博士論文

Studies on experimental visceral leishmaniasis

(実験的内臓型リーシュマニア症に関する研究)

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Abbreviations

bp	base pair(s)
CytB	cytochrome b
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
h	hour(s)
KO	knock out
min	minute(s)
PBS	phosphate buffer saline
PBS-T	phosphate buffer saline-tween
PCR	polymerase chain reaction

General introduction

Leishmaniasis is vector-borne and largely zoonotic disease caused by parasitic protozoan of the genus *Leishmania*. Leishmaniasis typically presents as one of the two forms, either cutaneous (Fig. 1A) or visceral (Fig. 1B). Cutaneous leishmaniasis (CL) produces skin ulcer with raised border which may last months to years and typically result in severe scarring (1, 2). In contrast, visceral leishmaniasis (VL) affects the internal organs especially the spleen, liver and bone marrow (3).

Leishmania parasites are transmitted by the bite of blood sucking sandfly vectors (4), either *Phlebotomus* species in the Old World or *Lutzomia* species in the New World, resulting in the parasite inoculation to the skin of mammalian hosts. The parasite has two developmental forms. The flagellated promastigote (Fig. 2A) is transmitted with the bite of the sandfly to the mammalian host where it transforms into the amastigote (Fig. 2B), which replicates in the phagolysosome of macrophage (Fig. 3).

Leishmaniasis is endemic in the tropical and subtropical regions of 88 countries, with an estimated total of 350 million people at risk (5, 6) (Fig. 4). The WHO estimates that 12 million cases exist worldwide. VL is the most severe form of the diseases, which is caused by members of the *Leishmania donovani* complex (*L. donovani*, *L. infantum*). Five hundred thousand new cases of VL occur each year and over 90% of the VL cases are concentrated in five countries, India, Bangladesh, Nepal, Sudan and Brazil (7-9). Based on transmission characteristics, there are two types of VL, zoonotic VL caused by *L. infantum*, and anthroponotic VL caused by *L. donovani* (10, 11). VL is a systemic infection clinically characterized by prolonged fever, profound cachexia, hepato-splenomegaly, hypergammaglobulinemia and debilitation (12, 13), with an incubation period varying between 2 and 6 months, and is the only fatal form in the

leishmaniasis (14).

The diagnosis of VL is based on parasite detection methods and antibody-detection tests. The visualization of the amastigote form of the parasite by microscopic examination of aspirates from the bone marrow or spleen for VL is the classical confirmatory test (15, 16). However, spleen aspiration for VL can be complicated by life-threatening hemorrhages in some individuals and therefore requires considerable technical expertise as well as facilities for nursing (17). The detection of parasites in the organs by culture or detection of parasites DNA by PCR is more sensitive than microscopic examination but these techniques remain restricted to referral hospitals and research centers (18). The most used serological methods for diagnosis of VL are direct agglutination test (DAT) (19) and enzyme linked immunosorbent assay (ELISA) (20, 21). Two types of antigens, crude soluble antigen obtained by destruction of live promastigotes and a recombinant antigen with 39-amino acid repeat which is part of a kinesin-related protein in *L. chagasi* (rK39), can be used for diagnosis of VL (22). An immuno-chromatographic strip using rK39 antigen is commonly used for diagnosis (23, 24). In Bangladesh, patients are usually diagnosed as VL with following four factors; 1: Living in the endemic area of VL, 2: long-lasting fever for more than two weeks, 3: hepato-splenomegaly and 4: *Leishmania* positive or rK39 dipstick positive.

The pathogenesis of VL has not been well elucidated. It is one cause that there is no good mouse model in VL. In experimental CL, the most widely studied model of CL is the BALB/c mouse infected with *L. major*. BALB/c mice are susceptible to *L. major* infection and develop an exacerbating skin lesion (Figs. 5A, B). This mouse model is widely used for studying of pathogenesis, immunological study and evaluating of candidate compounds of anti-leishmanial drugs and vaccines. Accordingly, this mouse

model is a good model for studying of CL. However, mice infected with *L. donovani*, the most commonly studied model of VL, do not develop overt, progressive disease. Accordingly, these mouse models showed self-healing (25, 26). Compared to mice, hamsters seem to a better model for progressive VL expressing clinical features like hepato-splenomegaly, immunosuppression, anemia, cachexia, and death, closely simulating VL (27-31). However, a major disadvantage of this hamster model of VL is that immunological studies in this model have been limited by lack of available tools (32, 33) like various monoclonal antibodies, gene knockout animals and so on. Consequently, to study the pathogenesis and immunological study of VL, it needs to develop mouse model for exacerbation during *L. donovani* infection that resembles VL in human patients.

Two functionally distinct T helper (Th) cell subsets, Th1 and Th2, have been defined based on the cytokine profile elicited by antigen stimulation (34). Th1 cells, which produce interferon-gamma (IFN-γ) and interleukin (IL)-2, are often associated with cell-mediated immune responses including macrophage activation, whereas Th2 cells, which produce IL-4, IL-5 and IL-10, are associated with overall enhanced humoral immunity (35). In experimental CL, inbred mouse strains with different genetic backgrounds have been utilized to elucidate immunological responses associated with resistance or susceptibility to *Leishmania* infection. The most well established model is *L. major* infection. BALB/c mice are susceptible to *L. major* infection and develop an exacerbating skin lesion (36-38). In contrast, C57BL/6 mice are relatively resistant to the infection (36-38). Comparison of inbred mice has revealed that antigen-specific Th1 and Th2 immune responses are associated with resistance and susceptibility to *L. major* infection, respectively (39-45) (Fig. 6). However, in experimental *L. donovani* infection,

there is no well-established mouse model representing contrasting disease outcomes during *L. donovani* infection. Therefore, the relationship between Th1/Th2 balance and susceptibility is less clear in experimental VL.

Since 1950s, pentavalent antimonials have been the recommended drugs for the treatment of leishmaniasis, although amphotericin B and its formulations have also been used. These therapies are not ideal due to toxicity, the long duration of administration and the high cost (46) (Table 1). Furthermore, reports have indicated that a large proportion of cases have become unresponsive to traditional chemotherapy (47). Recently, the emergence of antimony-resistant parasites has been reported (48-50), which has compelled the search for new antileishmanial agents. In previous reports, there are many reports about evaluating of a therapeutic compound for anti-leishmaniasis. However, these reports are used not severe infection models and evaluated only leishmanicidal effect of the compound (51-53). Experimental studies in *L. infantum* and *L. donovani*-infected Syrian hamsters (*Mesocricetus auratus*) often reveal several clinical signs of progressive VL that closely mimic active VL (27-31). Therefore, hamster model is a good model for evaluating therapeutic compounds and vaccines for anti-leishmaniasis. However, there is a lack of reagents for immunological analysis in the hamster model of VL (32, 33). Taking these factors into account, I consider the Syrian hamster to not be a suitable model for immunological studies and the evaluation of immunization strategies. Recently, there are some problems after treatment of VL. There are some cases of relapse of VL after chemotherapy in patients (54, 55), and some patients show post kala-azar dermal leishmaniasis (PKDL) (56, 57). PKDL is characterized by development of skin lesions after the treatment of kala-azar. Then, the immunological aspects are not less clear after chemotherapy of VL. To

evaluate activity of therapeutic candidate compounds and vaccines for anti-leishmaniasis accurately, and to study the immunological aspects after chemotherapy and immunization strategies, we need VL mouse model which is not self-limiting, showed severe infection and can be evaluated leishmanicidal effect, improvement of clinical manifestation and immunological analysis.

The goal of this investigation was to develop a mouse model of *L. donovani* infection that shows non-healing, progressive disease (Chapter 1). And I aimed at developing mouse model of *L. donovani* infection for distinct disease phenotypes in inbred mice, and demonstrated that distinct Th1/Th2 patterns are associated with the different disease outcomes in experimental VL (Chapter 2). Furthermore, I examined the usefulness of this mouse model for evaluating new therapeutic compounds for anti-leishmaniasis (Chapter 3).

Chapter 1

L. donovani-infected BALB/c mice reproduced exacerbating infection that resembles progressive visceral leishmaniasis.

Abstract

Visceral leishmaniasis (VL) is the only fatal form among the leishmaniases. However, the pathogenesis of VL has not been well elucidated. Mice infected with *L. donovani*, the most commonly studied model of VL, do not develop overt, progressive disease. To study the pathological and immunological aspects of VL, it needs to develop mouse model for exacerbation during *L. donovani* infection that resembles VL in human patients. Here, I examined infectivity of *L. donovani* D10 in BALB/c mice, and monitored organ weights and parasite loads. BALB/c mice were inoculated intraperitoneally with *L. donovani* promastigotes. In parasite burden, infected mice showed the continuous parasite growth in the spleen and liver and progressive hepato-splenomegaly over the 12 weeks of infection. BALB/c mice infected with *L. donovani* had approximately 8 times weight of the spleen compared with these of uninfected mice at 12 weeks post infection. I have successfully developed mouse model for exacerbation during *L. donovani* infection that resembles progressive VL.

1.1. Introduction

Visceral leishmaniasis (VL) is caused by members of the *Leishmania donovani* complex (*L. donovani*, *L. infantum*). Five hundred thousand new cases of VL occur each year and over 90% of the VL cases are concentrated in five countries: India, Bangladesh, Nepal, Sudan and Brazil (7-9). VL is a systemic infection clinically characterized by prolonged fever, profound cachexia, hepato-splenomegaly, hypergammaglobulinemia and debilitation, with an incubation period varying between 2 and 6 months (10, 11), and is the only fatal form in the leishmaniases. However, the pathogenesis of VL has not been elucidated. It is one cause that there is no a good mouse model in VL.

According to previous studies, the most widely studied model of VL is the BALB/c mice infected with *L. donovani*. There is an early increase parasite burden, but particularly over the course of 4-8 week the *Leishmania* parasites are eliminated in the liver and the infection is controlled by host immune response (25, 26). In other words, there is no mouse model for exacerbation during *L. donovani* infection that resembles active VL in human patients. In contrast to mice, Syrian hamsters (*Mesocricetus auratus*) are also widely used experimental model of VL. *L. donovani* and *L. infantum*-infected Syrian hamsters reproduce several clinical signs (hypergammaglobulinemia, hepato-splenomegaly, anemia, cachexia and immunodepression) that closely mimic VL (27-31). However, there is a lack of reagents for immunological analysis in the hamster model of VL (32, 33). Mice is possible to study more detail and various immunological analysis compared with hamsters. Therefore, to elucidate the pathogenesis and to analyze more detail immunological studies about VL, it needs to develop mouse model for progressive disease. In a previous report, Pandey et al. have reported that *L. donovani* D10 strain from a

Nepalese VL patient was infective to BALB/c mice (58). Here, I examined infectivity of *L. donovani* D10 in BALB/c mice, and monitored organ weights and parasite loads, and histopathological studies.

Leishmaniasis is vector-borne disease by the bite of blood-sucking female sand fly vectors. Therefore, parasitemia (amastigotes circulate in blood) with *L. donovani* is common in VL (Fig. 7). In previous reports, *Leishmania* amastigotes were detected in blood smear of VL patients (59, 60). However, mechanism of parasitemia in patients with VL is less clear. Furthermore, there is no report of demonstration of parasitemia in experimental VL. Consequently, to study the mechanism of parasitemia in patients with VL, it needs to experimental animal model which can be demonstrated parasitemia.

The goal of the chapter 1 was to develop a mouse model of *L. donovani* infection which shows non-healing, progressive disease. Especially, I tried to achieve continuous growth and severe infection in the spleen and liver, because that is what has not been achieved in existing mouse models. Furthermore, to demonstrate parasitemia in this mouse model, *Leishmania* parasites were isolated from peripheral blood of mice infected with *L. donovani*.

1.2. Materials and Methods

1.2.1. Mice

Male BALB/c mice were purchased from Japan Clea, Tokyo, Japan. All mice were maintained under specific pathogens-free (SPF) condition. Mice were used for experiments at the age from 6-8 weeks. All experimental and animal care procedures were approved by the guideline of the University of Tokyo.

1.2.2. Parasites

Promastigotes of *Leishmania donovani* D10 (MHOM/NP/03/D10) were obtained from Institute of Tropical Medicine Nagasaki University (NBRP), Nagasaki, Japan (58). Promastigotes of *L. donovani* were cultured in TC199 medium (Nissui Pharmaceutical, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Sciences, USA) and 25 mM HEPES buffer (MP biomedical, UK) at 25°C. Promastigotes in stationary phase were used for all experiments.

1.2.3. Infection of mice

For experimental infections, BALB/c mice were inoculated intraperitoneally with 1×10^8 *L. donovani* promastigotes. At 2, 4, 8 and 12 weeks post infection BALB/c mice were euthanized.

1.2.4. Determination of spleen and liver parasite burden

The spleen and liver were taken for evaluation of organ weight and determination of parasite burden. Spleen and liver multiple impression smears were prepared and stained

with Giemsa. Organ parasite burden, expressed as Leishman Donovan units (LDU) (25), were represented as the number of amastigotes/ host cell nuclei ×organ weight (mg). To further evaluate whether the spleen and liver contained live parasites, the parasite burden was quantified in these tissue by serial dilution assay (61). Briefly, a weighed piece of spleen or liver from infected mice was first homogenized in complete TC199 medium with 100 U/ml penicillin and 100 μg/ml streptomycin (Dainippon Pharma, Japan) and 50 U/ml gentamycin (Schering-Plough Pharma, Japan), and then diluted with the same medium to a final concentration of 1 mg/ml. Ten-fold serial dilutions of the homogenized tissue suspensions were then plated in 96-well plates (NUNC, Denmark) and incubated at 25°C for 2 weeks. Wells were examined for viable and motile promastigotes, and the reciprocal of the highest dilution that was positive for parasites was considered to be the parasite concentration per mg of organ. The total organ parasite burden was calculated using the weight of the respective organs.

1.2.5. rK39 immuno-chromatographic test (ICT)

At 12 weeks post infection, plasma was collected. Plasma of uninfected BALB/c mice was collected as controls. Antibody against rK39 was examined by rK39 immuno-chromatographic test (ICT) (Kala-azar Detect™ Rapid Test for Visceral leishmaniasis) (InBios International, Seatle, USA), as recommended by the manufacturer.

1.2.6. Histopathology

The spleens and livers were collected 12 weeks post infection and tissues were fixed with 20% neutral buffered formalin and then embedded in paraffin. The

paraffin-embedded tissues were sectioned at 4 μm thick. The tissues were stained with Mayer's hematoxylin solution (WAKO, Osaka, Japan) for 1 min, and rinsed in running tap water for 30 min. Next, the tissues were stained with eosin solution (MUTO PURE CHEMICALS CO., LTD., Tokyo, Japan) for 2 min.

1.2.7. Immunohistochemical staining

Paraffin-embedded sections from the spleen and liver were used for the staining with C11C monoclonal antibody (mAb) (mouse IgG1). C11C mAb was anti-*Leishmania* thiol-specific antigen (TSA) (62) mAb, was produced in our previous study (unpublished data). Paraffin-embedded tissues were cut at 4 μm in thickness. Sections of paraffin-embedded tissues were dewaxed with xylene (three times for 5 min each time) and then 100%, 90%, 80% and 70% (for 5 min each time) ethanol. Then sections were washed with PBS(-) for 10 min. Hydrated sections were then treated with methanol containing 0.3% H_2O_2 for 30 min. Sections were washed three times with PBS(-) for 2 min each time and with PBS-T for 1 min. The sections were incubated with Block Ace® (Dainippon Pharmaceutical, Suita, Japan) for 10 min prior to incubation with each primary antibody. After 1 h 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, Tokyo, Japan). After 1 h, sections were washed three times PBS(-) for 2 min each time and reacted with substrates, 3,3' -diaminobenzine (DAB; Nichirei) was used as a substrate for HRP. Then sections were counterstained with hematoxylin solution (WAKO, Osaka, Japan) for 10 sec, and rinsed in running tap water for 30 min.

1.2.8. Detection of *Leishmania* parasites DNA by *LSUrDNA* gene from blood of *L. donovani*-infected BALB/c mice

At 2, 4, 8 and 12 weeks post infection, peripheral blood was collected. Peripheral blood of uninfected BALB/c mice was collected as controls. For PCR analysis buffycoat from *L. donovani*-infected BALB/c mice were examined for detection of *Leishmania* parasites DNA. DNA was extracted by DNeasy®Blood and Tissue Kit (QIAGEN Group), as recommended by the manufacturer. For PCR for detection *Leishmania* DNA in peripheral blood samples of *L. donovani*-infected BALB/c mice, two sets of primers were designed using *L. donovani Large-Subunit rDNA* (*LSUrDNA*) gene. Nucleotide sequences of *L. donovani LSUrDNA* gene retrieved from GenBank from *L. donovani* HU3 (MHOM/ET/67/HU3) were aligned manually. The designed primers were: *L. donovani LSUrDNA* forward (5'-GGC GGG CAA CGA AGT GCA AGA AT -3') and *L. donovani LSUrDNA* reverse (5'-GCA CAC TCC AAC GCA ACC CAC GG -3'). PCR amplification was carried out 40 cycles using 2 µl of DNA sample as the template, 50 pmol of primers, 10×PCR buffer, 2 mM dNTPs, 25 mM $MgCl_2$ and 1.25 U Taq DNA polymerase (Applied Biosystems, USA) in the buffer recommended by manufacturer. The temperature of first denaturing was at 95°C for 5 min, followed by 40 cycles of denaturing at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR was carried out in a thermal cycler. Five micro liters of the PCR were analyzed in a 1.5% agarose gel containing ethidium bromide (0.25 µl/ml). Molecular size markers were the 1,000 bp markers from Invitrogen (USA). The PCR products were visualized under UV light in a transilluminator, and photographed with a digital camera.

1.2.9. Isolation of *Leishmania* parasites from peripheral blood of *L. donovani*-infected BALB/c mice

At 2, 4, 8 and 12 weeks post infection, peripheral blood was collected. The parasite burden in peripheral blood was quantified by limiting dilution culture. One hundred μl of peripheral blood from infected mice was diluted with complete TC199 medium with 100 U/ml penicillin and 100 μg/ml streptomycin, and 50 U/ml gentamycin. Ten-fold serial dilutions of the peripheral blood suspensions were then plated in 96-well plates and incubated at 25°C for 2 weeks. Wells were examined for viable and motile promastigotes, and the reciprocal of the highest dilution that was positive for parasites.

1.2.10. Statistical analysis

Data were expressed as mean with standard deviation. Student's *t* test was employed to assess the significance of differences between groups. Differences were considered statistically significant at *P* values of < 0.05.

1.3. Results

1.3.1. Continuous parasite growth and progressive hepato-splenomegaly in *L. donovani*-infected BALB/c mice

At 2, 4, 8 weeks and 12 weeks after infection, mice were sacrificed to take the spleen and liver. The parasite burden was determined by amastigotes levels in the spleen and liver and quantitated as LDU in organs stamp smear. Amastigotes were observed in the spleen and liver stamp smear of *L. donovani*-infected BALB/c mice (Fig. 8A). The LDU increased progressively in the both organs over this time period (Figs. 8B, C). The spleen LDU was 805±121 and the liver LDU was 3,139±489 at 12 weeks post infection. Furthermore, the parasite burden was determined by limiting dilution assay (Figs. 9A, B). The growth of parasites was progressive in both the spleen and the liver over the 12 weeks of infection. *L. donovani*-infected BALB/c mice had massively enlarged spleen and liver compared with these of the uninfected (Fig. 10A). Splenomegaly and hepatomegaly in BALB/c mice caused by *L. donovani* infection were progressive over the 12 weeks of infection (Figs. 10B, C). In infected mice, the spleen weights were approximately 8 times and the liver weights enlarged approximately 1.4 times compared with these of uninfected at 12 weeks post infection.

1.3.2. Detection of *Leishmania* specific antibody (antibody against rK39) by rK39 immuno-chromatographic test

Detection of *Leishmania* specific antibody in plasma samples of *L. donovani*-infected BALB/c mice was attempted by ICT. Plasma from uninfected BALB/c mice was used as negative control for ICT detection. At 12 weeks post infection, two bands were

detected from test and control line in all plasma samples of *L. donovani*-infected BALB/c mice (Fig. 11). *Leishmania* specific antibody was detected from all *L. donovani*-infected BALB/c mice. In uninfected BALB/c mice, one band was detected from control line.

1.3.3. Histopahological and immunohistchemistry analysis

In histopathological analysis, amastigotes were observed and increased in host cells of spleens and livers of *L. donovani*-infected BALB/c mice at 12 weeks post infection (Figs. 12A, B). In spleens, host cells invaded and increased in white and red pulp, and amastigotes were localized in red pulp macrophages. In liver, infiltration of mononuclear cells was observed in *L. donovani*-infected BALB/c mice (Fig. 12C), and amastigotes were localized in kupffer cells. Immunohistochemical staining was performed with C11C mAb. A large number of C11C positive cells (it means amastigotes) were found in the spleens and livers of infected BALB/c mice (Figs. 12D, E).

1.3.2. Demonstration of parasitemia in *L. donovani*-infected BALB/c mice

Detection of *Leishmania* parasites DNA in peripheral blood samples of *L. donovani*-infected BALB/c mice was attempted by PCR. *LSUrDNA* was selected for targeting gene. DNA from *L. donovani* D10 promastigotes was used as positive control, and DNA from uninfected BALB/c mice blood was used as negative control for PCR detection. At 4 weeks post infection, *Leishmania* DNA were detected in peripheral blood of *L. donovani*-infected BALB/c mice (Fig. 13A). Electrophoresis of the PCR product from all positive peripheral blood samples showed one expected band size of

approximately 310 bp in length. The intensity of the band showed gradually strong. Furthermore, the parasite burden in the blood of *L. donovani*-infected BALB/c mice was determined by limiting dilution culture assay. The lower limit of culture quantification was one parasite per 100 μl of blood. At eight weeks post infection, the promastigotes were detected in peripheral blood culture of *L. donovani*-infected BALB/c mice (Figs. 13B, C). The parasite burden in the blood increased between 8 and 12 weeks post infection.

