mM HEPES (MP Biomedicals, LLC, France) at 25°C.

Mice and inoculation

Male BALB/c mice of 8 weeks of age were purchased from the CLEA Japan, Inc. (Tokyo, Japan).Male recombination activating gene 2 knockout BALB/c mice ($RAG2^{-/-}$) were obtained from the Central Institute of Experimental Animals, Kawasaki, Japan. All mice were maintained under specific pathogen-free conditions. $RAG2^{-/-}$ mice were used for experiments at the age of 7-8 weeks.

For infection, promastigotes in a stationary phase were washed three times withand resuspended in Hanks' balanced salt solution (HBSS, Gibso Life Technologies).The mice were inoculated intraperitoneal with $1Ч10^8$promastigotesand were sacrificed at 0, 2, 4, 8 and 12 weeks post inoculation (PI).

All experimental and animal care procedures were approved by the guiding principles of The University of Tokyo and were conducted in accordance with the institution's guidelines for the care and use of laboratory animals under the SPF condition during the experiment.The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Tokyo.

Histopathological and immunohistochemistry analysis

Pieces of the spleen and liver were fixed with 10% buffered formalin (Sumitani Shoten Co., Ltd, Japan) and embedded in paraffin wax. Paraffin-embedded tissues were cut at 4 μm. For histology, sections were dewaxed with xylene, and then 100%, 90%, 80%, and 70% ethanol. The rehydrated sections were stained with hematoxylin and eosin (Wako, Japan) for subsequent light microscopy.

For immunohistochemistry, serial sections were deparaffinized, dehydrated, washed in distilled water for 1 min. The sections were treated with methanol containing 0.3% H_2O_2 for 30 min. The sections were washed with PBS (-) for 2 min each time and with PBS-Tween (PBS-T) for 1 min. The sections were incubated with 10% BlockAce (DS Pharma Biomedical Co., Ltd, Japan) for 10 min prior to incubation with each primary antibody which was anti-*Leishmania* monoclonal C11C antibody (2.5 μg/ml). The antibody C11C was developed against *Leishmania* amastigotes and has been shown to react with TSA (Thiol specific antioxidant) protein.

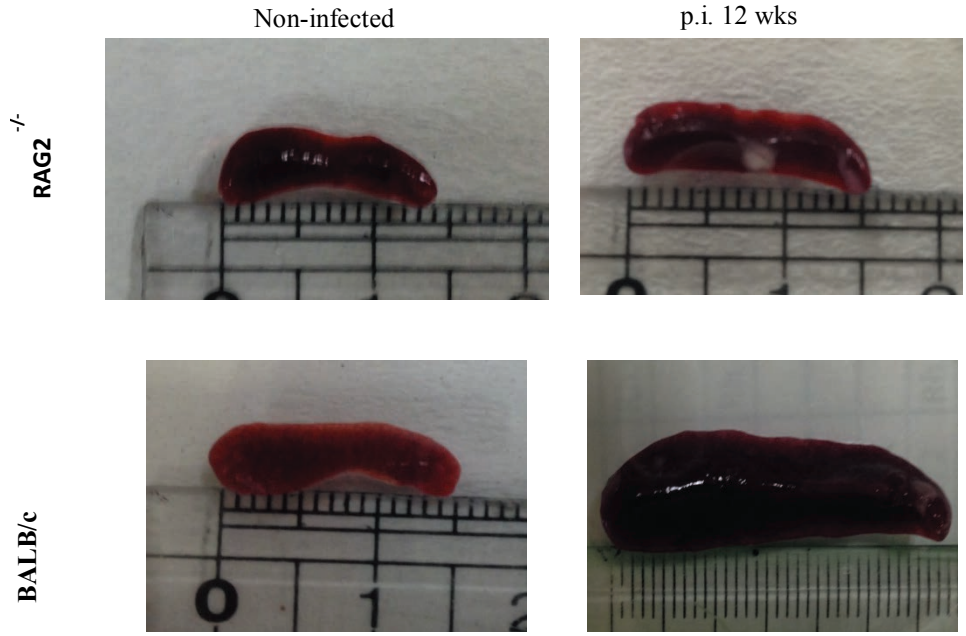
After 1 hour incubation with primary antibody, sections were washed three times with PBS (-) for 2 min each time and with PBS-T for 1 min. Then sections were incubated with HRP-conjugated goat polyclonal IgG Fab fragment against mouse IgG (Nichirei Biosciences, Tokyo, Japan). After 1 hour, sections were washed three times with PBS (-) for 2 min each time. Sections were developed for peroxidase activity in 3,3-diaminobenzidine tetrahydrochloride-DAB (Nichirei Biosciences, Tokyo, Japan). Sections were counterstained for 1 min with Mayer's hematoxylin (Wako, Japan), dehydrated and mounted in Mount quick (Daido Sangyo Co., Ltd, Japan).

