論文の内容の要旨

応用動物科学専攻

平成 28 年度博士課程進学

氏名　森本彩子

指導教員名　後藤康之

論文題目　Hemophagocytosis in visceral leishmaniasis

(内臓型リーシュマニア症における血球貪食)

Visceral leishmaniasis (VL), also known as kala-azar, is caused by parasitic protozoa of the genus *Leishmania*. Endemic countries of VL include India, Bangladesh, Nepal, Brazil, Ethiopia and Sudan. It is estimated that there are 300,000 new cases of VL and 20,000 deaths annually (WHO, 2012). In sand flies, the insect vector for *Leishmania*, the parasites develop as promastigotes in the midgut. Once transmitted to mammalian hosts through blood feeding by sand flies, these parasites proliferate as amastigotes within macrophages in the spleen, liver and bone marrow. VL is characterized by clinical manifestations such as fever, weight loss, hepatosplenomegaly and anemia. Anemia often occurs in infectious diseases, whereas the mechanisms of infection-associated anemia may be variable depending on the diseases. In VL, the mechanisms of anemia remained still elusive due to lack of appropriate animal model. I have established a mouse model of VL exhibiting anemia in my master thesis, and using this model it was demonstrated that hemophagocytosis in the spleen of infected mice is a possible cause of anemia during VL. The aim of Ph. D thesis is to examine the contribution of hemophagocytes to anemia during VL, molecular mechanisms of hemophagocytosis during VL and the role of hemophagocytosis in *Leishmania* survival using an experimental model.

First, more detailed analyses on anemia and hemophagocytosis during VL were performed. At 24 weeks of *L. donovani* infection, BALB/cA mice exhibited splenomegaly, hepatomegaly and anemia. Bone marrow histology and iron status of both infected and uninfected mice were examined but there were no significant histopathological changes and serum iron level or MCV of infected mice were comparable to that of naïve mice. On the other hand, serum indirect bilirubin increased in infected mice. There were no apparent signs of defective erythropoiesis in the infected mice but rather indication of up-regulated hemolysis. Histological analyses on the spleen of infected mice demonstrated macrophages phagocytosing erythrocytes. Autoantibodies to erythrocytes or damages on erythrocyte membrane were not detected, implying those are not related the induced hemophagocytosis. The spleen was the major place for hemophagocytosis when compared with the liver and bone marrow, the other major tissues for parasitization; there, multinucleated giant cells heavily infected with amastigotes were markedly observed and were the major cell type phagocytosing erythrocytes. In addition, hemophagocytosis in the spleen and anemia of the infected mice were improved by treatment of the mice with an anti-leishmanial drug, suggesting that direct infection by parasites is one of the key factors causing hyper-activation of host macrophages to engulf blood cells. On the other hand, Ld-infected nu/nu mice didn't show anemia and had quite low frequency of hemophagocytosis. From this result, it is suggested that secondary signals from T-cells are also required for the hyper-activation. Taken together, these results suggest that heavy infection of macrophages with *Leishmania* parasites in the spleen triggers phagocytosis of erythrocytes resulting in anemia during experimental VL. Up-regulated hemophagocytosis occurs in several infectious diseases or familial diseases. However, the detailed mechanisms of infection-associated hemophagocytosis are not fully understood. In order to identify essential factors for generation of hemophagocytosis directly, to reproduce hemophagocytosis in vitro by *L. donovani* only or in the presence of other factors. In my master thesis, in vitro *L. donovani* infection-induced hemophagocytosis cell culture model was reproduced. Next, the mechanism of hemophagocytosis during VL was analyzed using this model and mouse model. Contrary to the result from nude mice, without any extra signal, stimulation with *L. donovani* only caused hemophagocytosis in vitro. Phagocytosis assay for polystyrene beads was performed and phagocytic activity to beads was not up-regulated in Ld-infected macrophages. From this result, it is revealed that hemophagocytosis