1.4. Discussion

According to previous studies, BALB/c mice have been used for studies on *L. donovani* infection as VL mouse model. Although this strain is considered to be susceptible and the infection progresses during the first four weeks, the infection is then controlled by the host immune response (25, 26). However, VL presents a spectrum of clinical manifestations from a self-controlled infection to progressive disease. The mouse model is comparable to self-controlled oligosymptomatic cases and therefore is useful for the study of the protective immune response. In other words, there is no mouse model for progressive disease. These factors result in lack of understanding the pathogenesis of active human VL. Interestingly, inoculating BALB/c mice with Indian *L. donovani* strains AG83 leads to progressive VL with clinical manifestations of hepato-splenomegaly (63, 64). This mouse model does not show high parasite burden in the spleen and liver and severe hepato-splenomegaly. In contrast, present mouse model showed the continuous parasite growth and high parasite burden compared with previous studies in spleen and liver (Figs. 8B, C, 9A, B), progressive splenomegaly and hepatomegaly (Figs. 10B, C) and severe infection, at least up to 12 weeks post infection. I have successfully developed mouse model for exacerbation during *L. donovani* infection that resembles progressive VL in BALB/c mice. Accordingly, this mouse model can be useful to study the pathogenesis of progressive VL.

In detection of *Leishmania* specific antibody by rK39 ICT, all infected mice showed positive bands (Fig. 11). Therefore, present mouse model is useful for searching new specific *Leishmania* antigens for diagnosis. The rK39 antigen is specific for finding VL patients with high sensitivity (23, 24, 65-68). Currently reports, other kinesin-related

proteins (rK26 (65), rK9 (65), rKRP42 (69) and rKE16 (70)) are also available for diagnosis of VL. Furthermore, other recombinant antigens also were reported. For example, heat shock proteins (rHSP70 (71-73), rGRP78 (74) and rHSP83 (75, 76)), nuclear proteins (rlepp12 (77), rpaplee22 (78) and rH3 (79, 80)) and other antigens (rA2 (81, 82) and rPeroxiredoxin (83)) used for the diagnosis of VL. However, these recombinant antigens are available for finding the VL patients. It is quite important point for diagnosis tools, but it needs other types of diagnostic tools. Recently, there are some problems after treatment of VL, such as relapse of VL after chemotherapy in patients (54, 55). Therefore, it needs to develop diagnostic tools to distinguish complete cure and re-infection from relapse markers. I hope that present mouse model contributes for finding new diagnostic antigens and new diagnostic tools such as cure or relapse indicators.

Leishmaniasis is vector-borne disease by the bite of blood-sucking female sand fly vectors. Therefore, parasitemia with *L. donovani* is common in VL (Fig. 7). In previous reports, in India, amastigotes are seen in peripheral blood smear in 67 to 79% of patients (84, 85), and cultures of peripheral blood are positive in 96 to 97% of patients (86, 87). In China, at least one pre-treatment blood culture was positive in 31 of 34 patients, and at least one pre-treatment blood smear was positive in all nine patients studied (59). In these reports, parasitemia showed quietly high percentage in VL patients. However, mechanism of parasitemia in patients with VL is less clear. Furthermore, there is no report of parasitemia in experimental VL. Consequently, to study the mechanism of parasitemia in patients with VL, it needs to animal model that can be demonstrated parasitemia. *Leishmania* parasites were isolated from peripheral blood of *L. donovani*-infected BALB/c mice (Fig. 13B). *Leishmania* parasites were detected after 12 weeks

post infection. After 12 weeks post infection, *Leishmania* parasites were progressively increasing in spleen and liver, and *L. donovani*-infected BALB/c mice showed progressive hepato-splenomegaly. Therefore, it is suggested that parasitemia commonly occurs in progressive VL patients. But *Leishmania* parasites were also detected from peripheral blood after 8 weeks post infection in *L. donovani*-infected BALB/c mice. After 8 weeks post infection, *L. donovani*-infected BALB/c mice did not show high parasite growth and progressive hepato-splenomegaly. Therefore, it is suggested that parasitemia often occurs in VL patients who do not show progressive infection. To my knowledge, this is first report on parasitemia is demonstrated in experimental animals. Accordingly, this mouse model can be useful to study the mechanism of parasitemia in patients with VL.

Chapter 2

Susceptibility is correlated with Th2 response and resistance is correlated with Th1 response in experimental visceral leishmaniasis

Abstract

In experimental cutaneous leishmaniasis caused by *Leishmania major*, resistance in C57BL/6 mice is associated with a Th1 immune response, whereas progressive disease in BALB/c mice correlated with a Th2 response. However, in experimental *L. donovani* infection, the situation is different. According to previous studies, in both mouse strains, the infection is self-limiting. To understand immunological aspects of visceral leishmaniasis (VL), it needs to develop mouse model of *L. donovani* infection distinct disease phenotypes, susceptible to resistant. Here, I examined infectivity of *L. donovani* D10 in three inbred mouse strains, i.e., BALB/c, C57BL/6 and C3H/HeN, and monitored organ weights and parasite loads. BALB/c mice showed the continuous parasite growth in the spleen and the liver, associated with progressive hepato-splenomegaly over the 24 weeks of infection. In contrast, C57BL/6 mice did not show progressive parasite growth and hepato-splenomegaly. Moreover, there were no parasites detected in spleen and liver, and tissue enlargement was not found in C3H/HeN mice. To examine immune responses associated with the susceptibility/ resistance, interferon-gamma (IFN-γ) and interleukin (IL)-4 productions by spleen cells upon recall with *L. donovani* lysate antigen were measured at 12 weeks post infection. Spleen cells from BALB/c mice produced the lowest level of IFN-γ and the highest level of IL-4 among the tested. C57BL/6 and C3H/HeN mice showed Th1-dominant responses compared with BALB/c mice. To my knowledge, this is the first report on *L. donovani* infection which leads to clearly distinct disease outcomes in inbred mice with different backgrounds.

2.1. Introduction

Two functionally distinct T helper (Th) cell subsets, Th1 and Th2, have been defined based on the cytokine profile elicited by antigen stimulation (34). Th1 cells, which produce interferon-gamma (IFN-γ) and interleukin (IL)-2, are often associated with cell-mediated immune responses including macrophage activation, whereas Th2 cells, which produce IL-4, IL-5 and IL-10, are associated with overall enhanced humoral immunity (35). In experimental cutaneous leishmaniasis (CL), inbred mouse strains with different genetic backgrounds have been utilized to elucidate immunological responses associated with resistance or susceptibility to *Leishmania* infection. The most well established model is *L. major* infection. BALB/c mice are susceptible to *L. major* infection and develop an exacerbating skin lesion (36-38). In contrast, C57BL/6 mice are relatively resistant to the infection. Although *L. major* can cause a cutaneous lesion in C57BL/6 mice, the infection is self-limiting (36-38). Resistant mice develop a Th1 response characterized by production of IFN-γ and IL-2 and the mice heal their lesions, whereas susceptible mice exhibit a predominant Th2 phenotype, associated with IL-4, IL-5 and IL-10 production. Therefore, antigen-specific Th1 and Th2 immune responses are associated with resistance and susceptibility to *L. major* infection, respectively (39-45) (Fig.6). However, in experimental *L. donovani* infection, the situation is a little different. According to previous studies, BALB/c and C57BL/6 mice have been used for studies on *L. donovani* infection. In both mouse strains, there is an early increase parasite burden, but particularly over the course of 4-8 week the *Leishmania* parasites are eliminated in the liver and the infection is controlled by host immune response (25, 26, 88-91). Accordingly, in both mouse strains, the infection is self-limiting and is not

significantly different. In other words, there is no mouse model which shows clearly distinct disease outcomes in inbred mice with different backgrounds. Therefore, the relationship between Th1/Th2 balance and susceptibility is less clear in experimental VL.

The goal of the chapter 2 was to develop a mouse model of *L. donovani* infection for distinct disease phenotypes, susceptible to resistant in inbred mice, and demonstrated that distinct Th1/Th2 patterns are associated with the different disease outcomes in experimental VL.

2.2. Materials and Methods

2.2.1. Mice

Male BALB/c, C57BL/6 and C3H/HeN mice were purchased from Japan Clea, Japan. All mice were maintained under specific pathogens-free condition. Mice were used for experiments at the age from 6-8 weeks. All experimental and animal care procedures were approved by the guideline of the University of Tokyo.

2.2.2. Parasites

Leishmania donovani D10 (MHOM/NP/03/D10) were obtained from Institute of Tropical Medicine Nagasaki University (NBRP), Nagasaki, Japan (58). Promastigotes of *L. donovani* were cultured in TC199 medium (Nissui Pharmaceutical, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Sciences, USA) and 25 mM HEPES buffer (MP biomedical, UK) at 25°C. Promastigotes in stationary phase were used for all experiments.

2.2.3. Infection of mice

For experimental infections, BALB/c, C57BL/6 and C3H/HeN mice were inoculated intraperitoneally with 1×10^8 *L. donovani* promastigotes. At 12 weeks post infection BALB/c, C57BL/6 and C3H/HeN mice were euthanized, and at 24 weeks post infection BALB/c and C57BL/6 mice were euthanized.

2.2.4. Determination of spleen and liver parasite burden

The spleen and liver were taken for evaluation of organ weight and determination of

parasite burden. Multiple impression smears from the spleen and liver were prepared and stained with Giemsa. Organ parasite burden, expressed as Leishman-Donovan units (LDU) (25), were represented as the number of amastigotes/ host cell nuclei ×organ weight (mg). To further evaluate whether the spleen and liver contained live parasites, the parasite burden was quantified in these tissue by serial dilution assay (61). Briefly, a weighed piece of spleen or liver from infected mice was first homogenized in complete TC199 medium with 100 U/ml penicillin and 100 μg/ml streptomycin and 50 U/ml gentamycin, and then diluted with the same medium to a final concentration of 1 mg/ml. Ten-fold serial dilutions of the homogenized tissue suspensions were then plated in 96-well plates and incubated at 25°C for 2 weeks. Wells were examined for viable and motile promastigotes, and the reciprocal of the highest dilution that was positive for parasites was considered to be the parasite concentration per milligram of organ. The total organ parasite burden was calculated using the weight of the respective organs.

2.2.5. *In vitro* restimulation assay to determine production of IFN-γ and IL-4

Spleen cell suspensions were prepared from infected mice at 12 weeks post infection and uninfected mice. Cells were adjusted to final concentration of 1×10^6 cells/ml in RPMI1640 culture medium (Corning, USA) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 U/ml gentamycin, and plated at 0.2 ml in 96-well round-bottomed tissue culture plate (SIGMA-ALDRICH, USA). Spleen cell suspensions were received 10 μg *L. donovani* SLA/ml at the time of culture initiation. Supernatants were harvested 72 h later and were assayed for cytokine activity.

2.2.6. Analysis of cytokines by ELISA

Cytokine concentrations in the splenocyte culture supernatants of infected and uninfected mice, in response to *L. donovani* SLA stimulation, respectively, were determined by sandwich ELISA (eBioscience, USA), as recommended by the manufacturer. IFN-γ and IL-4 production were determined in infected and uninfected mice.

2.2.7. Statistical analysis

Data were expressed as mean with standard deviation. Student's *t* test was employed to assess the significance of differences between groups. Differences were considered statistically significant at *P* values of <0.05.

2.3. Results

2.3.1. Progressive infection in BALB/c mice and self-limiting infection in C57BL/6 mice

At 12 weeks and 24 weeks post infection, mice were sacrificed to harvest the spleen and liver. The parasite burden was determined by amastigotes levels in the spleen and liver and quantitated as LDU in organs stamp smear. Amastigotes were observed in the spleen and liver stamp smear of *L. donovani*-infected BALB/c and C57BL/6 mice. In BALB/c mice, the LDU increased progressively in the both organs over this time period (Figs. 14A, B). Furthermore, the parasite burden was determined by limiting dilution assay (Figs. 15A, B). The growth of parasites was progressive in both the spleen and liver over the 24 weeks of infection. BALB/c mice had massively enlarged spleen and liver compared with those of the uninfected. Splenomegaly and hepatomegaly in BALB/c mice caused by *L. donovani* infection were progressive over the 24 weeks of infection (Figs. 16A, B). In C57BL/6 mice, the spleen and liver LDU at 12 weeks was much lower than that of BALB/c mice (Figs. 14A, B). Moreover, the liver LDU declined between 12 and 24 weeks, and no parasites detected in the liver stamp smears at 24 weeks post infection (Fig. 14B). Although parasites in the spleen were detectable at 24 weeks in C57BL/6, the parasite burdens did not show any increase over time as seen in BALB/c mice. The spleen and liver weights were also changed over 24 weeks of *L. donovani* infection in both BALB/c mice and C57BL/6 mice. In conjunction with parasite burdens, development of pathology was also limited in C57BL/6J mice. For the spleen, BALB/c and C57BL/6 mice manifested enlargement of the tissue at 12 weeks. In contrast to BALB/c mice, however, C57BL/6 mice did not show progressive

splenomegaly at 24 weeks of infection (Fig. 16A). For the liver, tissue enlargement was not found at all in C57BL/6 mice during the experimental period (Fig. 16B).

2.3.2. C3H/HeN mice are the most resistant to *L. donovani* infection

At 12 weeks post infection, BALB/c, C57BL/6 and C3H/HeN mice were sacrificed to harvest the spleen and liver. The parasite burden was determined by amastigotes levels in the spleen and liver and quantitated as LDU in organs stamp smear. In contrast to the susceptible BALB/c mice and self-limiting C57BL/6 mice, there were no parasites in either the spleen or liver of C3H/HeN mice (Figs. 17A, B). Furthermore, there were no live parasites in both organs (Figs. 18A, B). The enlargement of the spleen and liver at 12 weeks post infection, BALB/c mice showed progressive hepato-splenomegaly and C57BL/6 mice showed transient splenomegaly. At 12 weeks of infection, BALB/c mice and C57BL/6 mice had spleen sizes 9 times and 4 times bigger than those of the uninfected mice, respectively. In clear contrast, either the spleen or liver of infected C3H/HeN mice was not significantly different from the uninfected mice, even at 12 weeks (Figs. 19A, B).

2.3.3. Cytokine production by spleen T cells

The cytokine patterns of spleen cells derived from *L. donovani*-infected BALB/c, C57BL/6 and C3H/HeN mice were analyzed at 12 weeks post infection. To explore immunological mechanisms behind the different disease outcomes in the three inbred mouse strains, spleen cells were prepared from mice at 12 weeks of *L. donovani* infection, and the production of IFN-γ and IL-4 by those cells upon antigen recall were analyzed. Cells from *L. donovani*-infected mice produced IFN-γ; there were remarkable

differences in their levels during *L. donovani* infection. C3H/HeN (5.34±1.18 ng/ml) and C57BL/6 (3.93±2.18 ng/ml) mice produced significantly higher IFN-γ levels than BALB/c (1.93±0.26 ng/ml) mice in response to *Leishmania* antigen (Fig. 20A). In *Leishmania* antigen-induced IL-4 production, BALB/c (47.31±18.84 pg/ml) mice produced significantly higher IL-4 levels than C3H/HeN (11.55±5.65 pg/ml) and C57BL/6 (3.06±2.46 pg/ml) mice in response to *Leishmania* antigen (Fig. 19B). Susceptible BALB/c mice showed the lowest IFN-γ and the highest IL-4 production. Self-limiting C57BL/6 mice and resistant C3H/HeN mice showed Th1-dominant responses compared with BALB/c mice.

2.4. Discussion

According to previous studies, the mouse model of CL caused by *L. major* has demonstrated that BALB/c mice showed susceptible model and C57BL/6 mice showed self-limiting model. The mouse model of CL has demonstrated that control of the disease severity is associated with the expression of IFN-γ, whereas progression is correlated with IL-4 and IL-10 production in the lymphoid tissues (39-45). These studies have indicated that Th1 cells are responsible for the development of a protective immune response, whereas the expansion of Th2 cells seems to be responsible for the progression of the disease. In contrast to the well-understood immunopathologic mechanisms in CL, the relationship between Th1/Th2 balance and susceptibility is less clear in experimental VL. According to previous VL mouse model; there is no mouse model which shows clearly distinct disease outcomes in inbred mice with different backgrounds. BALB/c and C57BL/6 mice have been used for studies on *L. donovani* infection. In both mouse strains, the infection progresses during the first four weeks, the infection is then controlled by host immune response (25, 26, 88-91). Accordingly, in both mouse strains, the infection is self-limiting and is not significantly different. In this study, I have successfully developed mouse model of *L. donovani* infection for distinct disease phenotypes, from susceptible to resistant in this study. As mentioned above, *L. donovani*-infected BALB/c mice showed the continuous parasite growth in the spleen and the liver and progressive hepato-splenomegaly over this time period (Figs. 14-16). In C57BL/6 mice, the spleen and liver parasite burden was lower than that of BALB/c mice (Figs. 14, 15). Moreover, the liver parasite burden declined between 12 and 24 weeks post infection. The spleen weight had increased 12 weeks after infection, but

C57BL/6 mice did not show progressive splenomegaly at 24 weeks of infection (Fig. 16A). It is suggested that *L. donovani*-infected C57BL/6 mice showed self-limiting infection. In C3H/HeN mice, *Leishmania* parasites were not detected from the spleen and the liver (Figs. 17, 18). Moreover, tissue enlargement was not found at all in the spleen and the liver (Fig. 19). Hence, I established susceptible, healing and resistant infection such as CL mouse model.

According to previous cytokine studies in experimental VL, while euthymic *L. donovani*-infected BALB/c mice are able to control infection with granuloma formation and IFN-γ and IL-2 production, nude BALB/c mice neither form granulomas nor produce IFN-γ (89). Furthermore, anti-IFN-γ antibody abolishes granuloma formation (26), confirming the importance of this cytokine in protection. The role of IL-4 as a cytokine related to susceptibility has been reported in experimental CL (39-45). However, most studies on experimental VL had raised this question about the role of IL-4 in susceptibility. In one study, besides predominant IFN-γ production in the initial and late phase of infection, IL-4 production was detected in the intermediary phase coinciding with peak of parasite burden in the susceptible strain, and no IL-4 production in the resistant mouse strain (92). Nevertheless other studies contradicted these findings. No IL-4 production was observed in inbred mice strains infected with *L. donovani* (93), and in liver, only IFN-γ RNA was detected by Northern blot, and both Th1 and Th2 cytokine mRNAs, IL-4, IL-10, IFN-γ and IL-2 mRNA were detected by PCR (88). Furthermore, mice treated with anti-IL-4 monoclonal antibodies (88) and mice with IL-4 gene disruption (94) did not show better control of the infection. In previous studies, the role of IL-4 as a cytokine related to susceptibility almost has not been reported in experimental VL. Because, I think that there is no mouse model for

exacerbation during *L. donovani* infection. In contrast to these reports, I demonstrated that distinct Th1/Th2 patterns are associated with the different disease outcomes (Fig. 21). IFN-γ is one of the indicators of Th1 response, which level of C3H/HeN and C57BL/6 mice is higher than BALB/c mice (Fig. 20A). In contrast, IL-4 is one of the indicators of Th2 response, which level of BALB/c mice is higher than C3H/HeN and C57BL/6 mice (Fig. 20B). Accordingly, self-limiting C57BL/6 mice and resistant C3H/HeN mice showed Th1-dominant responses compared with susceptible BALB/c mice. It is possible that these immune responses are just the result, and not the cause of the different outcomes. However, the fact that distinct disease outcomes have been achieved in *L. donovani* infection means immunological studies can be accelerated as they were for *L. major*.

In previous VL studies, there are used several cytokine knockout mice and depletion of cytokine expression using several monoclonal antibodies. *L. donovani*-infected IL-12 KO mice and *L. donovani*-infected mice which were treated anti-IL-12 antibody showed continuous parasite growth in the spleen and liver (90, 95, 96). From these reports, IL-12 is crucial cytokine for control the *L. donovani* infection. In contrast to these reports, other Th1-related cytokines (IFN-γ, IL-2 and TNF-α) was not showed crucial cytokines for control in previous reports. *L. donovani*-infected IFN-γ and TNF-α mouse and *L. donovani*-infected mice which were treated anti-IFN-γ and IL-2 antibodies showed higher parasite burden compared with naïve mice, but liver parasite burden declined from the peak and parasites were eliminated in the liver (26, 97-100). It is sure that these cytokines are related to suppress parasite growth. However, it is thought that more clear difference appears if my mouse model is used. Therefore, this mouse model can be useful to understand the crucial cytokines or other factors of control the *L.*

donovani infection.

Furthermore, new strategies of protective immunity were proposed in current CL studies. The degree of protection against *L. major* infection in mice is predicted by the frequency of $CD4^+$ T cells simultaneously producing IFN-γ, IL-2 and TNF-α (101). Notably, multifunctional effector cells generated by all vaccines tested are unique in their capacity to produce high amounts of IFN-γ. These data show that the quality of a $CD4^+$ T-cell cytokine response can be a crucial determinant in whether a vaccine is protective. It may provide a new and useful prospective immune correlate of protection for vaccines based on Th1 cells. Therefore, my mouse model can be useful to analyze various immunological studies and to propose new strategies of protective immunity of VL.

In summary, I established susceptible, self-limiting and resistant infection model. These mouse models can be useful to understand the factors of control the *L. donovani* infection, and may provide an improved platform to understand pathogenesis and protective immunity for *L. donovani* infection.

Chapter 3

Experimental *in vivo* mouse model for examining activity of anti-leishmanial drugs.

