論文の内容の要旨

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Study on the susceptibility of murine macrophages to *Leishmania major* infection
（マウスマクロファージのリーシュマニア原虫感受性に関する研究）

Macrophages are leukocytes which are present throughout the body. They develop from bone marrow precursors then mature and enter the bloodstream as monocytes. Monocytes are recruited into different tissues where they differentiate into tissue resident macrophages which perform important functions in the immune response. *Leishmania* is an intracellular protozoan parasite, and it causes leishmaniasis to humans and other mammalian animals. Phagocytic cells such as macrophages and dendritic cells are the host cells to *Leishmania* and the parasites proliferate inside of these cells. *Leishmania major*（*L. major*）causes cutaneous leishmnaiasis, and they develop lesion on the skin. Macrophages widely distribute in the body however, the infection is not observed in macrophages of every tissue. The skin, spleen and lymph nodes are major sites of parasite infection. Several experimental murine models demonstrated that the different susceptibility to *L. major* depends on the route of infection. Therefore, this site-specific immune response may define the susceptibility to *L. major*. However, it is still unclear what mechanisms would be involved in the variety immune responses to *L. major* in the same host. If the sites of infection determine the pathology and immune response induced by the infection then, it is required to know the mechanisms that might be involved in inducing the susceptibility to the infection. Hence, I hypothesized that macrophages may show tissue specific response to *L. major*, and the responses would relate to the parasite susceptibility.

In the first chapter, *L. major* infection was investigated using macrophages collected from two sites, lung alveoli and peritoneal cavity, in order to address if the susceptibility to *L. major* would be variety in macrophages distribute in different sites. The reason is that the infection is not reported in

the lung, while peritoneal macrophage（PMphi） are susceptible to *L. major* and the cells are used to experimental leihsmaniasis in murine model. Then, the collected macrophages were used to this study as an in vitro model. When macrophages were infected to *L. majo*r for four hr, 40 ± 5% of PMphi were infected by the parasites, and showed high levels of intracellular parasite. Interestingly, in contrast, fewer infected macrophages were observed in alveolar macrophages（AMphi）（26 ± 1%） and they displayed fewer intracellular parasites than that of PMphi. In addition, at the late phase of infection, 72 hr post-infection（p.i.）, infection rate and levels of intracellular parasite of PMphi were higher than those of AMphi. It is thus demonstrated that AMphi and PMphi had different susceptibility to *L. major*. Next, to evaluate the effect of the activation status on the resistance of macrophages, these macrophages were treated with lipopolisaccaride （LPS）, which one of the molecules stimulates macrophages. When macrophages were stimulated with LPS, PMphi showed higher levels of infection rate and held large number of intracellular parasites four hr p.i., compared to untreated control cells. On the contrary, in AMphi, LPS expose did not affect to the rate of infection and intracellular parasite. The regulation phagocytic activity of these macrophages was parasite specific, since identical uptake of latex beads was observed in both types of macrophages. In this chapter, different responses to *L. major* were observed in two different macrophages, AMphi and PMphi. These results suggest that macrophages distribute different sites would also show various response, and those variations might contribute to the establishment of tissue specific *L. major* infection.

The fact that macrophages distributed in two sites, lung alveoli and peritoneal cavity, showed different susceptibility to *L. major*, prompted to examine if macrophage derived factor（s） relate to the different susceptibility of macrophages to the parasites. IFN-γ was produced from macrophages, and identified as a cytokine required for not only Th1 cell differentiation but also macrophage activation to suppress the activity of infecting parasites. In an experimental murine leishmaniasis model, it was reported that the susceptibility of mice to *L. major* infection is due to the lack of Th1 responses related to IFN-γ production and the development of Interleukin（IL）-4 dominated Th2 responses. However, despite of the importance of IFN-γ in the resistance to *L. major* infection, several studies demonstrated that IFN-γ alone is not sufficient to control *L. major* and that additional factor（s） would be required for the development of protective immunity to *L. major*. IFN-γ is one of the macrophage-activating cytokine and activated macrophage is one of the principal source of IL-12. IL-12 is known as a cytokine involved in Th1 cell differentiation, and it stimulates NK cells to produce IFN-γ. Moreover, IL-12 induces autocrine macrophage activation and consequently IL-12 and IFN-γ production from the activated macrophages. Previously, it was reported the importance of IL-12 for resistance to *L. major* infection. It is therefore expected that both IFN-γ and IL-12 are directly related to behavior of *Leishmania* in infected macrophages. Cytokines such as IFN-γ enhanced nitric oxide（NO） production in macrophages by stimulating inducible nitric oxide synthase（iNOS） expression. NO plays an important role in clearance of various pathogens, and induction of NO production with IFN-γ from macrophages has been demonstrated in several pathogen including *Mycobacterium* and *Leishmnaia* to

control the growth of these intracellular pathogens. Therefore, it is expected that IFN-γ and IL-12 are directly related to the behavior of *Leishmania* in infected macrophages.

