

## 論文の内容の要旨

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論文題目 Studies on experimental visceral leishmaniasis in immunodeficient mice  
(免疫不全マウスにおける実験的内臓型リーシュマニア症の研究)

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania*. There are 12 million patients in 88 countries. Leishmaniasis can be divided into three major forms, cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis (VL also known as kala azar), according to the clinical manifestations. VL is the severest form of the disease, and its major symptoms include fever, anemia and hepatosplenomegaly. The disease is often fatal if left untreated. The visceral form is present in 70 countries. It is highly endemic in the Indian subcontinent and in East Africa, Brazil. An estimated 200 000 to 400 000 new cases of VL occur worldwide each year. Over 90% of new cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan.

Recently, VL has become a more significant issue in countries other than the five highly endemic ones. This is due to co-infection with HIV. Thirty five countries have reported cases of co-infection worldwide, and the co-infection now accounts for 2-12% of all VL cases. VL/HIV co-infection seems to present features different from VL in HIV-free patients. High incidence of disseminated leishmaniasis in AIDS patients has been reported, and atypical symptoms, for example gastrointestinal leishmaniasis representing diarrhea and gastrointestinal hemorrhage, are found in those patients. A recent study demonstrated lower incidence of fever and hepatosplenomegaly in HIV-positive VL patients than HIV-negative VL. This indicates the influence of acquired immunity to pathology of VL.

General objective of this study is to understand the influence of acquired immunity (T cell/B cell) to pathology by using an experimental VL model. As a model for acquired immunity deficiency, the recombination activating gene 2 knockout BALB/c mice (RAG2<sup>-/-</sup>) were chosen in this study. RAG2 gene is

one of the recombination activating genes responsible for the rearrangement and recombination of immunoglobulin and T cell receptors during the process of VDJ recombination, and RAG2<sup>-/-</sup> mice lack functional T and B cells. Our laboratory has been using RAG2<sup>-/-</sup> mice to understand the pathology of leishmaniasis. Goto et al., 2007 have reported that *L. major* infection causes the development of skin lesion in RAG2<sup>-/-</sup> mice, demonstrating T and B cell-independent mechanism for lesion development. However, immunopathological studies on experimental VL in RAG2<sup>-/-</sup> mice have not previously been performed, and such a study may lead to better understanding of human VL in immunodeficient condition. Therefore, immunopathology of experimental VL in RAG2<sup>-/-</sup> mice was studied.

In Chapter 1, pathological characterization of *L. donovani* infection in RAG2<sup>-/-</sup> mice was performed. One hundred million promastigotes were inoculated intraperitoneal to RAG2<sup>-/-</sup> mice as well as BALB/c mice, and tissues 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 at 12-week infected mice were as twice as that of naive mice. In contrast, such the enlargement was not observed in RAG2<sup>-/-</sup> mice. Tissue enlargement was also observed in the liver and kidney. Again, the enlargement was observed in BALB/c mice but not in RAG2<sup>-/-</sup> mice. Organ parasite burden were represented as the number of amastigotes per 1,000 host nuclei cells enumerated by microscopic observation. In BALB/c mice, the spleen and liver parasite burden were 740.25 ± 244.8 (mean ± standard deviation; SD) and 667 ± 163.5 at 12 weeks post infection (p.i.). The parasite burden in spleen and liver of infected RAG2<sup>-/-</sup> mice were 1033.75 ± 126.18 and 805.75 ± 159.81 at 12 weeks p.i. There was not a huge difference in parasite burdens between BALB/c mice and RAG2<sup>-/-</sup> mice. Also, RAG2<sup>-/-</sup> mice are also susceptible to *L. donovani*. Accumulations of mononuclear cells were found in the liver of infected BALB/c mice, whereas such the formations were less in RAG2<sup>-/-</sup> mice. These results suggest that development of hepatosplenomegaly during experimental VL is dependent on the presence of T cell or B cell rather than parasite burden. 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.