Abstract

The pentavalent antimonials are widely used to treat leishmaniasis. However, the use of these compounds is limited by toxicity to the host and the development of resistance by the parasites. Therefore, new drug candidates are urgently needed. To evaluate activity of anti-leishmanial drugs and therapeutic candidate compounds accurately, we need VL mouse model which is not self-limiting, showed severe infection and can be evaluated leishmanicidal effect and improvement of clinical manifestation. The main goal of this study was to examine the usefulness of this mouse model for evaluating new therapeutic compound for anti-leishmaniasis. I focused on quinone-terpenoids derived from *Sargassum yamadae*. First, the growth inhibition was evaluated against promastigotes about ten quinone-terpenoids. Sargaquinoidatic acid remarkably showed 100% growth inhibition. The in vivo experiments were carried out about sargaquinoidatic acid using BALB/c mice infected with *Leishmania donovani*. The infected mice were treated with sargaquinoidatic acid by intraperitoneally route. The treated and untreated mice were monitored parasite loads and organ weights. In treated group, amastigotes were not detected in the spleen and liver stamp smear. Furthermore, live parasites were not isolated from treated group by culture method. The untreated mice showed progressive hepato-splenomegaly. In contrast, organ weights decreased before treatment, and hepato-splenomegaly was healed in treated mice. From these results indicated that leishmanicidal effect showed against amastigotes and clinical manifestations improved. Accordingly, this mouse model can be useful to evaluate candidate compounds of antileishmanial drugs.

3.1. Introduction

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis, characterized by prolongs fever, splenomegaly, hepatomegaly, pancytopenia, progressive anemia and debilitation. If untreated, VL is uniformly fatal. Thus, improvements to the therapeutic regimen, the quality of diagnosis and disease control are needed.

In light of the current clinical scenario, the development of new drugs is desirable. For more than 50 years, heavy metal derivatives, mainly pentavalent antimonials, have been used as standard drugs for the treatment of leishmaniasis. Among them, the most representative are sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime). Other types of drugs, amphotericin B and its formulations, aminosidine and pentamidine have also been used. These therapies are not ideal due to toxicity, the long duration of administration and the high cost (46) (Table 1). Furthermore, reports have become unresponsive to traditional chemotherapy (47). Recently, the emergence of antimony-resistant parasites has been reported (48-50). In addition, the participation of the host immune response may negatively affect treatment and disease progression. Several new antileishmanial compounds are under development, although a drug with the capacity to completely cure this infection has yet to be discovered (102). Therefore, there is an urgent need to discover novel agents, as the efficacy of the currently available drugs is declining. In previous reports, there are many reports about evaluating of a therapeutic compound for anti-leishmaniasis. However, these reports are used not severe infection models and evaluated only leishmanicidal effect of the compound (51-53). To evaluate activity of anti-leishmanial drugs and therapeutic candidate compounds accurately, we need VL mouse model which is not self-limiting, showed

severe infection and can be evaluated leishmanicidal effect and improvement of clinical manifestation.

In an effort to find new drugs for leishmaniasis, my research has focused on quinone-terpenoids (**a**-**j**) (Fig. 22) derived from *Sargassum yamadae*, a brown alga Sargassaceae species. The growth inhibition was evaluated against promastigotes about ten quinone-terpenoids. Sargaquinoidatic acid (**c**) showed highest growth inhibition *in vitro*.

The goal of the chapter 3 was to examine the usefulness of this mouse model for evaluating new therapeutic compound for anti-leishmaniasis, I examined in vivo efficacy of sargaquinoidatic acid in *L. donovani*-infected BALB/c mice.

3.2. Materials and methods

3.2.1. Parasites

Leishmania donovani D10 (MHOM/NP/03/D10) were obtained from Institute of Tropical Medicine Nagasaki University (NBRP), Nagasaki, Japan (58). Promastigotes of *L. donovani* and were cultured in TC199 medium (NISSUI Pharmaceutical, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Sciences, USA) and 25 mM HEPES buffer (MP biomedical, UK) at 25°C. Promastigotes in stationary phase were used for all experiments.

Expression vector, p6.5 was constructed by Dr. Chang KP., University of Health Science, Chicago Medical School. The PCR products of GFP cDNA were inserted into the *Bam*H I site of p6.5, which contains a tunicamycin-resistance gene, and designated as p6.5-GFP. The p6.5-GFP expression vector was transfected into *L. donovani* D10 promastigotes by electroporation. Promastigotes of GFP-expressing *L. donovani* (*L. donovani/egfp*) were cultivated in 199 medium (NISSUI Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum, 25 mM Hepes buffer (MP biomedical, UK) and 10 μg/ml tunicamycin (SIGMA-ALDRICH, inc., USA).

3.2.2. Compounds and drug

Quinone-terpenoid compounds (**a**-**j**) derived from marine plants *Sargassum yamadae*, a brown alga Sargassaceae species (Fig. 22). Quinone-terpenoid compounds were extracted by Dr. Junji Kimura., Aoyama Gakuin University, Kanagawa, Japan. Quinone-terpenoid compounds were dissolved in tissue culture-grade dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml and then diluted at 10 μg/ml with

culture medium. Culture medium containing 1 % DMSO was used as a control medium. Amphotericin B was used as a control to compare the quinone-terpenoid compounds, and solubilized in DMSO and then diluted at 10 μg/ml with culture medium.

3.2.3. Anti-leishmanial assay *in vitro*

To each well of 96-well plates containing 100 μL of *L. donovani/egfp* suspension with 1×10^6 cells/mL, 100 μL of test solution (sample dissolved in DMSO) was added, and the plates were incubated at 25°C for 72 h. Fluorescence signals of *L. donovani/egfp* promastigotes in 96-well black microplate (NUNC, Denmark) were measured with a fluorescence microplate reader (Fluoroscan Ascent FL, Dainippon Pharmaceutical Co., Japan) with excitation at 485 nm and emission at 538 nm. To determine the growth inhibition rate of *L. donovani/egfp* by quinone-terpenoid compounds, *L. donovani/egfp* were cultured at 1×10^6 cells/mL with 10 μg/ml compounds, and their fluorescence was measured after 72 h incubation.

3.2.4. Mice

Male BALB/c mice were purchased from Japan Clea, Japan. All mice were maintained under specific pathogens-free condition. Mice were used for experiments at the age from 6-8 weeks. All experimental and animal care procedures were approved by the guideline of the University of Tokyo.

3.2.5. Infection and treatment regiment

BALB/c mice were inoculated intraperitoneally with 1×10^8 *L. donovani* promastigotes. At 49 days post infection, the mice received a single daily dose of the sargaquinoidatic

acid (**c**) intraperitoneally. The treatment lasted for 30 days. First 14 days were used 1 mg/head; the remaining 16 days were used 2 mg/head. Liposomal amphotericin B (AmBisome®; Dainippon Pharmaceutical, Japan, 200 μg/head) was used as a control to compare with the sargaquinoidatic acid. Animals in the negative control group received the same volume of the vehicle (saline/DMSO). Animals were sacrificed next day after the completion of treatment (79 days post infection). At 49 and 79 days post infection the animals were euthanized, and the spleens and livers were taken for evaluation of organ weight and determination of parasite burden.

3.2.6. Determination of the spleen and liver parasite burden

The spleens and livers were taken for evaluation of organ weight and determination of parasite burden. Multiple impression smears of the spleen and liver were prepared and stained with Giemsa. Organ parasite burden, expressed as Leishman Donovan units (LDU) (25), were represented as the number of amastigotes/ host cell nuclei ×organ weight (mg). To further evaluate whether the spleen and liver contained live parasites, the parasite burden was quantified in these tissue by serial dilution assay (61). Briefly, a weighed piece of spleen or liver from infected mice was first homogenized in complete TC199 medium with 100 U/ml penicillin and 100 μg/ml streptomycin (Dainippon Pharma, Japan) and 50 U/ml gentamycin (Schering-Plough Pharma, Japan), and then diluted with the same medium to a final concentration of 1 mg/ml. Ten-fold serial dilutions of the homogenized tissue suspensions were then plated in 96-well plates (NUNC, Denmark) and incubated at 25°C for 2 weeks. Wells were examined for viable and motile promastigotes, and the reciprocal of the highest dilution that was positive for parasites was considered to be the parasite concentration per milligram of organ. The

total organ parasite burden was calculated using the weight of the respective organs.

3.2.7. Detection of *Leishmania* parasites DNA by *LSUrDNA* gene from the spleen, liver and blood of sargaquinodatic acid treated mice

At 49 and 79 days post infection, the spleens, livers and peripheral blood were collected, and these samples of uninfected BALB/c mice were collected as controls. For PCR analysis spleens, livers and buffycoat from sargaquinodatic acid treated mice were examined for detection of *Leishmania* parasites DNA. DNA was extracted by DNeasy®Blood and Tissue Kit (QIAGEN Group), as recommended by the manufacturer. For PCR for detection *Leishmania* DNA in the spleen, liver and peripheral blood samples of sargaquinodatic acid treated mice, and two sets of primers were designed using *L. donovani Large-Subunit rDNA* (*LSUrDNA*) gene. Nucleotide sequences of *L. donovani LSUrDNA* gene retrieved from GenBank from L. donovani HU3 (MHOM/ET/67/HU3) were aligned manually. The designed primers were: *L. donovani LSUrDNA* forward (5'-GGC GGG CAA CGA AGT GCA AGA AT -3') and *L. donovani LSUrDNA* reverse (5'-GCA CAC TCC AAC GCA ACC CAC GG -3'). PCR amplification was carried out 40 cycles using 2 µl of DNA sample as the template, 50 pmol of primers, 10×PCR buffer, 2 mM dNTPs, 25 mM $MgCl_2$ and 1.25 U Taq DNA polymerase (Applied Biosystems, USA) in the buffer recommended by manufacturer. The temperature of first denaturing was at 95°C for 5 min, followed by 40 cycles of denaturing at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR was carried out in a thermal cycler. Five micro liters of the PCR were analyzed in a 1.5% agarose gel containing ethidium bromide (0.25 µl/ml). Molecular size markers were the 1,000 bp markers from

Invitrogen (USA). The PCR products were visualized under UV light in a transilluminator, and photographed with a digital camera.

3.2.8. Histopathology

The spleens and livers were collected 49 days and 79 days post infection and tissues were fixed with 20% neutral buffered formalin and then embedded in paraffin. The paraffin-embedded tissues were sectioned at 4 μm thick. The tissues were stained with Mayer's hematoxylin solution (WAKO, Osaka, Japan) for 1 min, and rinsed in running tap water for 30 min. Next, the tissues were stained with eosin solution (MUTO PURE CHEMICALS CO., LTD., Tokyo, Japan) for 2 min.

3.2.9. Statistical analysis

Data were expressed as mean with standard deviation. Student's *t* test was employed to assess the significance of differences between groups. Differences were considered statistically significant at *P* values of < 0.05.

3.3. Results

3.3.1. *In vitro* antileishmanial evaluation

To evaluate growth inhibition against promastigotes for ten quinone-terpenoids, growth inhibition of these compounds at 10 μg/ml was examined by measuring fluorescence intensity using *L. donovanir/egfp* promastigotes (Fig. 23A). Six among the ten samples had over 50% growth inhibition activity, and sargaquinoidatic acid (**c**) remarkably showed 100% growth inhibition equal to amphotericin B against *L. donovani/egfp* promastigotes (Fig. 23B).

3.3.2. Treatment with sargaquinoidatic acid causes complete clearance of spleen and liver parasite burden of *L. donovani*-infected mice

To evaluate antileishmanial activity of sargaquinoidatic acid, the spleens and livers were harvested for determination of parasite burden. The parasite burden was determined by amastigotes levels in the spleen and liver and quantitated as LDU in organs stamp smear. The LDU increased progressively in the both organs at 49 days post infection. At 79 days post infection, in all mice of untreated group, the spleen and liver LDU increased progressively compared with at 49 days post infection. In contrast, amastigotes were not detected from sargaquinoidatic acid and AmBisome treated group (Figs. 24A, B). To detect the live parasites in these organs of treated mice, these organs were harvested for determination of parasite burden using culture method. In all mice of untreated group, spleen and liver parasite number increased compared with at 49 days post infection. In contrast, live parasites were not isolated from sargaquinoidatic acid and AmBisome treated groups (Figs. 25A, B).

3.3.3. Detection of *Leishmania* parasites DNA by *LSU rDNA* gene from the spleen, liver and blood of sargaquinoidatic acid treated mice

Detection of *Leishmania* parasites DNA in the spleen, liver and peripheral blood samples of sargaquinoidatic acid treated mice was attempted by PCR. *LSUrDNA* was selected for targeting gene. DNA from *L. donovani* D10 promastigotes was used as positive control. In initiation of treatment (Day 49), *Leishmania* DNA was detected in the spleen, liver and peripheral blood of all groups. At 79 days post infection, in non-treatment group, *Leishmania* DNA was detected all samples. The intensity of the band showed gradually strong. In contrast, in sargaquinoidatic acid and AmBisome treatment groups, *Leishmania* DNA was not detected from spleen, liver and blood samples (Figs. 26-28).

3.3.4. Histopathological analysis of the spleen and liver in mice with sargaquinoidatic acid treatment

In histological analysis, in initial of treatment (Day49), macrophages infected *Leishmania* parasites were observed in spleen and liver of *L. donovani*-infected mice. At 79 days post infection, in non-treatment group, macrophages infected amastigotes were observed. In contrast, in sargaquinoidatic acid and AmBisome treatment groups, macrophages infected with *Leishmania* parasites were not observed in the spleen (Fig. 29) and liver (Fig. 30).

3.3.5. Complete cure is induced by sargaquinoidatic acid treatment in the spleen and liver of *L. donovani*-infected BALB/c mice

To evaluate improvement clinical manifestations, the spleen and liver weights were

measured. At 49 days post infection, infected mice had enlarged spleen and liver compared with the uninfected. At 79 days post infection, in all mice of untreated group, these organ weights increased compared with at 49 days post infection. In contrast, spleen (Figs. 31A, B) and liver (Fig. 31C) weights decreased before treatment in sargaquinoidatic acid and AmBisome treated mice.

3.4. Discussion

The visceral form of leishmaniasis remains therapeutic challenges and serious public health problems. Pentavalent antimonials are the most commonly used drugs for the treatment of leishmaniasis, although the existence of drug-resistant infections and the fact that these drugs need to be administered parenterally represent major limitations for leishmaniasis chemotherapy. Unfortunately, ideal therapies for leishmaniasis have yet to be identified (46).

In this study, I focused on quinone-terpenoids (**a**-**j**) derived from *Sargassum yamadae*, a blown alga Sargassaceae species. *In vitro* antileishmanial evaluation, growth inhibition of these compounds at 10 μg/ml was examined. Six among the ten samples showed over 50% growth inhibition activity (Figs. 23B). It is suggested that these compounds from this seaweed have high inhibition activity against *Leishmania* parasites. Then, sargaquinoidatic acid (**c**) remarkably showed 100% growth inhibition. To evaluate growth inhibition against amastigotes, *L. donovani*-infected BALB/c mice were treated with sargaquinoidatic acid. In sargaquinoidatic acid treated group, amastigotes were not observed in the spleen and liver stamp smears (Figs. 24A, B), and live parasites were not isolated from treated group by culture method (Figs. 25A, B). Furthermore, in sargaquinoidatic acid treated group, even *Leishmania* parasites DNA was not detected from spleen, liver and blood (Figs. 26-28). In histological analysis, in sargaquinoidatic acid treated group, macrophages infected with *Leishmania* parasites were not observed in spleen (Fig. 29) and liver (Fig. 30). These results indicated that leishmanicidal effect showed against amastigotes. Therefore, it is suggested that sargaquinoidatic acid treatment effected complete clearance of parasites in the spleen and liver of *L.*

donovani-infected BALB/c mice. The untreated mice showed progressive hepato-splenomegaly. In contrast, spleen and liver weights decreased before treatment, and hepato-splenomegaly was healed in sargaquinoidatic acid treated mice (Figs. 31A, B), and progressive hepato-splenomegaly improved. From these results, it is suggested that complete cure is induced by sargaquinoidatic acid treatment in the spleen and liver of *L. donovani*-infected BALB/cA mice. Quinone-terpenoids derived from *Sargassum yamadae* showed high growth inhibition *in vitro*, and sargaquinoidatic acid showed effective growth inhibition *in vivo*. Accordingly, sargaquinoidatic acid and these derivatives are promising as new drug candidate compounds for leishmaniasis.

In previous reports, there are many reports about evaluating of a therapeutic compound for anti-leishmaniasis. However, these reports were used mouse model which is shown healing and not severe infection. Furthermore, these reports evaluated only leishmanicidal effect of the compounds (51-53). To evaluate activity of anti-leishmanial drugs and therapeutic candidate compounds accurately, we need better VL animal model which is not self-limiting, showed severe infection and can be evaluated leishmanicidal effect and improvement of clinical manifestation. In contrast, hamsters seem to a better model for progressive VL expressing clinical features and death (27, 28). The hamster model is more suitable to evaluate activity of therapeutic candidate compounds. However, hamster model is difficult to analysis immunological aspects. There are some problems after treatment of VL. There are some cases of relapse (54, 55), and some patients show post kala-azar dermal leishmaniasis (PKDL) (56, 57). PKDL is characterized by development of skin lesions after the treatment of kala-azar. Then, the immunological aspects are not less clear after chemotherapy of VL. Also hamster model is suitable to evaluate efficacy of vaccines. However, it needs not only to evaluate

efficacy of vaccines but also to evaluate immunization strategies in aspect of development of vaccines. Therefore, not only to evaluate activity of therapeutic candidate compounds and vaccines for anti-leishmaniasis accurately but also to study the immunological aspects after chemotherapy and vaccination, we need VL mouse model better than hamster model. Then, my mouse model is not healed, showed progressive manifestation and severe infection. From results of this study, my mouse model can be evaluated leishmanicidal effect and improvement of clinical manifestations. Accordingly, this mouse model can be useful to evaluate candidate compounds of antileishmanial drugs and vaccines, and to study the immunological aspects after chemotherapy and vaccination.

Conclusion

Visceral leishmaniasis (VL) is the only fatal form among the leishmaniasis (14). However, the study of VL has not been well elucidated, and VL is not controlled at all. Because, it is one of the causes that there is no good mouse model in VL. According to previous studies, the most widely studied model of VL is the BALB/c mice infected with *L. donovani*. There is an early increase parasite burden, but particularly over the course of 4-8 week the *Leishmania* parasites are eliminated in the liver and the infection (25, 26). In other words, there is no mouse model for exacerbation during *L. donovani* infection that resembles progressive VL in human patients. If the good mouse model which shows progressive disease is developed, it is suggested that mouse model widely contributes to studies of the VL (understanding the pathogenesis, immunological studies and development of new candidate antileishmanial drugs and vaccines). In this study, the useful mouse model which contributes for studying the progressive VL was developed.

In chapter 1, I have successfully developed mouse model for exacerbation during *L. donovani* infection that resembles progressive VL in BALB/c mice. *L. donovani*-infected BALB/c mice showed the continuous parasite growth in spleen and liver, progressive hepato-splenomegaly, at least up to 24 weeks post infection. Leishmaniasis is vector-borne disease by the bite of blood-sucking female sandfly vectors. Therefore, parasitemia is common feature in VL. However, there is no report of parasitemia in experimental animals. Then, to demonstrate parasitemia in this mouse model, *Leishmania* parasites were isolated from peripheral blood of *L. donovani*-infected BALB/c mice. To my knowledge, this is first report on progressive VL achieved in

inbred mice, and demonstrated parasitemia in experimental animals.

In chapter 2, I revealed that antigen-specific Th1 and Th2 immune responses are associated with resistance and susceptibility to *L. donovani* infection. In experimental CL, the most well established model is *L. major* infection. BALB/c mice are susceptible to *L. major* infection. In contrast, C57BL/6 mice are relatively resistant to the infection. Comparison of inbred mice has revealed that antigen-specific Th1 and Th2 immune responses are associated with resistance and susceptibility to *L. major* infection, respectively (39-45). However, there is no well-established mouse model representing contrasting disease outcomes during *L. donovani* infection. Therefore, the relationship between Th1/Th2 balances is less clear in experimental VL. In this study, I developed clearly susceptible and resistant or healing mouse model. *L. donovani*-infected BALB/c mice showed the continuous parasite growth and progressive hepato-splenomegaly over this time period. In C57BL/6 mice, *Leishmania* parasites were detected from each organ, but the parasite number declined in the time between 12 and 24 weeks post infection. The spleen weight had increased 12 weeks post infection, but the spleen weight did not increase between 12 and 24 weeks post infection. It is suggested that *L. donovani*-infected C57BL/6 mice showed healing infection. Furthermore, in C3H/HeN mice, *Leishmania* parasites were not detected from both organ and both organ weights did not increase. In cytokine production studies, IFN-γ is one of the indicators of Th1 response, which level of C3H/HeN and C57BL/6 mice is higher than BALB/c mice. In contrast, IL-4 is one of the indicators of Th2 response, which level of BALB/c mice is higher than C3H/HeN and C57BL/6 mice. Accordingly, resistance is correlated with Th1 and susceptibility is correlated with Th2 in experimental VL. To my knowledge, this is first report on *L. donovani* infection which leads to clearly distinct disease

outcomes in inbred mice with genetic backgrounds.

In chapter 3, to examine the usefulness of this mouse model for evaluating new therapeutic candidate compounds for anti-leishmaniasis, I examined in vivo efficacy of sargaquinoidatic acid in *L. donovani*-infected BALB/c mice. In sargaquinoidatic acid treated group, amastigotes were not observed in the spleen and liver stamp smears, and live parasites were not isolated by culture method. Furthermore, in sargaquinoidatic acid treated group, spleen and liver weights decreased before treatment. From these results, it is suggested that complete clearance of parasites and complete cure induced by sargaquinoidatic acid treatment in *L. donovani*-infected BALB/c mice. This mouse model shows progressive and severe infection, and can be evaluated leishmanicidal effect and improvement of clinical manifestation. Accordingly, this mouse model can be useful and more accurate to evaluate candidate compounds of antileishmanial drugs. Furthermore, it is suggested that this mouse model can be evaluated not only antileishmanial drugs but also vaccines for VL.

In summary, I developed VL mouse model for the progressive disease observed in human patients, and mouse model representing contrasting disease outcomes during *L. donovani* infection. This mouse model can be useful to study the basic researches such as studying of pathological and immunological aspects, and the applied researches such as development of diagnosis and evaluating new candidate antileishmanial drugs and vaccines. Hence, I believe that this mouse model can be accelerated study of VL, and I hope that this mouse model contributes for the control on human VL.