during VL is not up-regulation of universal phagocytosis. Alteration of gene expression levels in macrophages caused by *L. donovani* infection was analyzed by RNA-Seq to seek hemophagocytosis-related molecules. Expression levels of genes involved in familial hemophagocytin lymphohistiocytosis were not changed by the infection. Although the results from phagocytic activity assay implied that infected macrophages fail to recognize self RBC correctly, neither the mRNA levels of phagocytosis promoting nor inhibiting gene cluster was significantly changed after infection. Independent analysis of molecules involved in phagocytosis inhibiting was performed and Immunoblot of naïve/infected RAW264.7 demonstrated that SIRPα; one of the phagocytosis inhibiting receptors, was down-regulated in infected macrophages. Immunohistochemistry analysis revealed that in addition to in vitro *Leishmania*-infected RAW264.7, in MGCs in infected mice, SIRPα expression was also lower than other splenic monocytes/macrophages. Since contrary to down-regulation of SIRPα protein, mRNA level of *sirpα* is comparable between infected/uninfected macrophages, the mRNA levels of protease family which is responsible for SIRPα proteolysis were examined by RNA-Seq. although Adam10 which is known to proteolyse human SIRPα is up-regulated, Adam17 for mouse SIRPα is rather down-regulated. In order to examine the involvement of these protease to SIRPα proteolysis in infected macrophages, analysis at protein expression level or activity status is needed. From the results, It is suggested that *Leishmania donovani* infection down-regulate SIRPα in macrophages subsequently misrecognition of RBCs leading to hemophagocytosis by infected macrophages. However, whether if hemophagocytosis is harmful or beneficial to parasite remained elusive. Therefore, the survival of *Leishmania* in hemophagocytes was compared to those in non-hemophagocytes. By treat RBCs with glutaraldehyde, hemophagocytosis by RAW264.7 were artificially induced. These hemophagocytes and normal RAW264.7 were infected with *L. donovani*, and after 72h the number of amastigotes in both cells were compared. Both the ratio of infected macrophages with *L. donovani* in total macrophages and the number of amastigotes per infected macrophage were higher in the artificially induced hemophagocytes. In order to examine if RBCs in infected macrophages were digested and contributed to parasites survival, degradation status of RBCs was also tested on tissue section. Besides intact RBCs, there were the fragmented and colorless RBC or black depositions in MGCs, suggesting RBCs were considered to be degraded after engulfment by MGCs. In order to identify the

structure of black depositions, histopathological analysis was performed. Prussian blue staining was performed for ferrous iron, and the depositions were not stained. They were also not stained with Hall staining for biliverdin whereas disappeared by treatment with hydrogen peroxode. As a result, they were suggested to be not ferrous iron, biliverdin or other endogenous pigment (e. g. melanin pigment). In addition, since they have polarization which is corresponded with hemozoin or formalin pigment, the black deposition seems to have similar composition with heme. Next, In order to explore the change in environment of hemophagocytes between naïve RAW264.7 cells affects the survivability of *Leishmania*, mRNA level of one of the antioxidant molecules, *hmox1* was tested using artificially introduced hemophagocytosis assay. In RBC-supplemented and Ld-infected macrophages, the mRNA level of hmox1 was higher than Ld-only infected culture. These results implied that hemophagocytosis is beneficial for *Leishmania* survival by providing nutrients and low oxidative stress. Together, through the presented study, it was suggested that hemophagocytosis in VL has influence on symptoms of the disease through direct mechanism (i.e., anemia) or through indirect mechanisms by offering *Leishmania* parasites an incubator for growth. Further studies on this topic may contribute to not only understanding of symptoms other than anemia during VL but also elucidation of hemophagocytosis due to other causes. Moreover, in the era that antileishmanial chemotherapy is the main stream of treatment for VL, this study will offer the alternative choice for disease management by revealing molecular mechanisms of immunopathology for targets of symptom relief drugs.