RESULTS

Measurement of visceral organ weight

At 2, 4, 8 and 12 weeks after infection, tissues were harvested from the animals and examined for tissue weights.

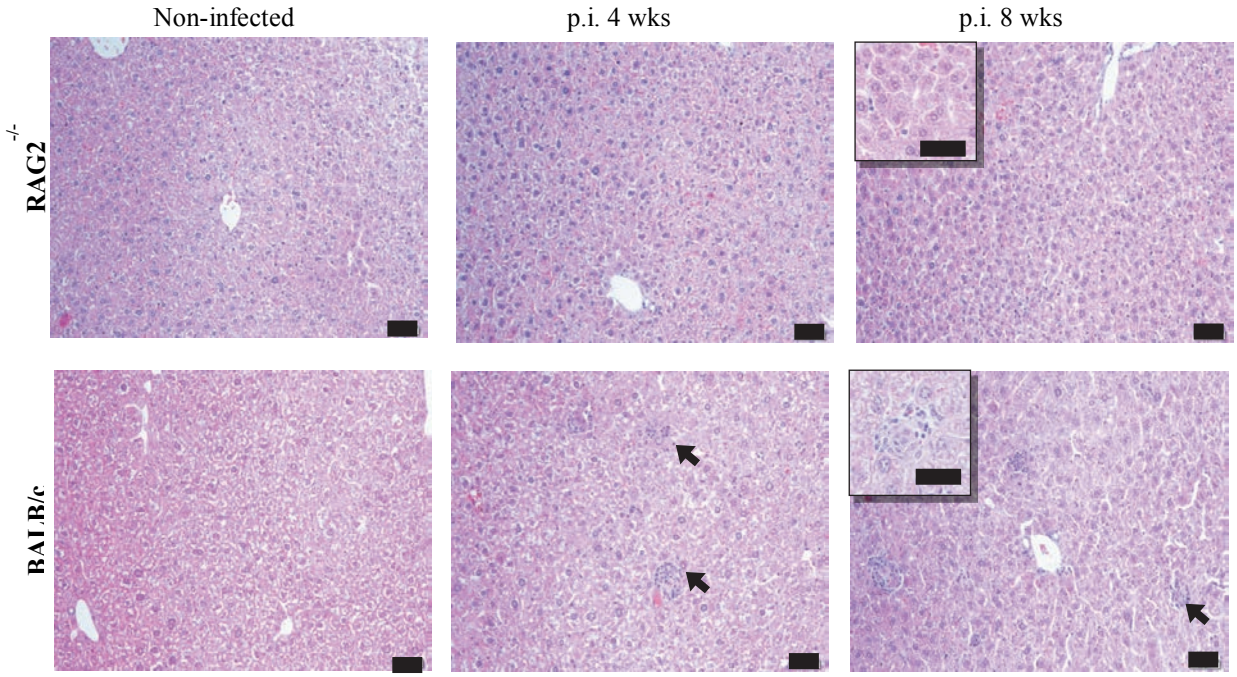
Significant splenomegaly was observed in infected BALB/c mice after 12 weeks of infection. In contrast, such the enlargement of the spleen was not observed in infected RAG2$^{-/-}$ mice (Pic.1).



Picture 1. Prominent enlargement of the spleen in BALB/c mice. Representatives of the spleen from uninfected and infected mice.