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Table

Table 1. Drugs used to treat visceral leishmaniasis and their principal limitations

Pentavalent antimonials Sodium stibogluconate (Pentostam) Meglumine antimonate (Glucantime)	Toxicity (liver and pancreas, especially in HIV patients) Drug resistance (up to 65% in Bihar, India) Cost (US$ 120-150) Parenteral administration (i.m. or i.v.) Prolonged treatment (up to 4 weeks)
Polyene antibiotics Amphotericin B Desoxycholate formulation (Fungizone) Lipid liposomal formulation (AmBisome)	High toxicity (hypokalaemia, renal failure, metabolic acidosis, cardiotoxicity) Cost (Fungizone US$ 60-150;AmBisome > US$ 1000) Parenteral administration (slow i.v. infusion) Prolonged hospitalization
Aminoglycoside antibiotics Paromomycin sulphate (Aminosidine)	Toxicity (nephrotoxicity, nerve deafness) Cost (US$ 50) Parenteral administration (i.m.) Prolonged treatment (up to 3 weeks)
Ether lipid analogues Hexadecylphosphorylcholine (Miltefocine)	Toxicity (teratogenicity: contraindicated in pregnant women) Cost (US$ 50) Ease of resistance (long half life of drug)

Figure legends

Fig. 1

Clinical manifestation of leishmaniasis. Ulcer formation on cutaneous leishmaniasis patient (A). Splenomegaly of visceral leishmaniasis patient (B).

Fig. 2

Two morphological forms of *Leishmania* parasites. Promastigotes form in the insect (A). Amastigotes form in mammalian host (B). Stamp smears were stained with Giemsa. Scale bar: 10 μm

Fig. 3

Life cycle of *Leishmania* parasites. *Leishmania* parasites exist as an extra cellular flagellated promastigote in the sandfly vector and an intracellular amastigote of in the macrophage of the susceptible vertebrate host.

Fig. 4

Worldwide distribution of leishmaniasis. Blue area: distribution of visceral leishmaniasis. Red area: distribution of cutaneous leishmaniasis.

Fig. 5

BALB/c mice are susceptible to *L. major* infection and develop an exacerbating skin lesion. Skin lesion from *L. major*-infected BALB/c mice (A). Scale bar: 1 cm. Progression of skin lesion size in BALB/c mice infected *L. major* (B).

Fig. 6

Susceptibility is correlated with Th2 and resistance is correlated with Th1 in experimental cutaneous leishmaniasis.

Fig. 7

Presence of amastigotes (parasitemia) in blood of visceral leishmaniasis patients.

Fig. 8

The spleen and liver parasite loads in *L. donovani*-infected BALB/c mice. Spleen and liver stamp smear of *L. donovani*-infected BALB/c mice (A). Stamp smears were stained with Giemsa. Scale bars: 10 μm. Arrows: amastigotes. The spleen (B) and liver (C) parasite burden by Leishman Donovan units (LDU) in *L. donovani*-infected BALB/c mice. LDU was represented as the number of amastigotes/ host cell nuclei ×organ weight (mg). Means and SD in each group are shown. *P<0.01.

Fig. 9

The spleen and liver parasite loads in *L. donovani*-infected BALB/c mice. The spleen (A) and liver (B) parasite burden were determined by limiting dilution assay. Total parasite number was represented as the reciprocal of highest dilution positive for promastigote (the parasite concentration per milligram of tissue) × total weight (mg).

Fig. 10

Progressive hepato-splenomegaly in *L. donovani*-infected BALB/c mice. Spleen from uninfected mouse and a 12-week infected mouse (A). Changes in the spleen (B) and

liver (C) weights over the course of infection in BALB/c mice infected with *L. donovani*. Means and SD in each group are shown. * $P < 0.05$, ** $P < 0.01$.

Fig. 11

Detection of *Leishmania* specific antibody by rK39 ICT. Detection of antibody against rK39 in plasma samples of *L. donovani*-infected BALB/c mice. The upper band is control line and the lower band is test line. Plasma of *L. donovani*-infected BALB/c mice showed two bands. In contrast, uninfected BALB/c mice showed just control line.

Fig. 12

Light micrographs of spleen (A) and liver (B, C) in BALB/cA mice during infection with *L. donovani*. Paraffin-embedded sections were prepared at the 12 weeks post infection and stained with hematoxylin and eosin. Scale bar: 50 μm (insets: 10 μm). Arrows indicated amastigotes (A, B). Infiltration of mononuclear cells was observed in liver of *L. donovani*-infected BALB/c mice (C). Arrows indicated infiltration of mononuclear cells (C). Detection of amastigotes in the spleen (D) and liver (E) of *L. donovani*-infected BALB/c mice. The spleen and liver of infected BALB/c mice were stained immunochemically with C11C mAb, followed by counterstained with hematoxylin. Scale bar: 20 μm.

Fig. 13

Detection of parasitemia in *L. donovani*-infected BALB/c mice. Detection of *Leishmania* parasites DNA by *LSUrDNA* gene from blood infected BALB/c mice with *L. donovani* (A). The 310 bp fragment corresponds to *Leishmania* amplified DNA. In

PCR assay *CytB* gene was used for detection of mammalian DNA as false negative controls. Isolation of *Leishmania* parasites from *L. donovani*-infected BALB/c mice peripheral blood (B). Blood parasite loads of *L. donovani*-infected BALB/c mice. Two, four, eight and twelve weeks after inoculation of parasites, blood were collected for parasite quantification by limiting dilution quantitative culture. Means and SD in each group are shown. n.d., not detected. Promastigote from culture medium derived peripheral blood of *L. donovani*-infected BALB/c mice (C). Scale bar: 10 μm.

Fig. 14

The spleen and liver parasite loads in *L. donovani*-infected BALB/c and C57BL/6 mice. The spleen (A) and liver (B) parasite burden by Leishman Donovan units (LDU) in *L. donovani*-infected BALB/c and C57BL/6J mice. LDU was represented as the number of amastigotes/ host cell nuclei ×organ weight (mg). Means and SD in each group are shown. *P<0.01.

Fig. 15

The spleen and liver parasite loads in *L. donovani*-infected BALB/c and C57BL/6 mice. The spleen (A) and liver (B) parasite burden were determined by limiting dilution assay. Total parasite number was represented as the reciprocal of highest dilution positive for promastigote (the parasite concentration per milligram of tissue) × total weight (mg).

Fig. 16

Enlargement of spleen and liver weight in *L. donovani*-infected BALB/c and C57BL/6 mice. Changes in the spleen (A) and liver (B) weights over the course of infection in

BALB/c and C57BL/6 mice infected with *L. donovani*. Means and SD in each group are shown. *$P < 0.01$.

Fig. 17

The spleen and liver parasite loads in *L. donovani*-infected BALB/c, C57BL/6 and C3H/HeN mice 12 weeks after infection of parasites. The spleen (A) and liver (B) parasite burden by LDU in BALB/c, C57BL/6 and C3H/HeN mice infected with *L. donovani*. LDU was represented as the number of amastigotes/ host cell nuclei ×organ weight (mg). Means and SD in each group are shown. *$P < 0.01$, n.d., not detected.

Fig. 18

The spleen and liver parasite loads in *L. donovani*-infected BALB/c, C57BL/6 and C3H/HeN mice 12 weeks after infection of parasites. The spleen (A) and liver (B) parasite burden were determined by limiting dilution assay. Total parasite number was represented as the reciprocal of highest dilution positive for promastigote (the parasite concentration per milligram of tissue) × total weight (mg).

Fig. 19

Enlargement of spleen and liver weight in *L. donovani*-infected BALB/c, C57BL/6 and C3H/HeN mice 12 weeks after infection of parasites. Weight ratio (organ weight of infected mice/ organ weight of uninfected mice) of the spleen (A) and liver (B) was compared among BALB/c, C57BL/6 and C3H/HeN mice. Means and SD in each group are shown. *$P < 0.01$.

Fig. 20

Leishmania antigen-induced IFN-γ (A) and IL-4 (B) production by spleen cells from BALB/c, C57BL/6 and C3H/HeN mice at 12 weeks after experimental *L. donovani* infection and non-infection. At 72 hours, culture supernatants were assayed for IFN-γ and IL-4 by ELISA. Means and SD in each group are shown. n.d., not detected.

Fig. 21

Susceptibility is correlated with Th2 and resistance is correlated with Th1 in experimental visceral leishmaniasis.

Fig. 22

Structure of quinone-terpenoid compounds (**a-j**) derived from *Sargassum yamadae,* a brown alga Sargassaceae species.

Fig. 23

Inhibition of *L. donovani/egfp* in vitro by quinone-terpenoid compounds derived from *Sargassum yamadae*. Promastigotes of transgenic *L. donovani* expressing enhanced green fluorescent protein (*L. donovani*/ egfp) (A). Scale: 10 μm. The growth inhibition rate of *L. donovani/ egfp* promastigotes by quinone-terpenoid compounds (B).

Fig. 24

Complete clearance of parasites by sargaquinoidatic acid treatment in the spleen and liver of *L. donovani*-infected BALB/c mice. The spleen (A) and liver (B) parasite burden by Leishman Donovan units (LDU) in treated and untreated mice. LDU was

represented as the number of amastigotes/ host cell nuclei ×organ weight (mg). Means and SD in each group are shown.

Fig. 25

Complete clearance of parasites by sargaquinoidatic acid treatment in the spleen and liver of *L. donovani*-infected BALB/cA mice. The spleen (A) and liver (B) parasite burden in treated and untreated mice were determined by limiting dilution assay. Total parasite number was represented as the reciprocal of highest dilution positive for promastigote (the parasite concentration per milligram of tissue) × total weight (mg).

Fig. 26

Detection of *Leishmania LSU rDNA* gene by PCR from spleen in mice treated with sargaquinoidatic acid. The 310 bp fragment corresponds to *Leishmania* amplified DNA. In PCR assay *CytB* gene was used for detection of mammalian DNA as false negative controls.

Fig. 27

Detection of *Leishmania LSU rDNA* gene by PCR from liver in mice treated with sargaquinoidatic acid. The 310 bp fragment corresponds to *Leishmania* amplified DNA. In PCR assay *CytB* gene was used for detection of mammalian DNA as false negative controls.

Fig. 28

Detection of *Leishmania LSU rDNA* gene by PCR from peripheral blood in mice treated

with sargaquinoidatic acid. The 310 bp fragment corresponds to *Leishmania* amplified DNA. In PCR assay *CytB* gene was used for detection of mammalian DNA as false negative controls.

Fig. 29

Histopathological analysis of spleen in mice treated with sargaquinoidatic acid. Paraffin-embedded sections were prepared at the 49 and 79 days post infection and stained with hematoxylin and eosin. Scale bar: 50 μm (insets: 20 μm). Arrows indicated amastigotes.

Fig. 30

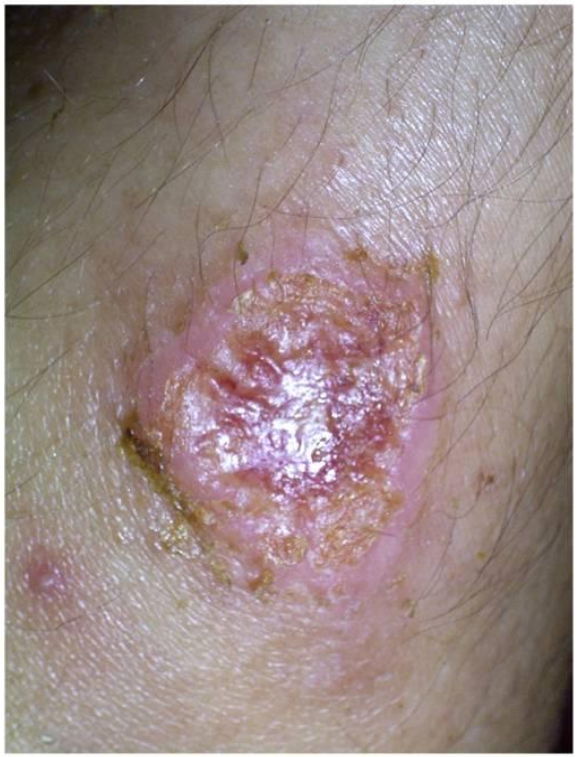
Histopathological analysis of liver in mice treated with sargaquinoidatic acid. Paraffin-embedded sections were prepared at the 49 and 79 days post infection and stained with hematoxylin and eosin. Scale bar: 50 μm (insets: 20 μm). Arrows indicated amastigotes.

Fig. 31

Complete cure is induced by Sargaquinoidatic acid treatment in the spleen and liver of *L. donovani*-infected BALB/cA mice. Spleen from uninfected mouse, sargaquinoidatic acid treated mouse, AmBisome treated mouse and untreated mouse (A). Changes in the spleen (B) and liver (C) weights in treated and unteated mice. Means and SD in each group are shown. $*P < 0.01$.

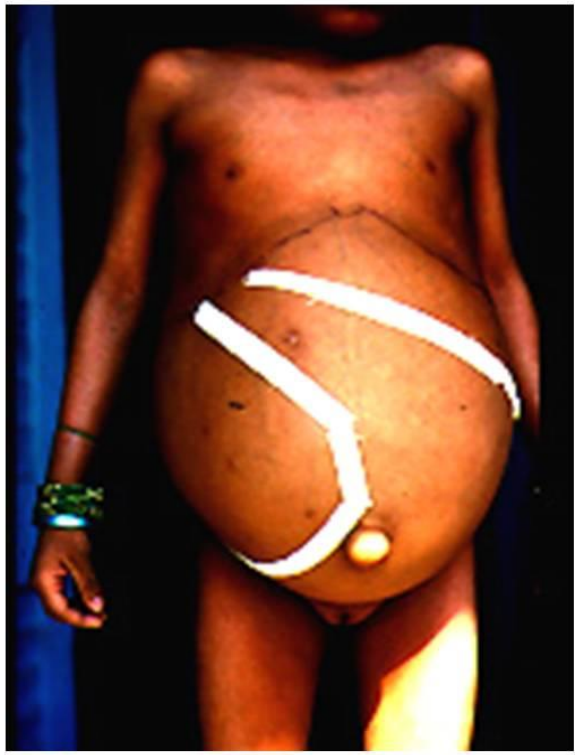
Figures

A



http://www.who.int/leishmaniasis/cutaneous_leishmaniasis/en/index.html

B



http://www.icp.ucl.ac.be/~opperd/parasites/images/WHO1.jpg

Fig. 1.

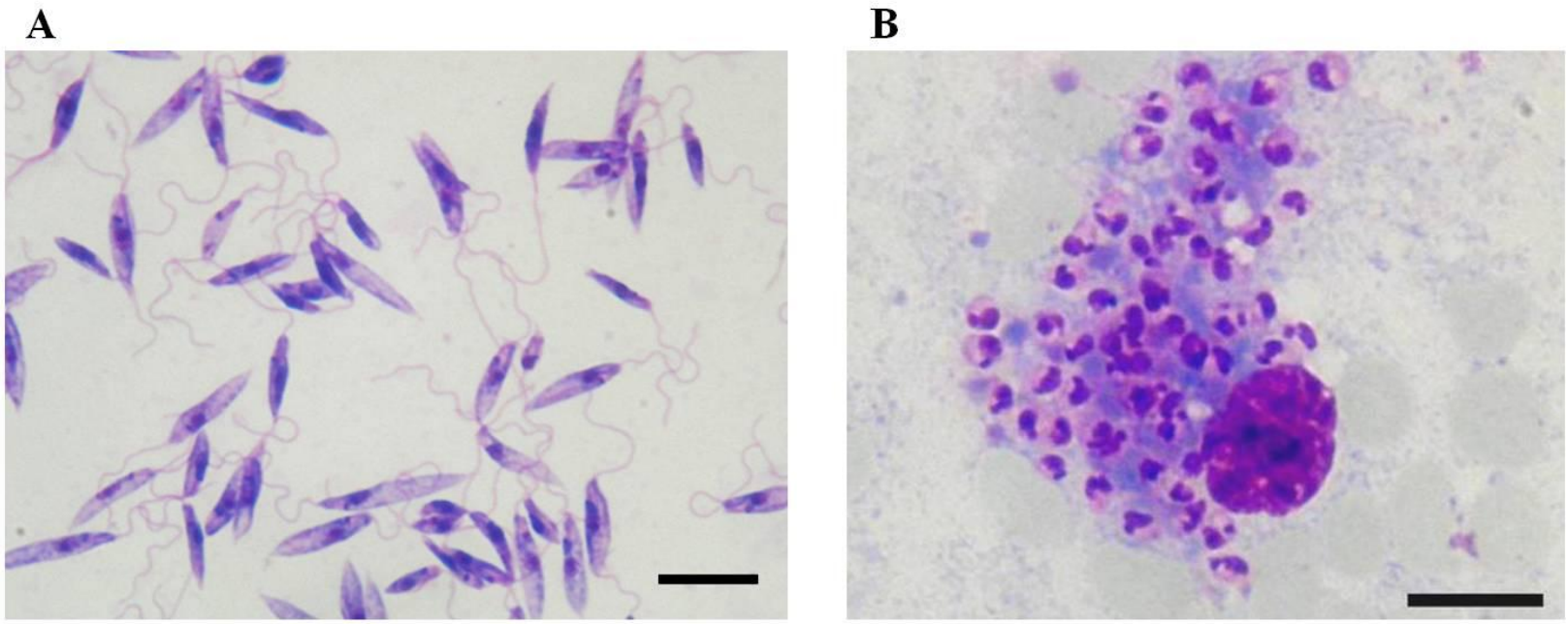


Fig. 2.

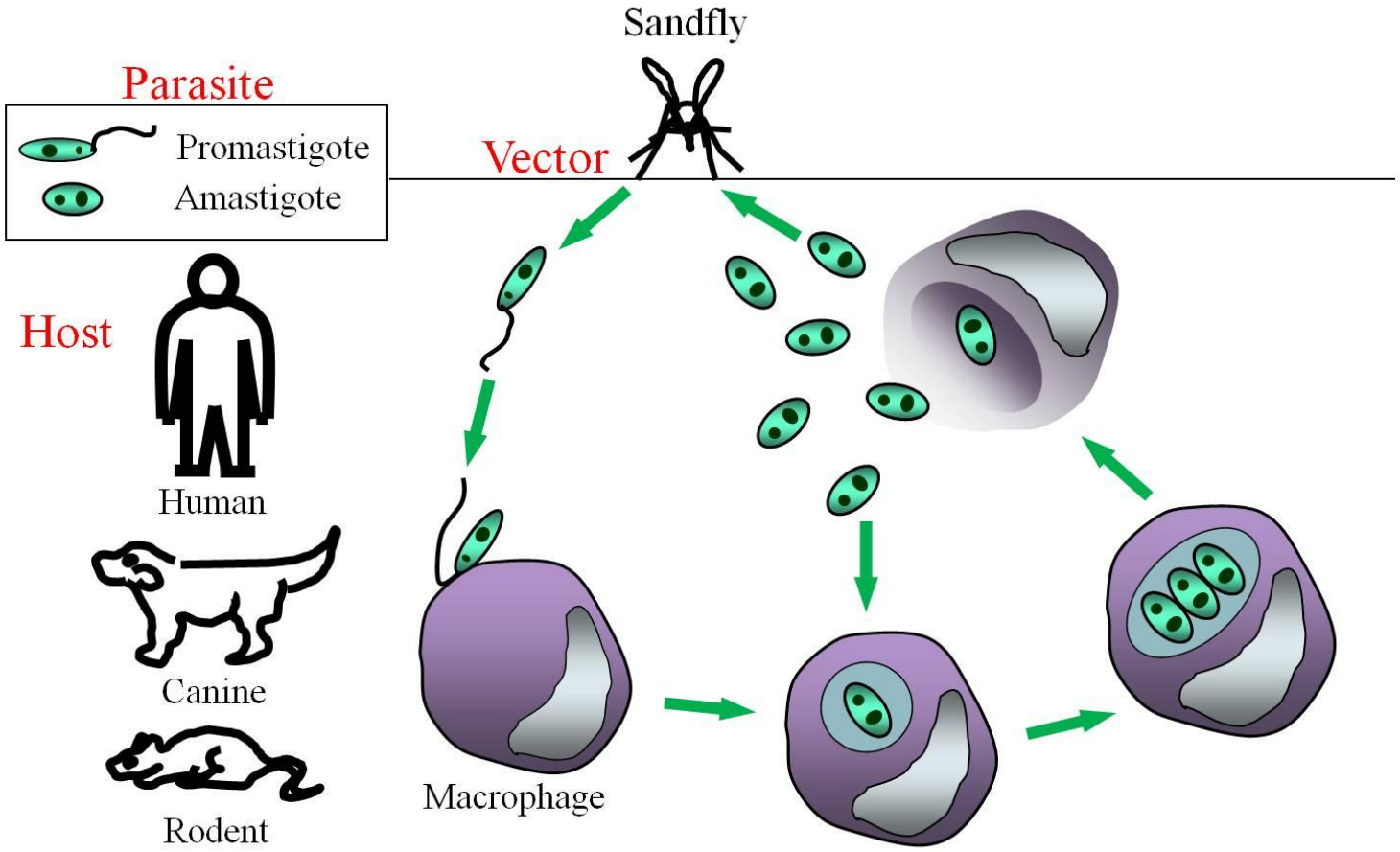
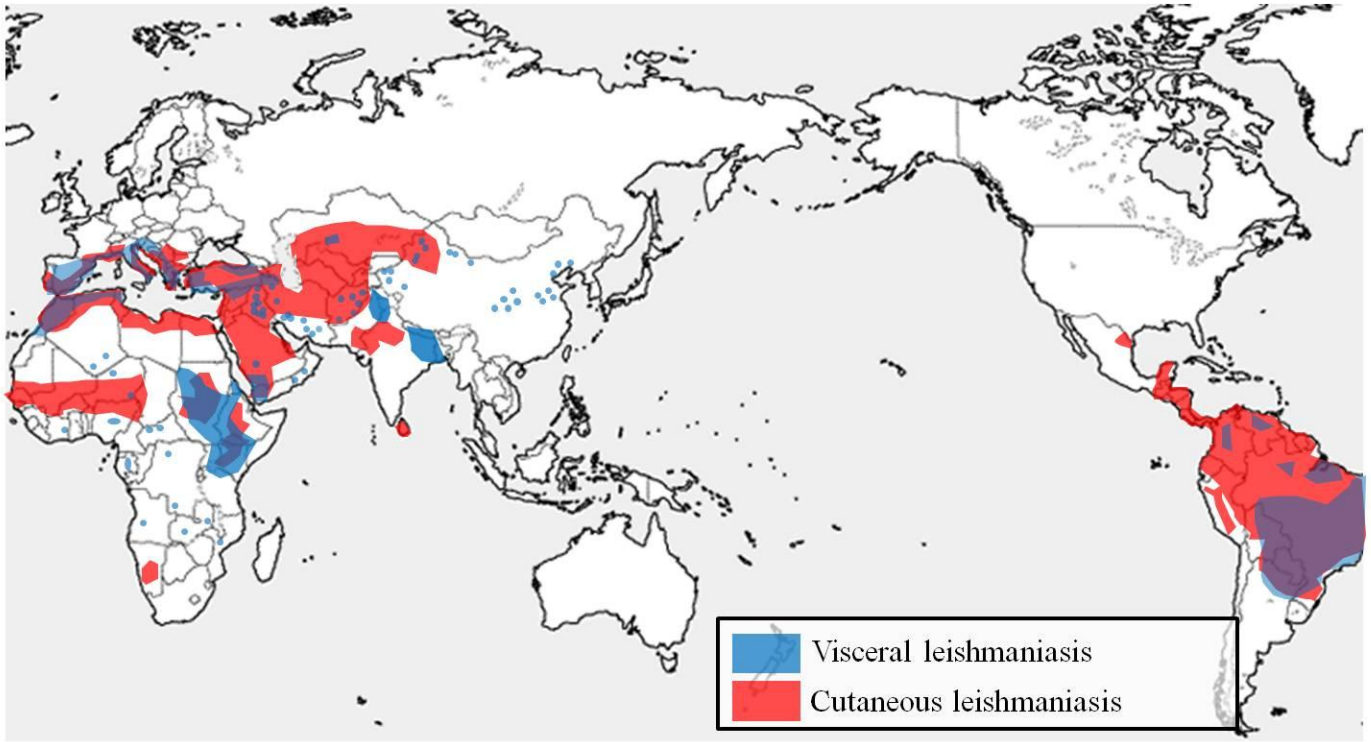

Sandfly
Parasite
Promastigote
Amastigote
Vector
Host
Human
Canine
Rodent
Macrophage

Fig. 3.



http://www.who.int/leishmaniasis/leishmaniasis_maps/en/index.html (Adapted from WHO report, 2010)

Countries affected ： 88 countries		
Number of patients (annual incidence)	visceral leishmaniasis	500,000
Population at risk ： 350 million people		

Fig. 4.