Because cell accumulation was observed in the enlarged tissues of infected BALB/c mice in Chapter 1, those cells may be related to the different pathology observed between BALB/c mice and RAG2<sup>-/-</sup> mice. In Chapter 2, characterization of immune cells during experimental VL in BALB/c mice and RAG2<sup>-/-</sup> mice was performed. Protocol for infection and tissue harvest was same as described in Chapter 1. For cell population analyses, immunohistochemical staining using antibodies to mouse proteins were performed. Used antibodies were CD3ε, B220, MRP8 and MRP14. In both the spleen and liver of infected BALB/c mice, increase of CD3ε and B220 positive cells was observed. In contrast, increase of those cells was not observed in infected RAG2<sup>-/-</sup> mice. In the case of MRP8 and MRP14 cells, increase of positive cells over the course of infection was observed in BALB/c mice and RAG2<sup>-/-</sup> mice, whereas the intensity of macrophage accumulation was higher in BALB/c mice. These results suggest that *L. donovani* infection

causes increase of T cells and B cells in BALB/c mice, and that macrophage accumulation to the spleen and liver is also affected by T cells or B cells. Because hepatosplenomegaly was observed in BALB/c mice but not in RAG2<sup>-/-</sup> mice, those increased T cells or B cells themselves as well as macrophages under the control of acquired immunity seem to be responsible for development of such the pathology. Results from Chapters 1 and 2 demonstrate that hepatosplenomegaly occurs in BALB/c mice but not in RAG2<sup>-/-</sup> mice, and so does T cell and B cell accumulation. Therefore, it is indicated that these two cells are more important than macrophages when it comes to development of hepatosplenomegaly.

Results from Chapters 1 and 2 demonstrated the differences in pathology and cellular kinetics during *L. donovani* infection between BALB/c mice and RAG2<sup>-/-</sup> mice. It is easily hypothesized that such the differences in pathology and cellular kinetics are caused by difference in molecules such as cytokines. In Chapter 3, therefore, cytokine expression in the spleen and liver during experimental VL was examined in BALB/c mice and RAG2<sup>-/-</sup> mice. Here, four cytokines, interferon gamma (IFN- $\gamma$ ), tumour necrosis factor alpha (TNF- $\alpha$ ), interleukin-4 (IL-4) and interleukin-10 (IL-10) were examined for mRNA expression levels at 0, 4, 8 and 12 weeks of infection by RT-PCR. In BALB/c mice, mRNA expressions of all the cytokines examined were increased over the course of infection. Especially, IL-4 and IL-10 were significantly increased. Increase of IL-10 was observed as early as 4 weeks and the expression was higher at 12 weeks. Increase of IL-4 was observed as early as 8 weeks and the expression was higher at 12 weeks. Increase of IFN- $\gamma$  was observed as early as 8 weeks and the expression was higher at 12 weeks. Increase of TNF- $\alpha$  was observed also, but the degree was smaller than the other cytokines. In contrast, changes in mRNA expression levels were observed in RAG2<sup>-/-</sup> mice for any cytokines. In the liver, some cytokines were detectable at certain time points. However, the expression levels in the liver were overall much lower than those in the spleen. The results that mRNA levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-4 and IL-10 in the spleens were increased over the course of *L. donovani* infection in BALB/c mice but not in RAG2<sup>-/-</sup> mice suggests that T cells or B cells are indispensable for such the increased expression of the cytokines in the spleen during experimental VL. Because these cytokines are known to relate with protection/exacerbation of *Leishmania* infection, further studies on mechanisms for expression of these cytokines by T cells or B cells may lead to better understanding of pathology during VL.

This study has demonstrated that BALB/c and RAG2<sup>-/-</sup> mice show a progressive parasite burden without any signs of heal. In contrast, the hepatosplenomegaly occurs in BALB/c mice but not in RAG2<sup>-/-</sup> mice. These results suggest that pathological mechanisms during VL are not very simple; although parasite burden is important for pathology, it is not the sole factor affecting the severity of the disease. Rather, acquired immunity regulated by T or B cells may have a more significant role in development of hepatosplenomegaly during VL. The mechanisms by which T or B cells contribute to the pathology may include cytokines, whereas how those cytokines induce hepatosplenomegaly remains to be studied. Immune responses are often 'a double-edged sword', the protective immunity against pathogen often serves as a pathological factor. This work demonstrated that pathology of VL, especially enlargement of

the spleen and the liver is also under influence of acquired immunity. Because such acquired immunity is important for elimination of parasites, this study may serve as the important first step to understand the watershed of protection/pathology by acquired immunity and will lead to development of better interventions for VL.