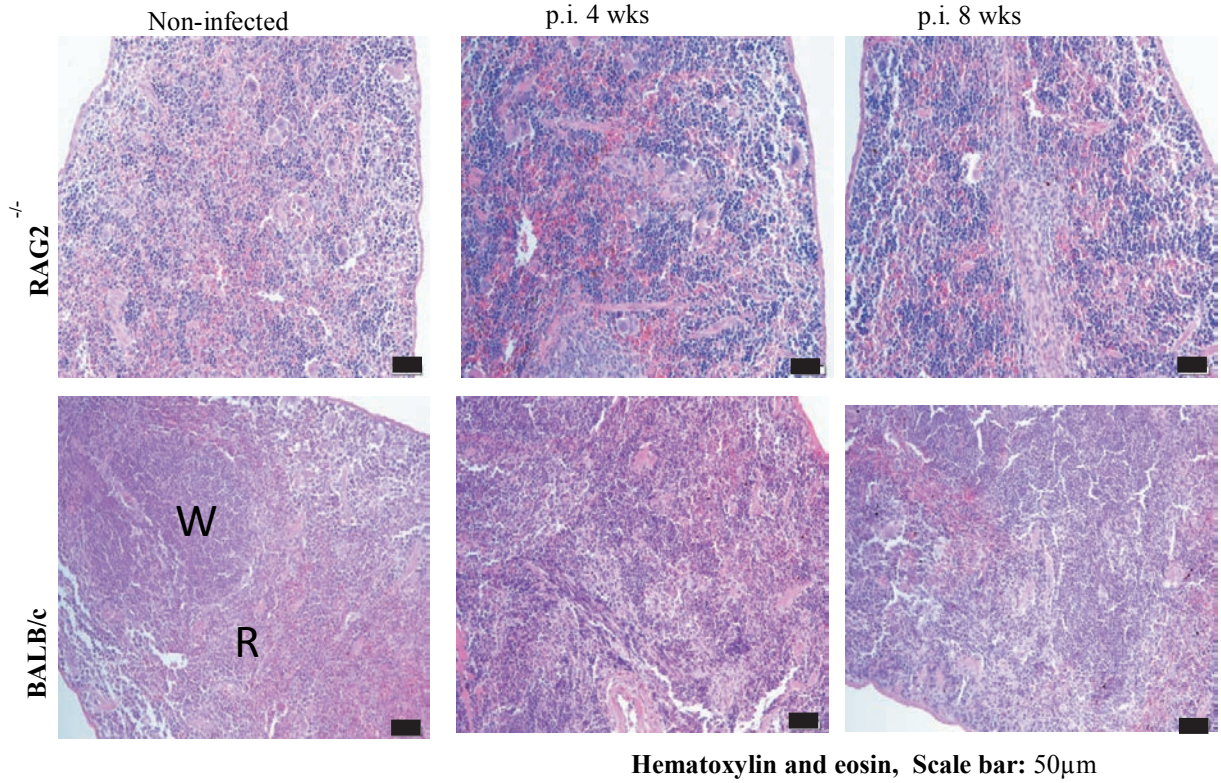
Histopathological and immunohistochemical analysis

The liver and spleen sections were stained with hematoxylin and eosin (HE). After 4 weeks of infection, accumulations of mononuclear cells were found in the liver of BALB/c mice. In contrast, such the mononuclear cells were less in RAG2$^{-/-}$ mice (Pic.2).



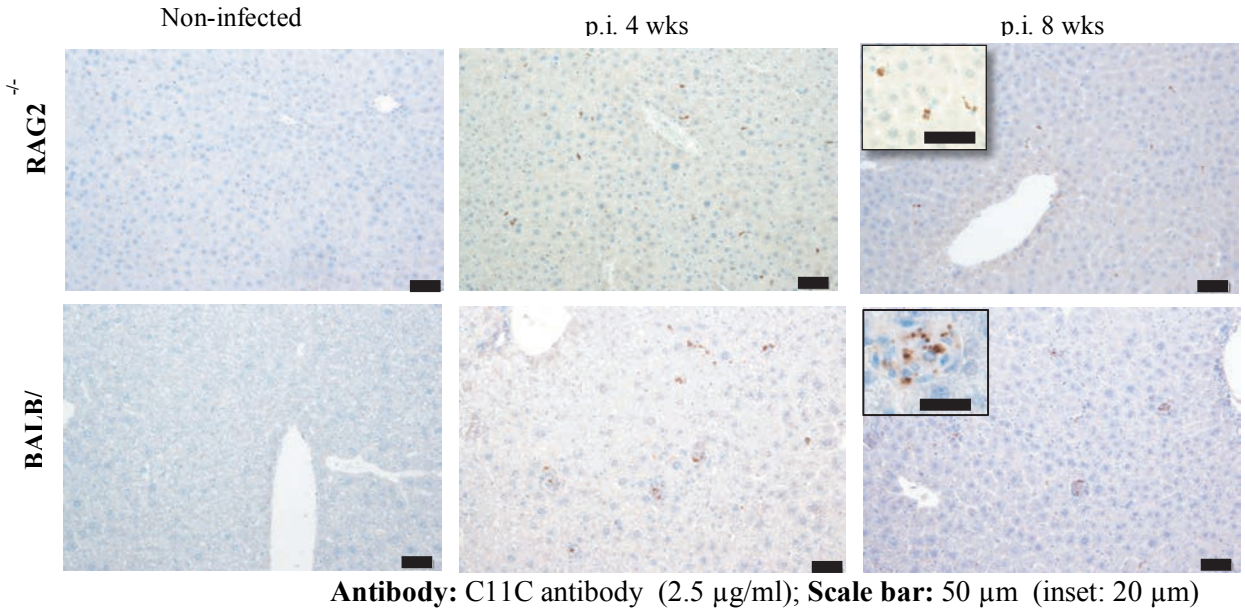
Picture 2. Histological analysis of the liver of RAG2$^{-/-}$ and BALB/c mice infected with *L. donovani*. The liver sections were stained with hematoxylin and eosin. Arrows indicate the mononuclear cells.