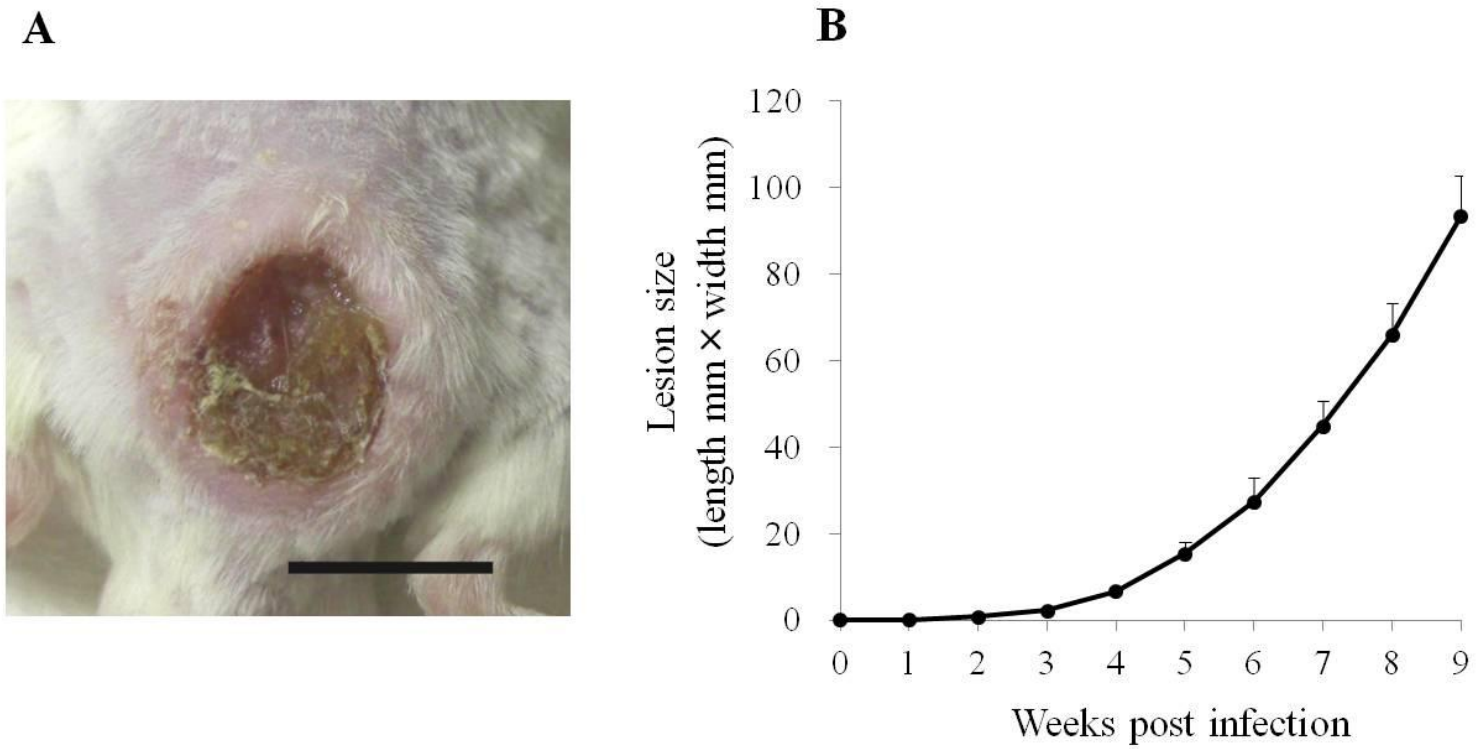

A
B
120
100
80
60
40
20
0
Lesion size
(length mm × width mm)
0 1 2 3 4 5 6 7 8 9
Weeks post infection

Fig. 5.

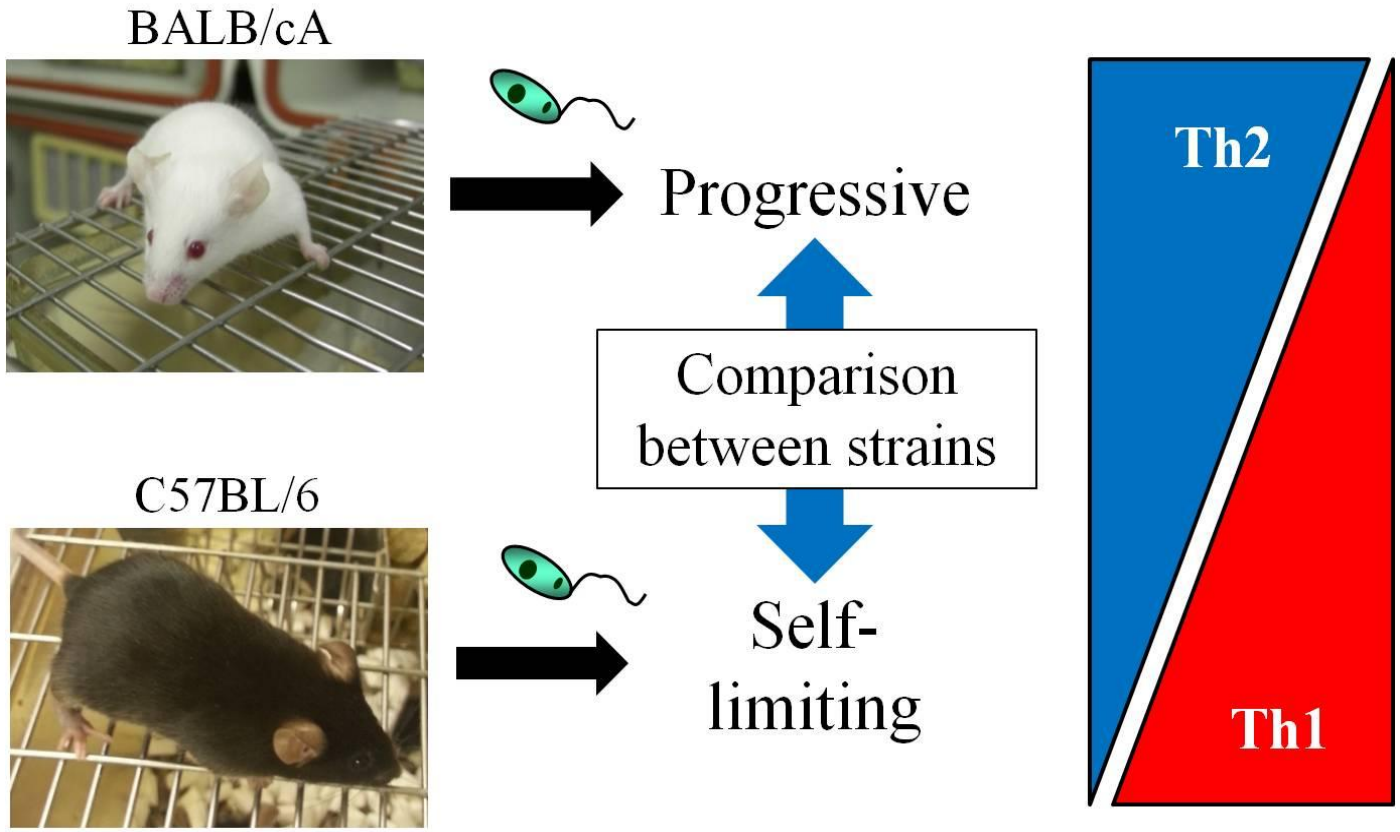

BALB/cA
Progressive
Comparison between strains
C57BL/6
Self-limiting
Th2
Th1

Fig. 6.

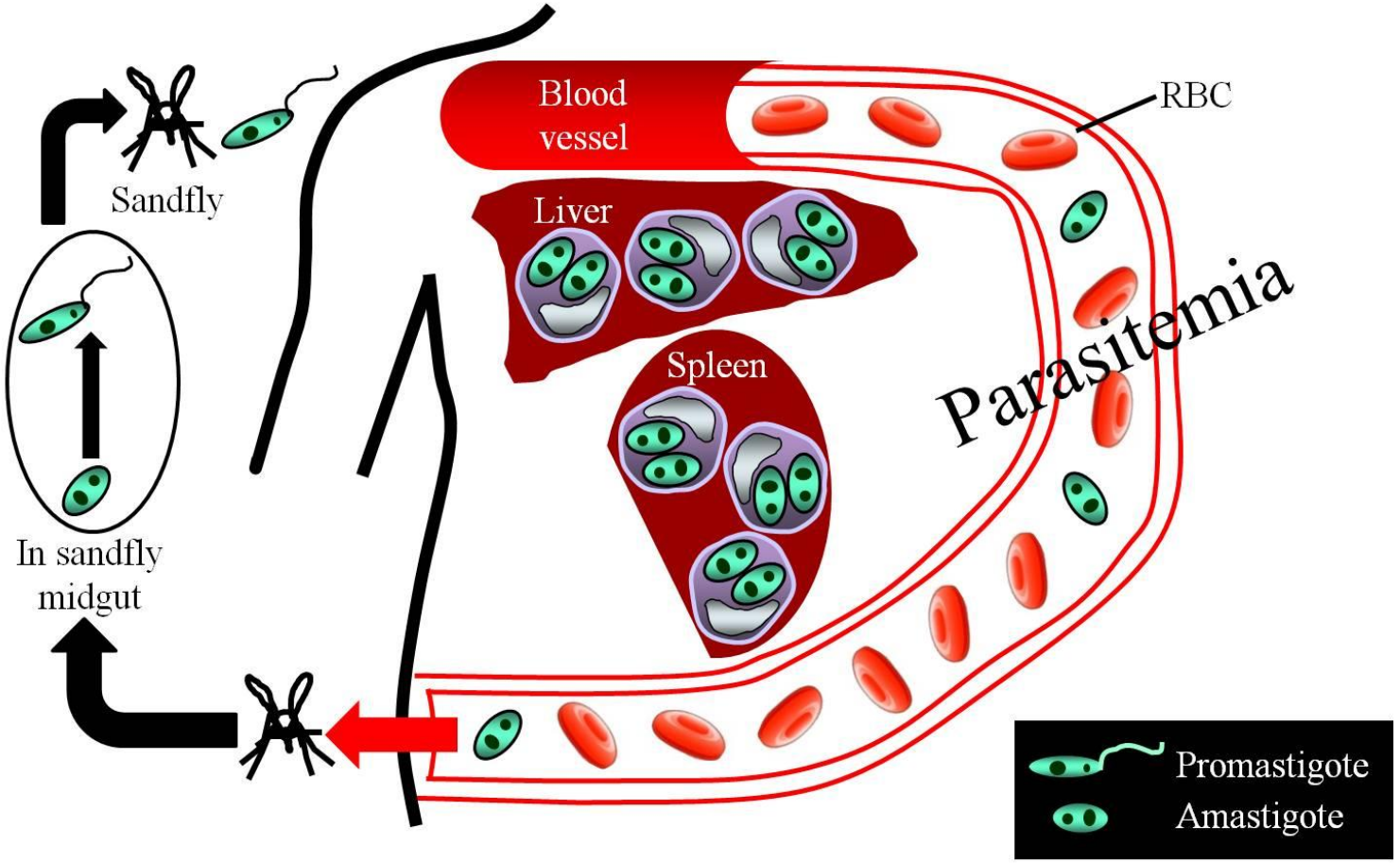

Blood
vessel
RBC
Liver
Spleen
Parasitemia
Sandfly
In sandfly
midgut
Promastigote
Amastigote

Fig. 7.

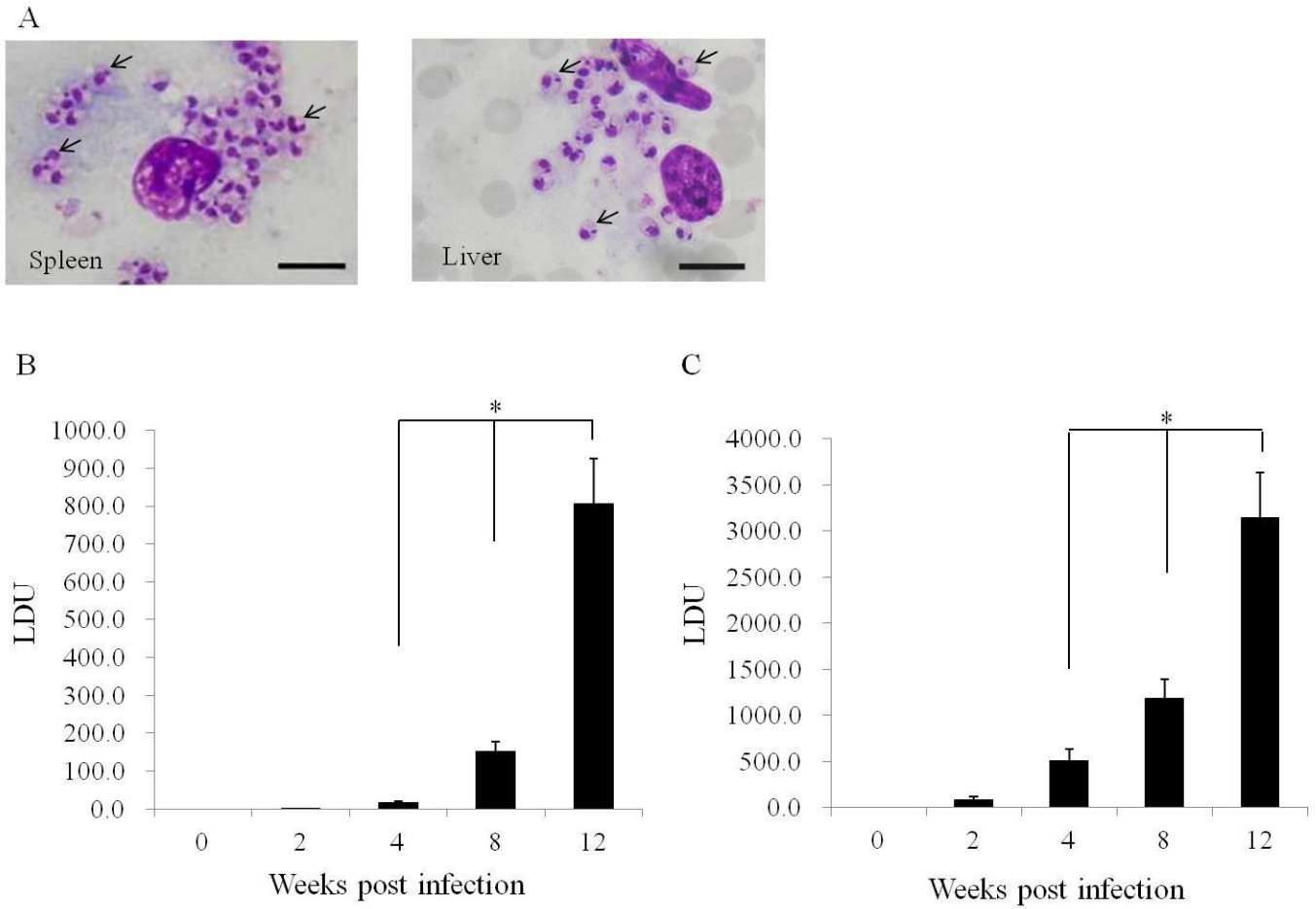

A
Spleen
Liver
B
LDU
1000.0
900.0
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
*
0
2
4
8
12
Weeks post infection
C
LDU
4000.0
3500.0
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
*
0
2
4
8
12
Weeks post infection

Fig. 8.

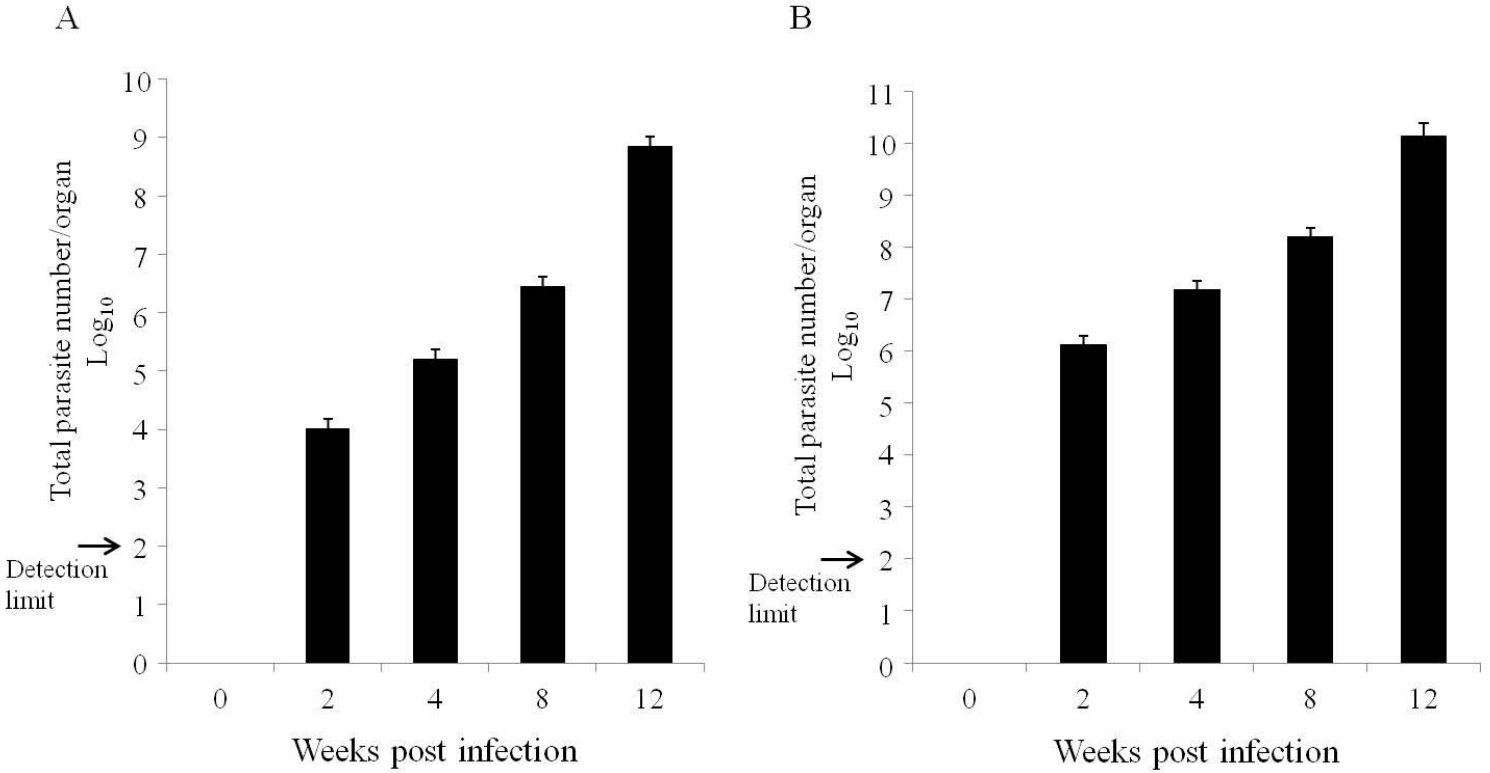

A
Total parasite number/organ
Log10
Detection limit
Weeks post infection
B
Total parasite number/organ
Log10
Detection limit
Weeks post infection

Fig. 9.