In the second chapter, to investigate the effect of IFN-γ and IL-12 on the susceptibility of macrophage to *L. major* infection, susceptible PMphi pretreated with IFN-g and/or IL-12 were infected with the parasites. Four hr p.i., PMphi treated with the combination of IFN-γ and IL-12（IFN-γ/IL-12） showed significantly lower levels of the rate of infection and intracellular parasite than those of the nontreated cells. However, PMphi treated with either IFN-γ or IL-12 did not show resistance to *L. major* infection. In addition, 72 hr p.i., the IFN-γ/IL-12-treated and IFN-γ-treated PMphi showed significantly lower levels of infection rate and intracelular parasite than those of the nontreated cells, and higher levels of resistance was observed in the IFN-γ/IL-12-treated PMphi than in the IFN-γ-treated PMphi. Although IFN-γ/IL-12 treatment of PMphi prior to the infection led to the induction of resistance, as described above, this resistance was not induced when these cytokines and the parasites were added simultaneously to PMphi culture. These results suggest that IFN-γ/IL-12 treatment prior to the infection restricts the early phase of the infection.

At the early point of infection, PMphi treated with IFN-γ/IL-12 showed low levels of NO production and few *L. major* infection. On the other hand, nontreated control PMphi produced little NO at similar levels as cytokine-treated cells, and they showed higher infection rate and intracellular parasite compared to cytokine treated cells. Since the NO production of nontreated PMphi was comparable levels as cytokine treated cells, the susceptibility of untreated PMphi at the early phase of infection seemed to be due to mechanisms that were not associated with the parasiticidal NO activity. The amount of NO would be at base level so that it would not be associated to parasite elimination. At the late phase of infection, cytokine treated PMphi produced high levels of NO comparing to untreated cells, and this production might relate to the resistance to *L. major* infection in the cytokine-treated cells. Hence, the cytokine-induced resistance and NO production would correlate to lead PMphi to the resistance to *L. major* at this late phase. In the second chapter, it is demonstrated that treatment of IFN-γ/IL-12 induce resistance to *L. major*, and the results indicated that the involvement of additional mechanisms in inducing resistance to *L. major* infection.

In the second chapter, it is showed that the combination of IFN-γ and IL-12 induced the resistance to *L. major* at the early phase of infection. Since the event occurring at the early phase is the invasion of the parasite to macrophages, receptor molecules associated to the binding between parasite and macrophages were focused on next study. Macrophages have a pivotal role in both recognition and response to the infection at the early phase, primarily due to their ability to phagocytose. They eliminate pathogen, and affect the activity of other cells that critical in the immune response. Phagocytosis involves receptors on macrophages and it is known that the receptor expression levels are variable in each macrophage. The entry of *L. major* into macrophages is mediated by the recognition of specific parasite ligands by receptors. Complement receptor 3（CR3）is highly expressed on a subset of macrophages. The surface of promastigotes is dominated by phosphoglycans（LPG）and the

glycoprotein, gp63, and these molecules have been shown to be important in binding, invasion and intracellular survival of the parasites in macrophages. *L. major* promastigotes bind macrophages through these molecules mediated by CR3. Hence, CR3 would be strongly involved in parasite binding to macrophages in serum dependent and independent mechanism. It has been demonstrated that some cytokine affect receptor expression on macrophages**,** however, it is not known if IFN-γ and IL-12 affect expression of the receptors that involved in parasite binding to macrophages and the resistance to the infection.

In the third chapter, to investigate association of the receptor expression levels and the susceptibility to *L. major* infection, CR3 expression on macrophages were examined under normal and cytokine treated condition. Surface expression of CR3 on AMphi and PMphi were examined using fluorescence activated cell sorting（FACS）. PMphi showed high CR3 expressing phenotype, and as expected, quite low levels of CR3 expression was observed on AMphi. Next, to examine if the resistance of cytokine-treated macrophages associates to CR3 expression level, the expression of CR3 on macrophages were observed following their treatment with IFN-γ and IL-12. Treatment with both IFN-γ and IFN-γ/IL-12 did not affect the expression of CR3 on PMphi comparing to nontreated PMphi. These results suggest that intracellular mechanisms, which regulate expression of other surface molecules, would be associated to inhibition of the parasite invasion that was induced by the cytokines. The result by flow cytometric analysis suggests the possibility that the different susceptibility to *L. major* infection in AMphi and PMphi would be related to the expression levels of CR3 on these macrophages. And the resistance of cytokine treated PMphi would associate to other factor derived from macrophages.

In this study, it is demonstrated that macrophages distributed in different site showed various susceptibility to *L. major*. Macrophages distributed in the lung where *L. major* infection is not documented, displayed resistance to the infection with low expression of CR3 on its surface. Susceptible macrophages resistance to *L. major* by the treatment of IFN-γ/IL-12 prior to the infection, the resistance was not involved CR3 mediated mechanisms. Direct association of the receptor and the infection has not been demonstrated. However, the resistance in each macrophage would be mediated by different mechanisms.

The mechanisms of site-specific parasite infection remain poorly understood. In this study, it was found that macrophages distributed in different sites show different susceptibility to *L. major* infection, and AMphi were resistant comparing to PMphi. Also, susceptible PMphi induced resistance to the parasite by the treatment with IFN-γ/IL12 prior to the infection. However, the observed resistance would involve different mechanisms. Further study on cytokine production and receptor expression in the tissues including the lung would provide better understanding of the factors critical for development of protective immunity and establishment of site-specific infection. And that will help to know the mechanisms of development of the susceptibility and the resistance of macrophages to *Leishmania* infection.