Histological analyses revealed that both red pulp and white pulp were enlarged in BALB/c mice. As the spleen weight was unchanged by *L. donovani* infection in RAG2$^{-/-}$ mice, there was no significant change in the cross-sectional area of the spleen in these mice (Fig.3).



Picture 3. Histological analysis of RAG2$^{-/-}$ and BALB/c mice infected with *L. donovani*. The spleen sections stained with hematoxylin and eosin.

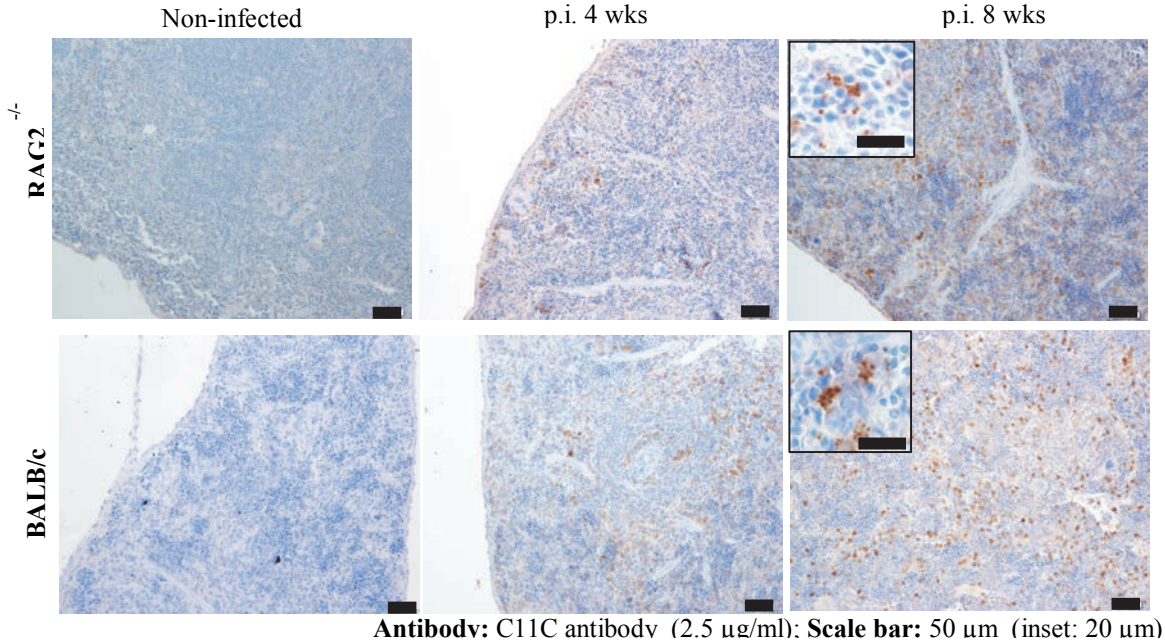
The parasites were also confirmed by immunohistochemistry using anti-*Leishmania* monoclonal antibody, C11C. By staining with C11C, positive signals were found in the tissues from infected mice. C11C staining also showed the similar results as analysis on Giemsa-stained samples, in other words, increased numbers of positive staining were observed over the course of infection in RAG2$^{-/-}$ mice and BALB/c mice(Fig.4).