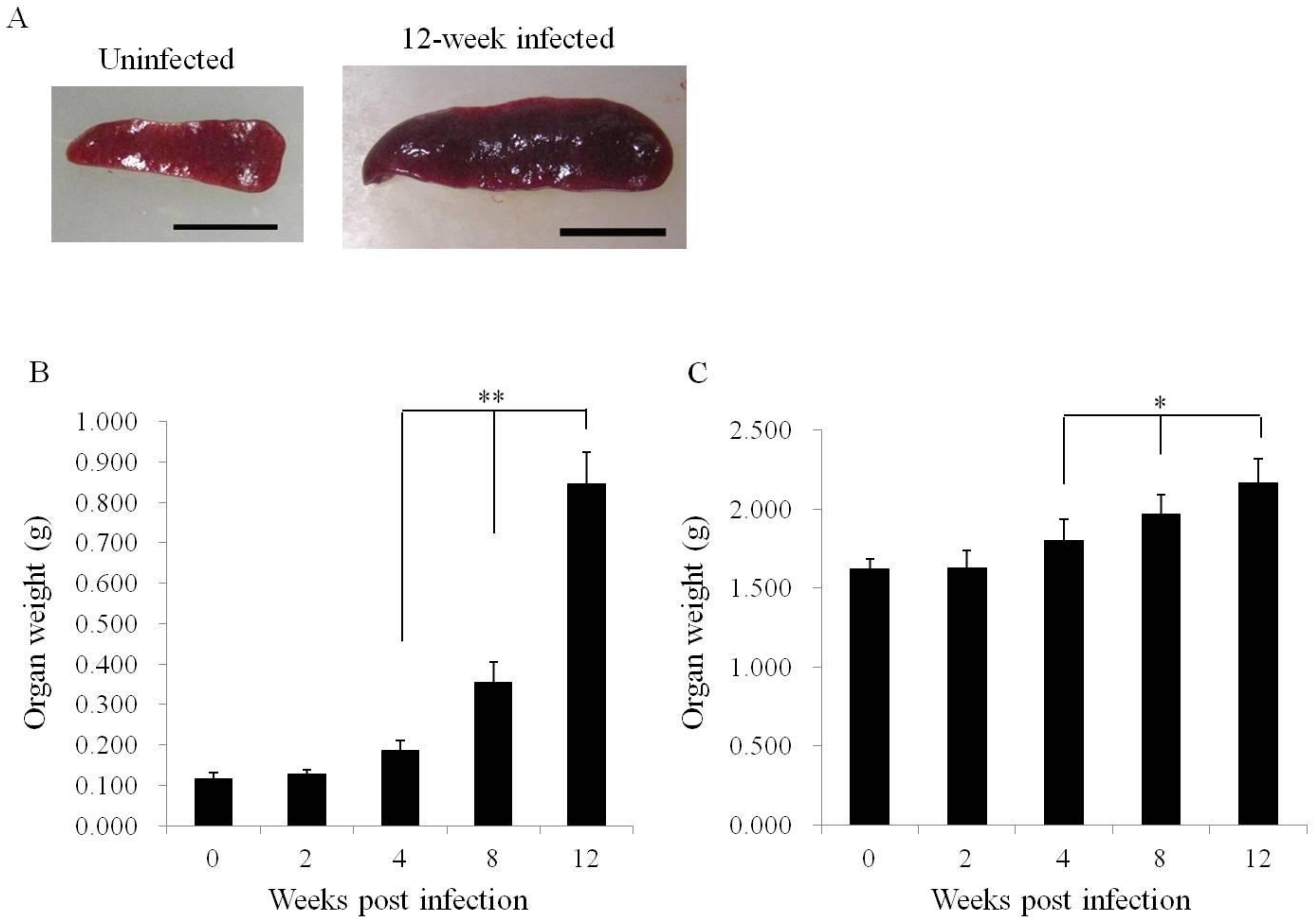

A
Uninfected
12-week infected
B
**
Organ weight (g)
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
2
4
8
12
Weeks post infection
C
*
Organ weight (g)
2.500
2.000
1.500
1.000
0.500
0.000
0
2
4
8
12
Weeks post infection

Fig. 10.

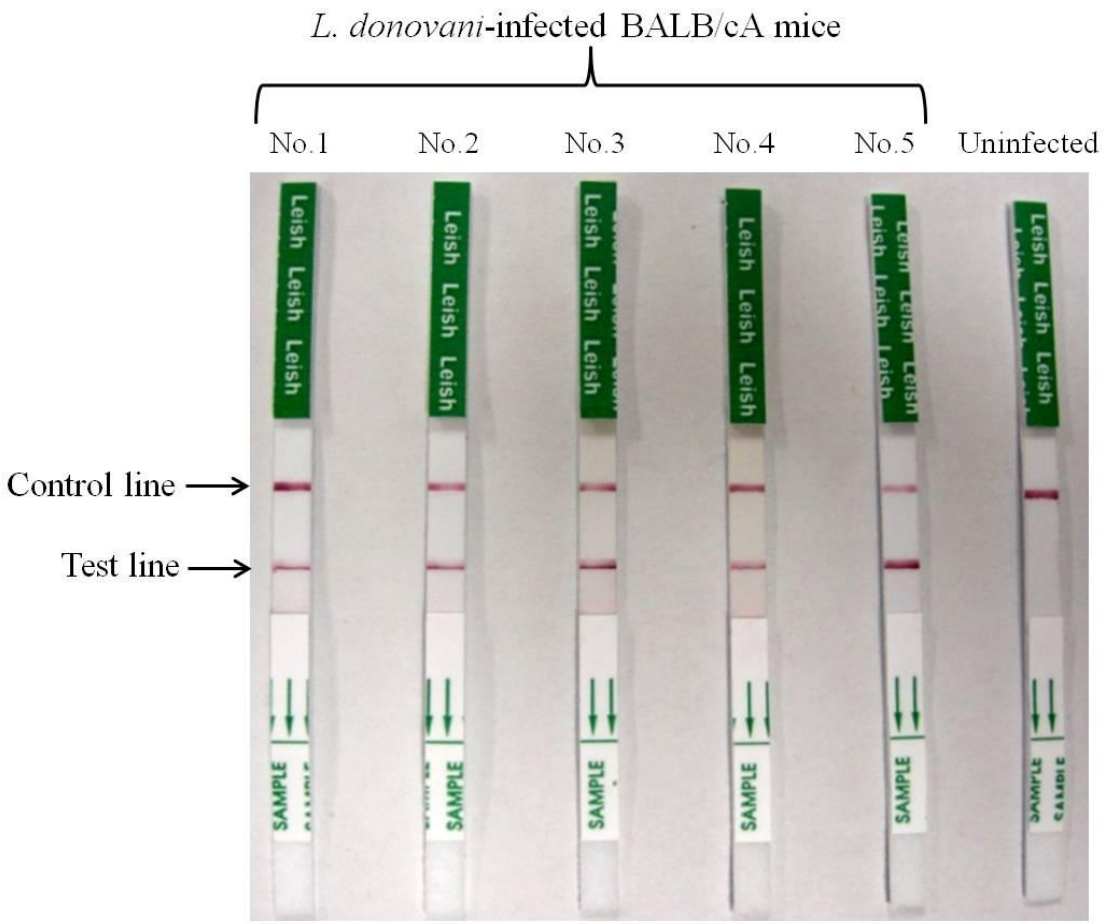

L. donovani-infected BALB/cA mice
No.1
No.2
No.3
No.4
No.5
Uninfected
Control line
Test line
Leish
SAMPLE

Fig. 11.

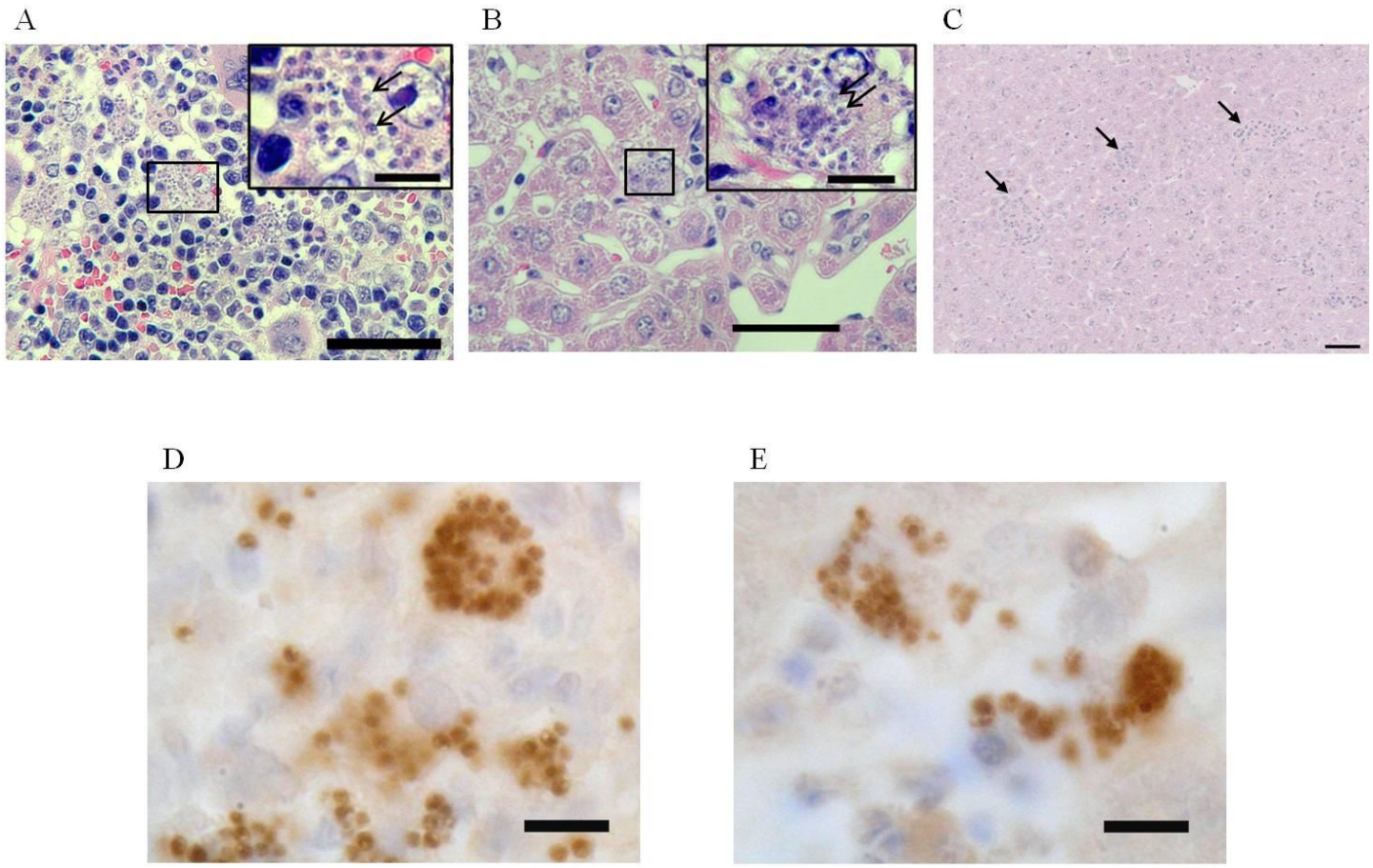
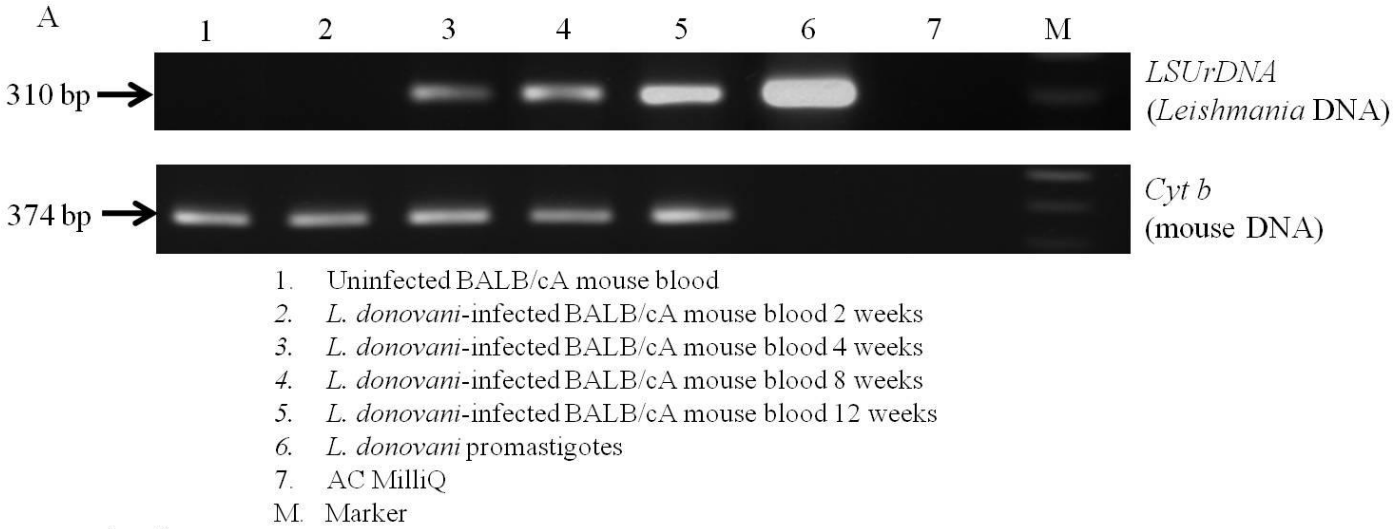
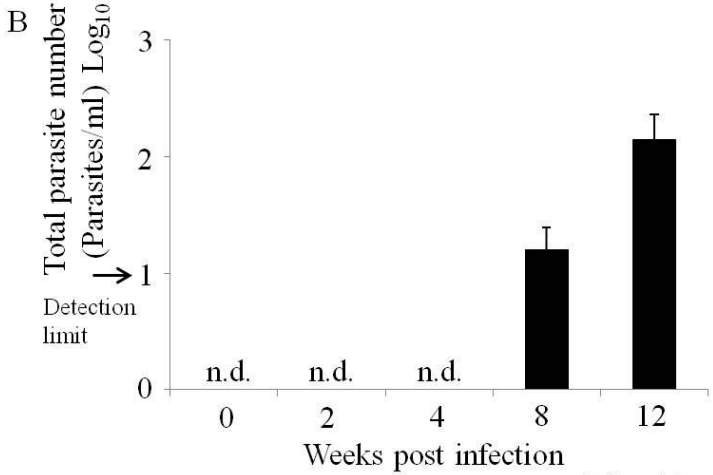


Fig. 12.



1. Uninfected BALB/cA mouse blood
2. *L. donovani*-infected BALB/cA mouse blood 2 weeks
3. *L. donovani*-infected BALB/cA mouse blood 4 weeks
4. *L. donovani*-infected BALB/cA mouse blood 8 weeks
5. *L. donovani*-infected BALB/cA mouse blood 12 weeks
6. *L. donovani* promastigotes
7. AC MilliQ

M. Marker



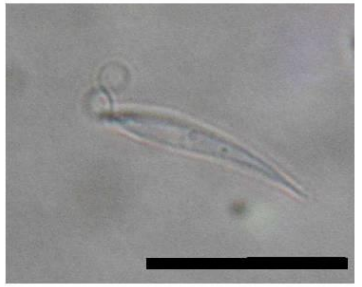


Fig. 13.

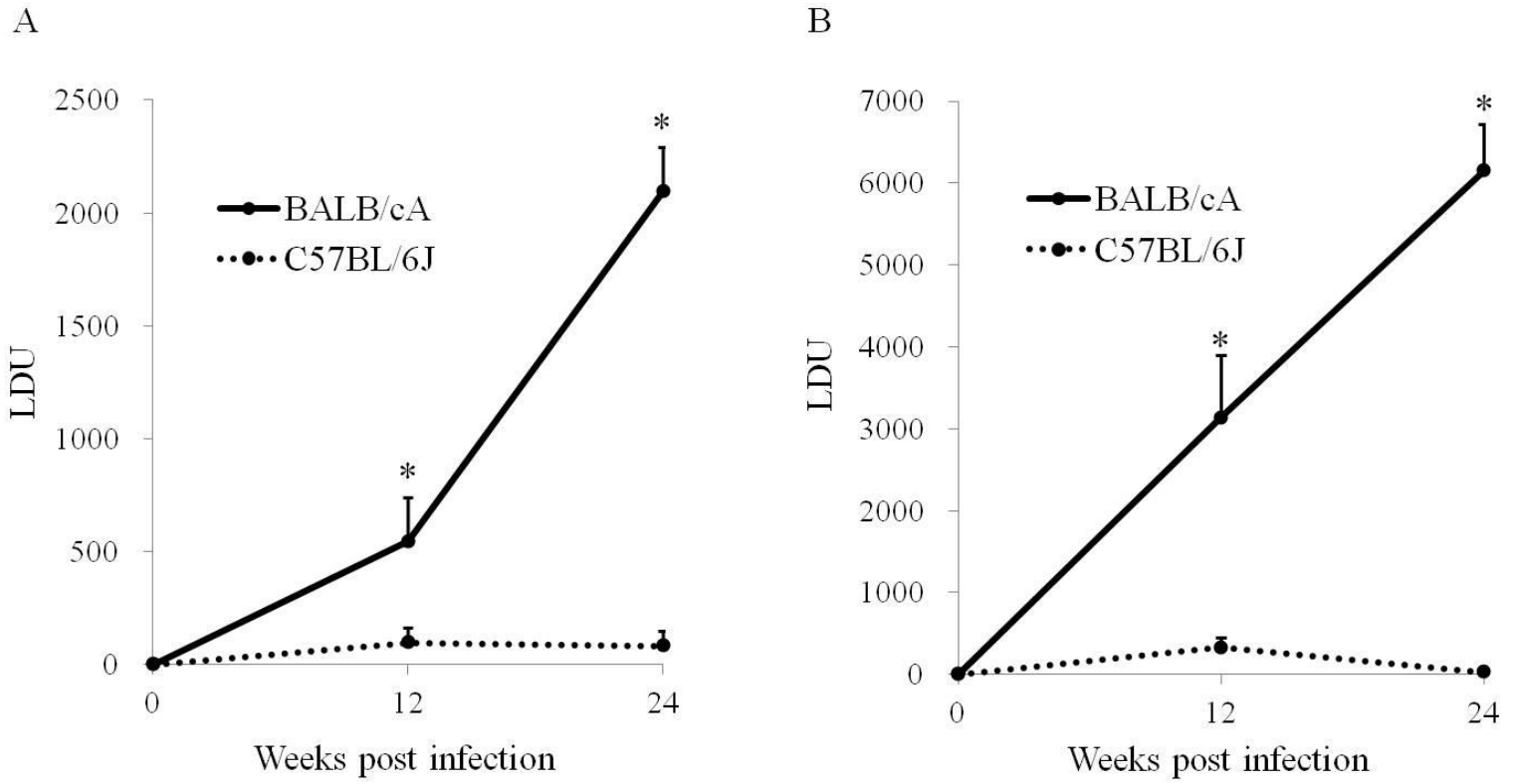

A
2500
2000
1500
1000
500
0
LDU
BALB/cA
C57BL/6J
*
*
0
12
24
Weeks post infection
B
7000
6000
5000
4000
3000
2000
1000
0
LDU
BALB/cA
C57BL/6J
*
*
0
12
24
Weeks post infection

Fig. 14.

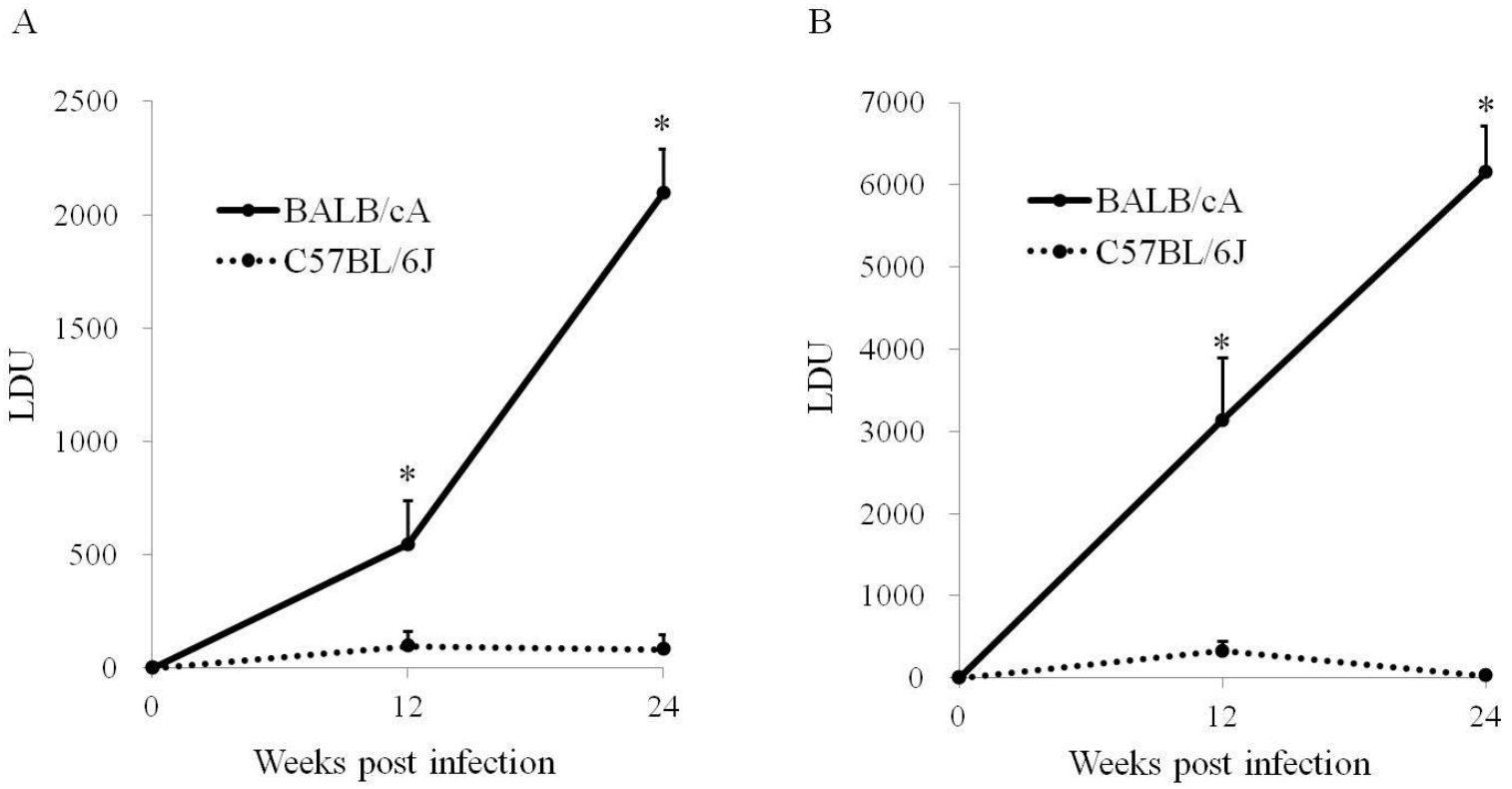

A
2500
2000
1500
1000
500
0
LDU
BALB/cA
C57BL/6J
*
*
0
12
24
Weeks post infection
B
7000
6000
5000
4000
3000
2000
1000
0
LDU
BALB/cA
C57BL/6J
*
*
0
12
24
Weeks post infection

Fig. 14.

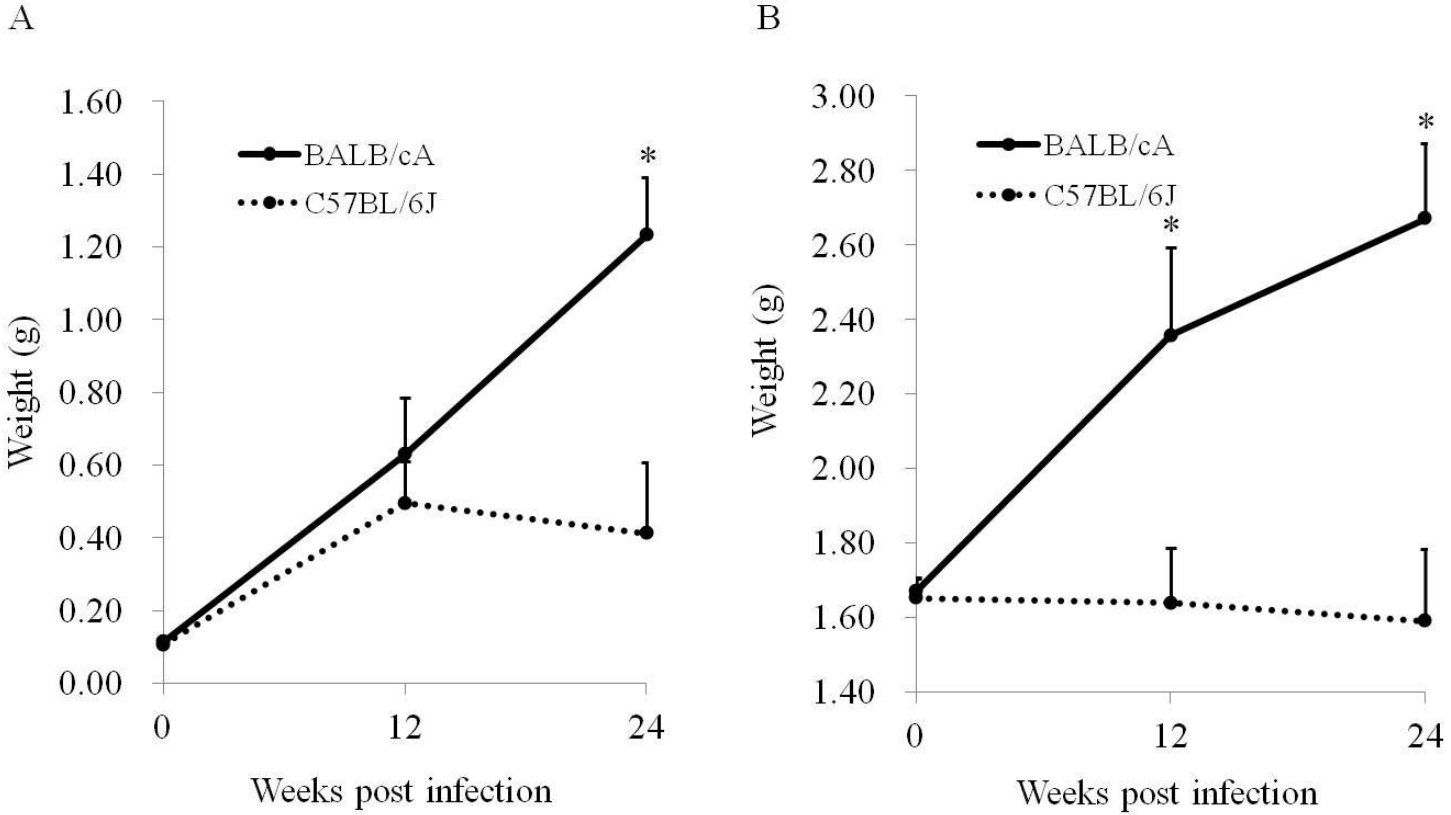

A
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Weight (g)
BALB/cA
C57BL/6J
*
0
12
24
Weeks post infection
B
3.00
2.80
2.60
2.40
2.20
2.00
1.80
1.60
1.40
Weight (g)
BALB/cA
C57BL/6J
*
*
0
12
24
Weeks post infection

Fig. 16.

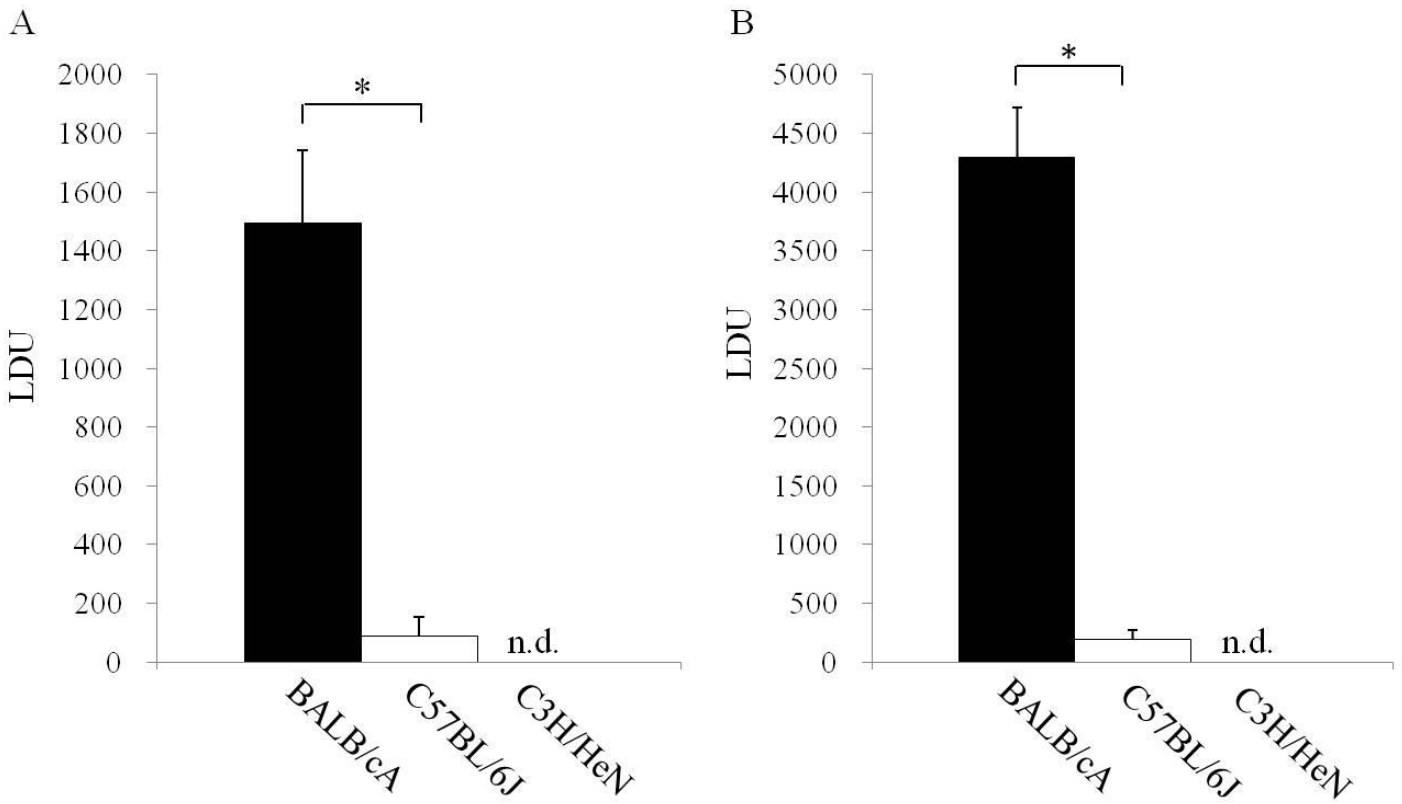

A
LDU
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
n.d.
BALB/cA
C57BL/6J
C3H/HeN
B
LDU
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
n.d.
BALB/cA
C57BL/6J
C3H/HeN

Fig. 17.

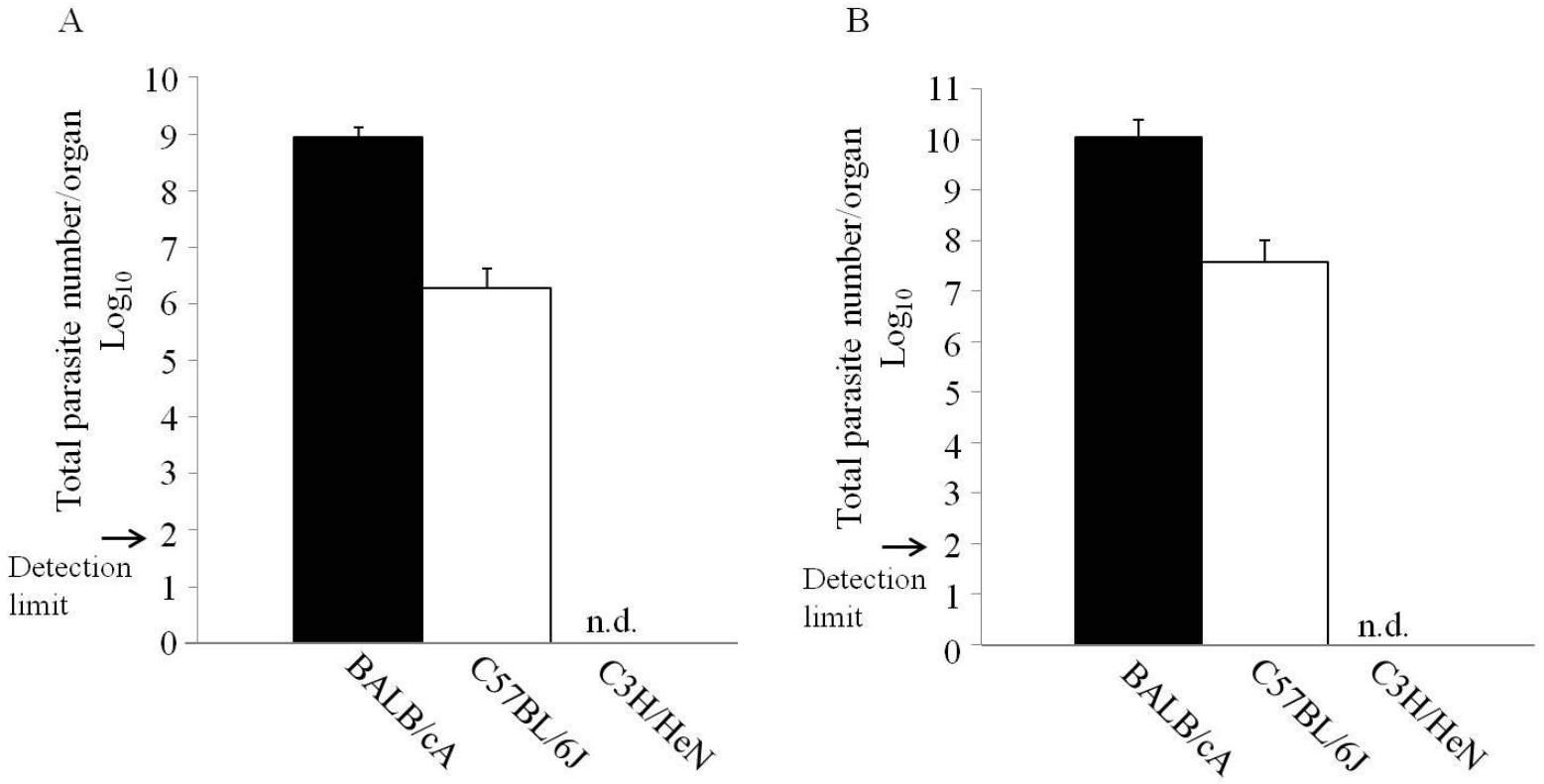

A
Total parasite number/organ
Log10
10
9
8
7
6
5
4
3
2
1
0
Detection limit
n.d.
BALB/cA
C57BL/6J
C3H/HeN
B
Total parasite number/organ
Log10
11
10
9
8
7
6
5
4
3
2
1
0
Detection limit
n.d.
BALB/cA
C57BL/6J
C3H/HeN

Fig. 18.

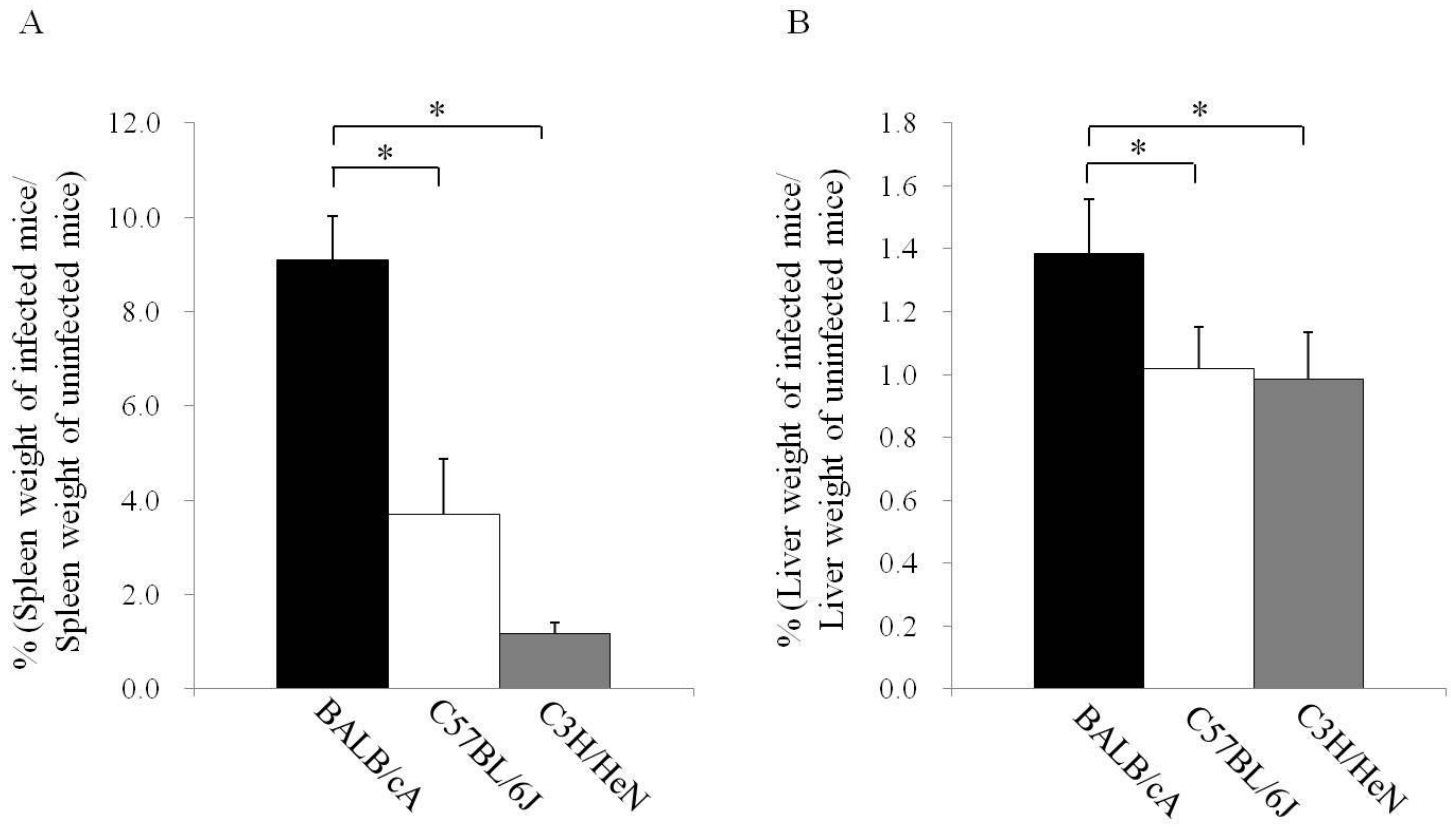

A
B
% (Spleen weight of infected mice/ Spleen weight of uninfected mice)
% (Liver weight of infected mice/ Liver weight of uninfected mice)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
BALB/cA
C57BL/6J
C3H/HeN

Fig. 19.

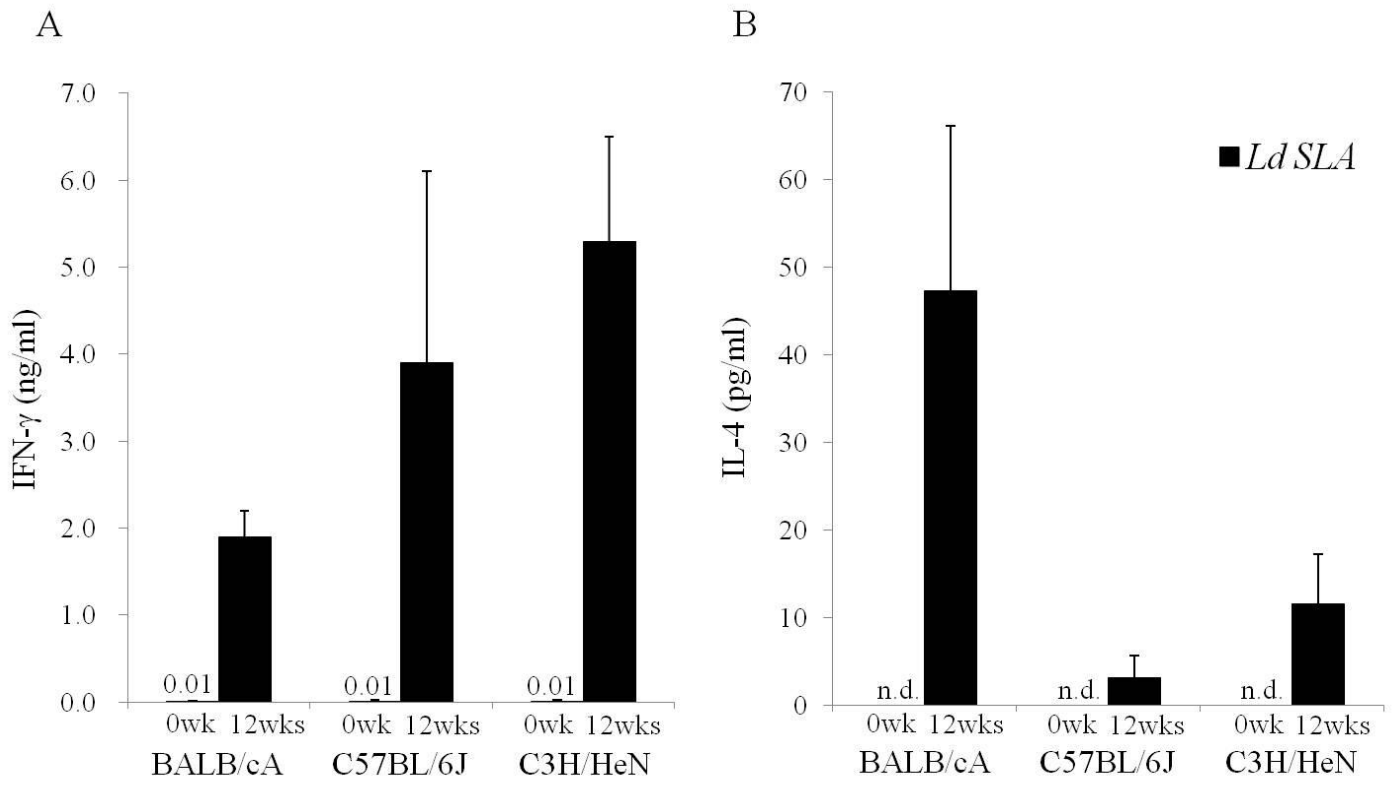

A
IFN-γ (ng/ml)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.01
0.01
0.01
0wk 12wks
0wk 12wks
0wk 12wks
BALB/cA
C57BL/6J
C3H/HeN
B
IL-4 (pg/ml)
70
60
50
40
30
20
10
0
n.d.
n.d.
n.d.
Ld SLA
0wk 12wks
0wk 12wks
0wk 12wks
BALB/cA
C57BL/6J
C3H/HeN

Fig. 20.