Antibody: C11C antibody (2.5 µg/ml); **Scale bar:** 50 µm (inset: 20 µm)

Picture 4. Immunohistochemical detection of amastigotes in the liver of RAG2$^{-/-}$ and BALB/c mice infected with *L. donovani*. The liver sections stained with anti-*Leishmania* monoclonal antibody, C11C (2.5 µg/ml). Scale bar: 50 µm (inset: 20 µm).

The spleen was also analyzed by immunohistochemistry using C11C, and again, increased numbers of positive staining were observed over the course of infection in RAG2$^{-/-}$ mice and BALB/c mice (Fig.5)



Antibody: C11C antibody (2.5 µg/ml); **Scale bar:** 50 µm (inset: 20 µm)

Picture 5. Immunohistochemical detection of amastigotes in the spleen of RAG2$^{-/-}$ and BALB/c mice infected with *L. donovani*. The spleen sections stained with anti-*Leishmania* monoclonal antibody, C11C (2.5 µg/ml). Scale bar: 50 µm (inset: 20 µm).

DISCUSSION

Intraperitoneal inoculation of RAG2$^{-/-}$ and BALB/c mice with *L. donovani* promastigotes was shown to induce progressive infection accompanied with increasing parasite load in the spleen, liver and bone marrow during the study period up to 12 weeks following the infection. These results suggested that BALB/c and RAG2$^{-/-}$ mice were showed susceptibility to *L. donovani* infection. Other groups also reported the infectivity of *L. donovani* to immunodeficient mice. Parasite burdens in SCID mice were found to be lower up to day 14 of infection, whereas the mice had between two and threefold higher parasite burdens than BALB/c mice by 28 days(4). Taken together, it is suggested that immunodeficient mice are susceptible to *L. donovani*. When it comes to pathology, however, the outcome was completely different between BALB/c mice and RAG2$^{-/-}$ mice. Hepatosplenomegaly is a hallmark of VL symptoms in humans, and it was reproduced in the present experimental VL. However, only BALB/c mice manifested hepatosplenomegaly by *L. donovani* infection, but the manifestation was not apparent in RAG2$^{-/-}$ mice. This result suggests that the pathology is dependent on acquired immunity, rather than the sole parasite burden. The dependence on acquired immunity varies at individual manifestations during leishmaniasis. For example, *L. major* infection causes the development of skin lesionin RAG2$^{-/-}$ mice comparable to BALB/c mice, demonstrating T and B cell-independent mechanism for lesion development (5). In contrast, ulceration of the skin lesion in *L. amazonensis* infection is CD4^{+} T cell-dependent (20-22). Together, it is considered that the pathological mechanisms under various manifestations in leishmaniasis are not uniform, and understanding the mechanisms is important for the control of individual symptoms to develop new interventions.

Histological examinations revealed the accumulations mononuclear cells in the liver of BALB/c mice after 4 weeks of infection. In contrast, such the accumulation mononuclear cells were less intense in RAG2$^{-/-}$ mice. Intense cellular accumulation was also observed in the spleen of infected BALB/c mice but not in that of RAG2$^{-/-}$ mice. These results suggest that cellular accumulation is associated with hepatosplenomegaly and is largely dependent on the presence of T cells or B cells. A recent study demonstrated lower incidence of hepatosplenomegaly in HIV-positive VL patients than HIV-negative VL patients(1), indicating that acquired immunity has important roles in development of hepatosplenomegaly also in human VL.It is reported that in BALB/c and C57BL/6 mice, the inflammatory mononuclear cells reaction around infected kupffer cells is developed and the infection is resolved by 4–8 weeks after infection (11).Cellular and molecular interactions mediated by kupffer cells, monocytes, CD4^{+} and CD8^{+} T cells and a number of cytokines and chemokines are required for effective hepatic granuloma formation (3, 8, 11, 12, 19). Although previous studies have identified B cells in hepatic cell infiltrations and functional studies in B cell-deficient mice have suggested a role for B cells in the control of experimental visceral leishmaniasis, little is known about the behavior of B cells in the mononuclear cells microenvironment. The hepatic B cell population in infected mice, where ≈60% of B cells are located within mononuclear cells, with that of naпve mice (9). Taken together, characterization of cells accumulated in the enlarged spleen and liver of infected BALB/c mice may help understand the mechanisms of hepatosplenomegaly during VL.

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