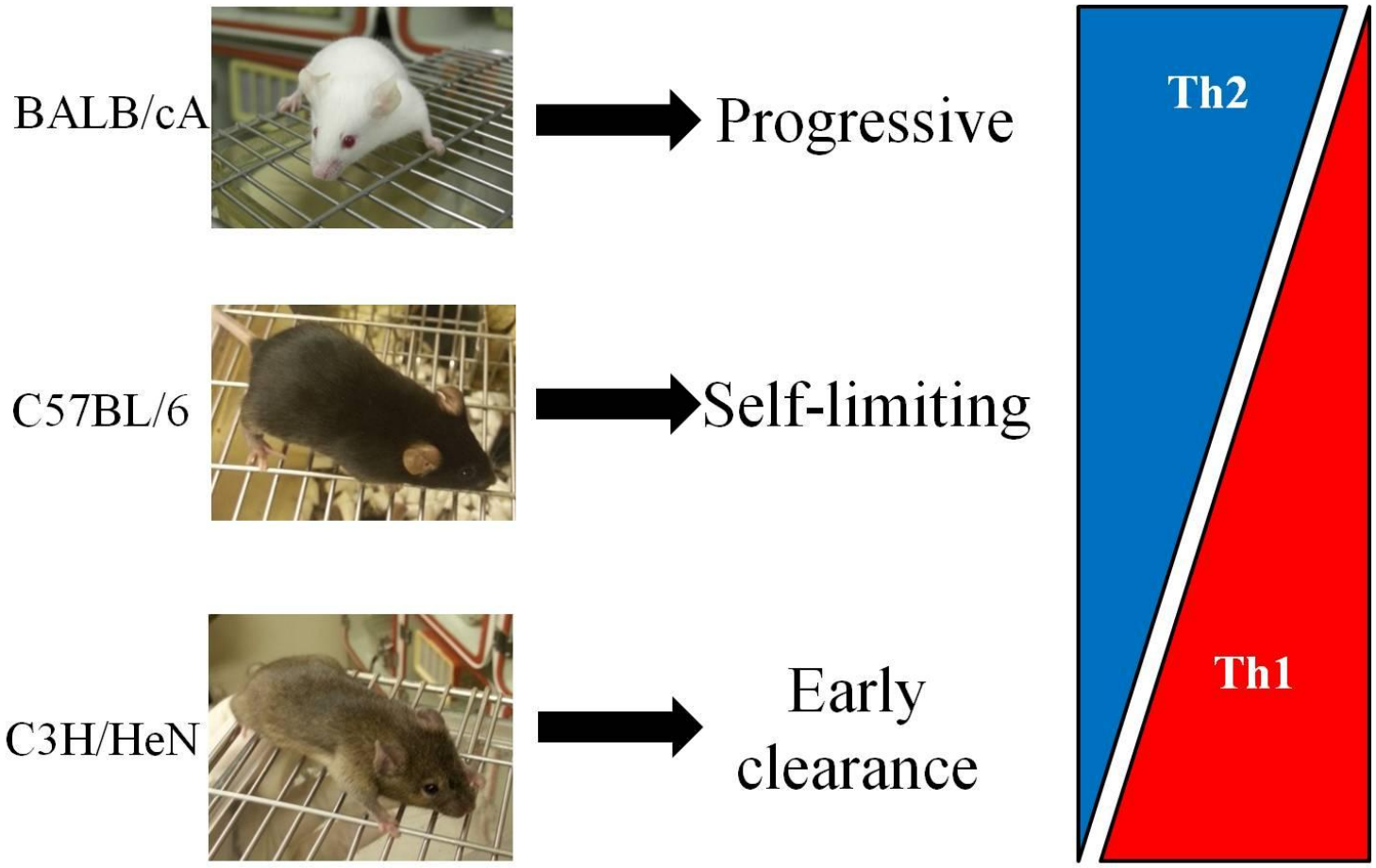

BALB/cA
Progressive
C57BL/6
Self-limiting
C3H/HeN
Early clearance
Th2
Th1

Fig. 21.

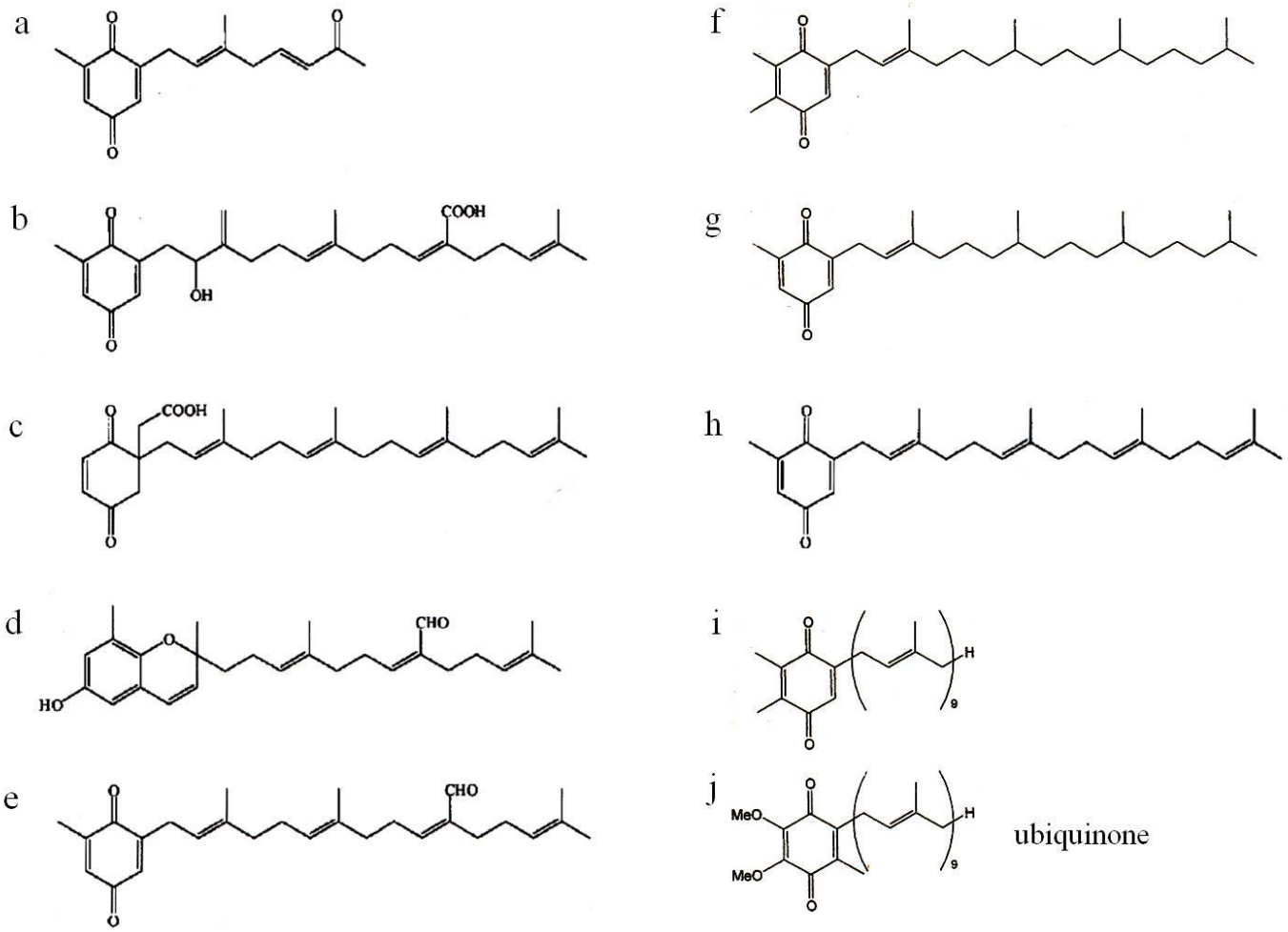
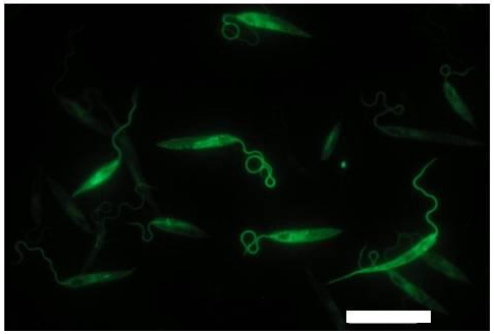


Fig. 22.

A



B

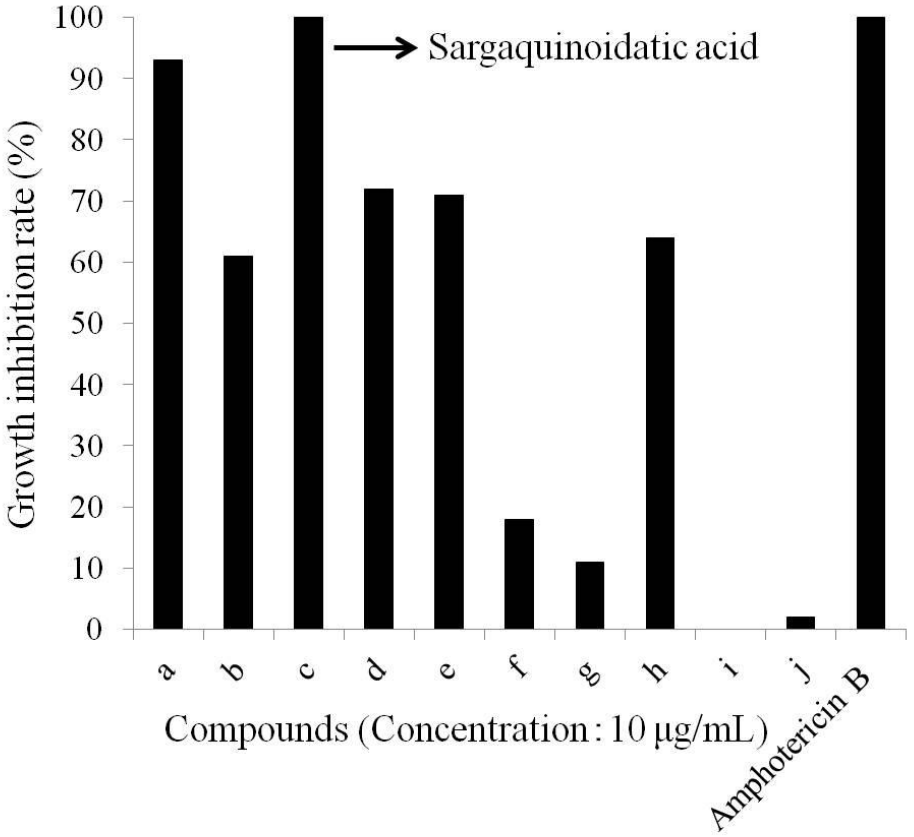

Growth inhibition rate (%)
100
90
80
70
60
50
40
30
20
10
0
Sargaquinoidatic acid
a
b
c
d
e
f
g
h
i
j
Amphotericin B
Compounds (Concentration : 10 μg/mL)

Fig. 23.

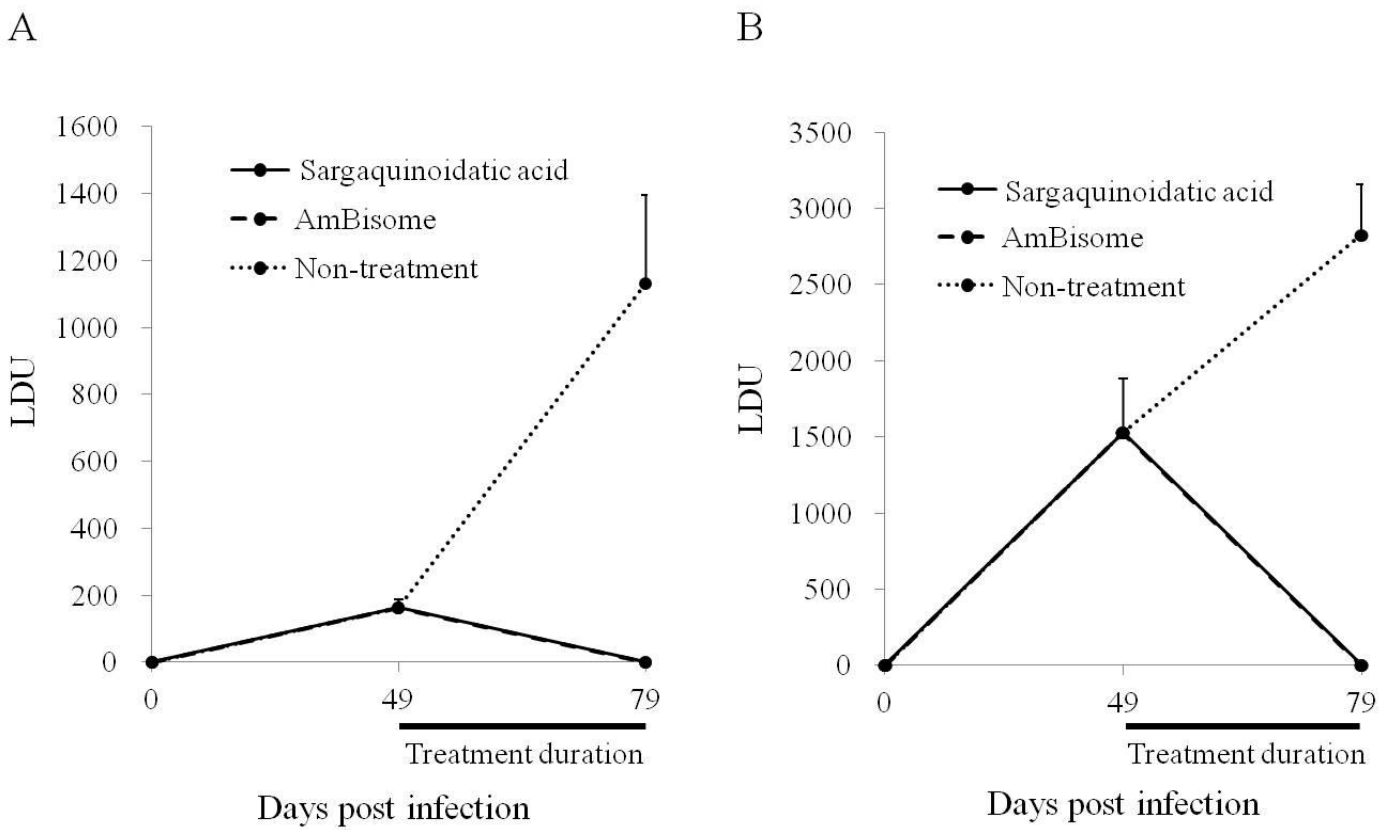

A
B
1600
1400
1200
1000
800
600
400
200
0
LDU
Sargaquinoidatic acid
AmBisome
Non-treatment
0
49
79
Treatment duration
Days post infection
3500
3000
2500
2000
1500
1000
500
0
LDU
Sargaquinoidatic acid
AmBisome
Non-treatment
0
49
79
Treatment duration
Days post infection

Fig. 24.

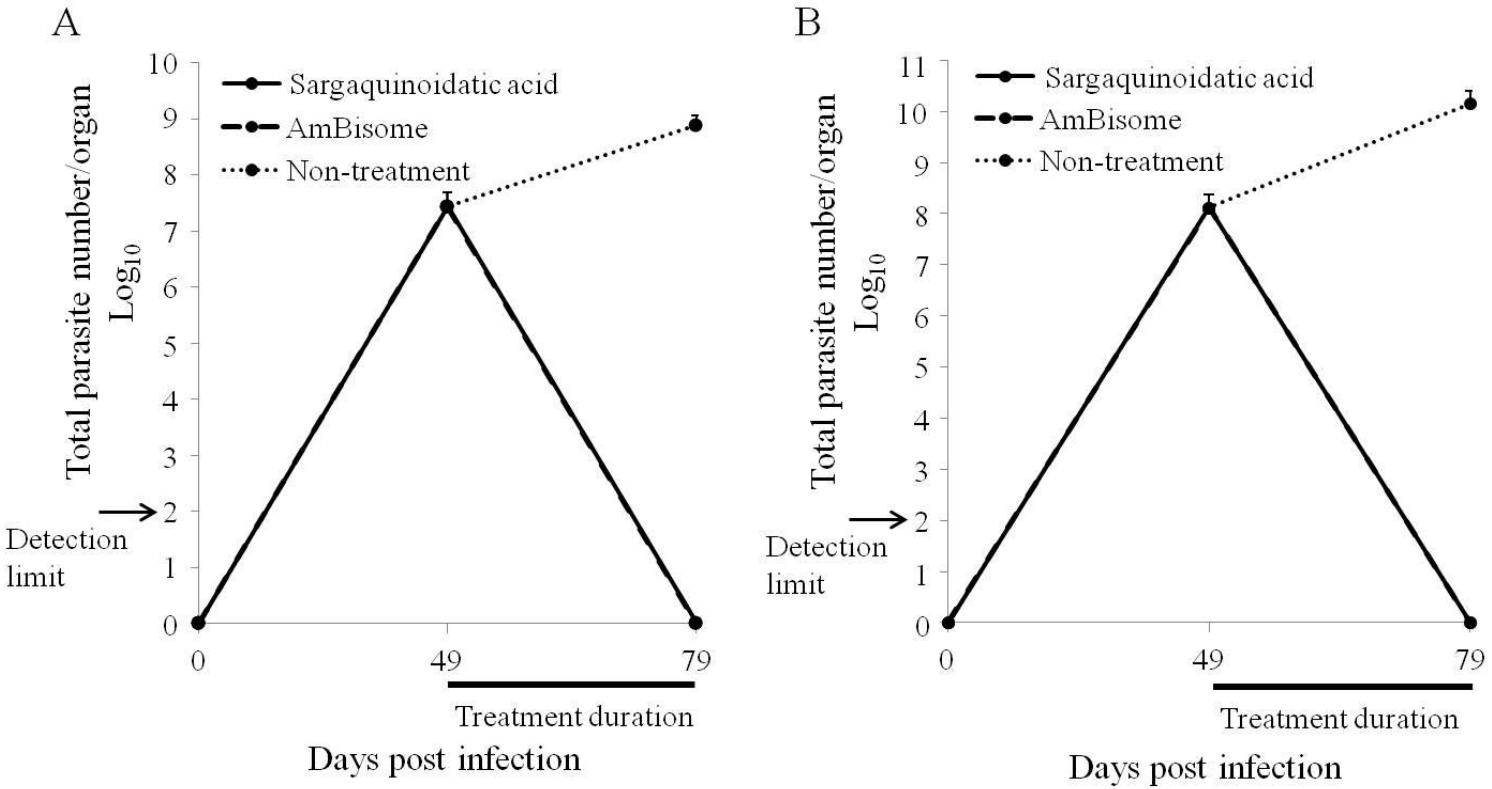
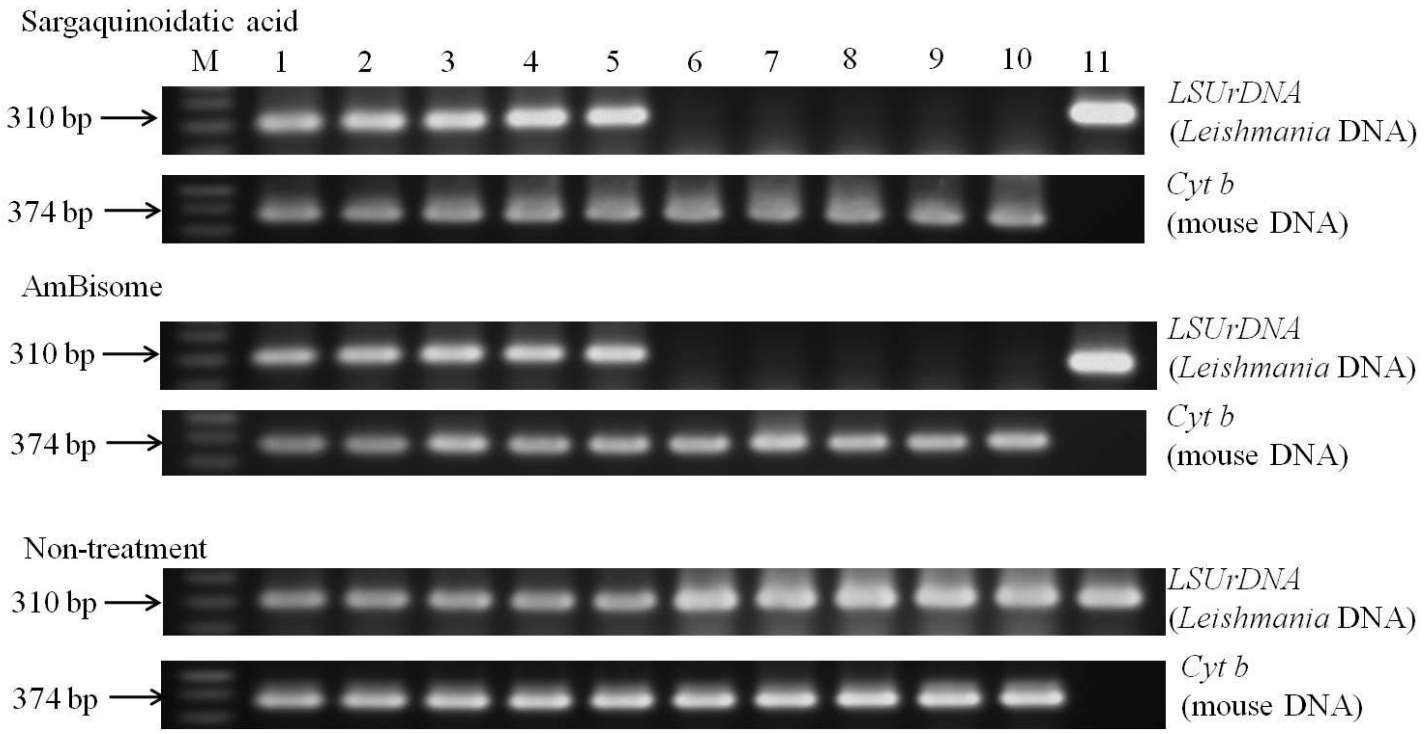
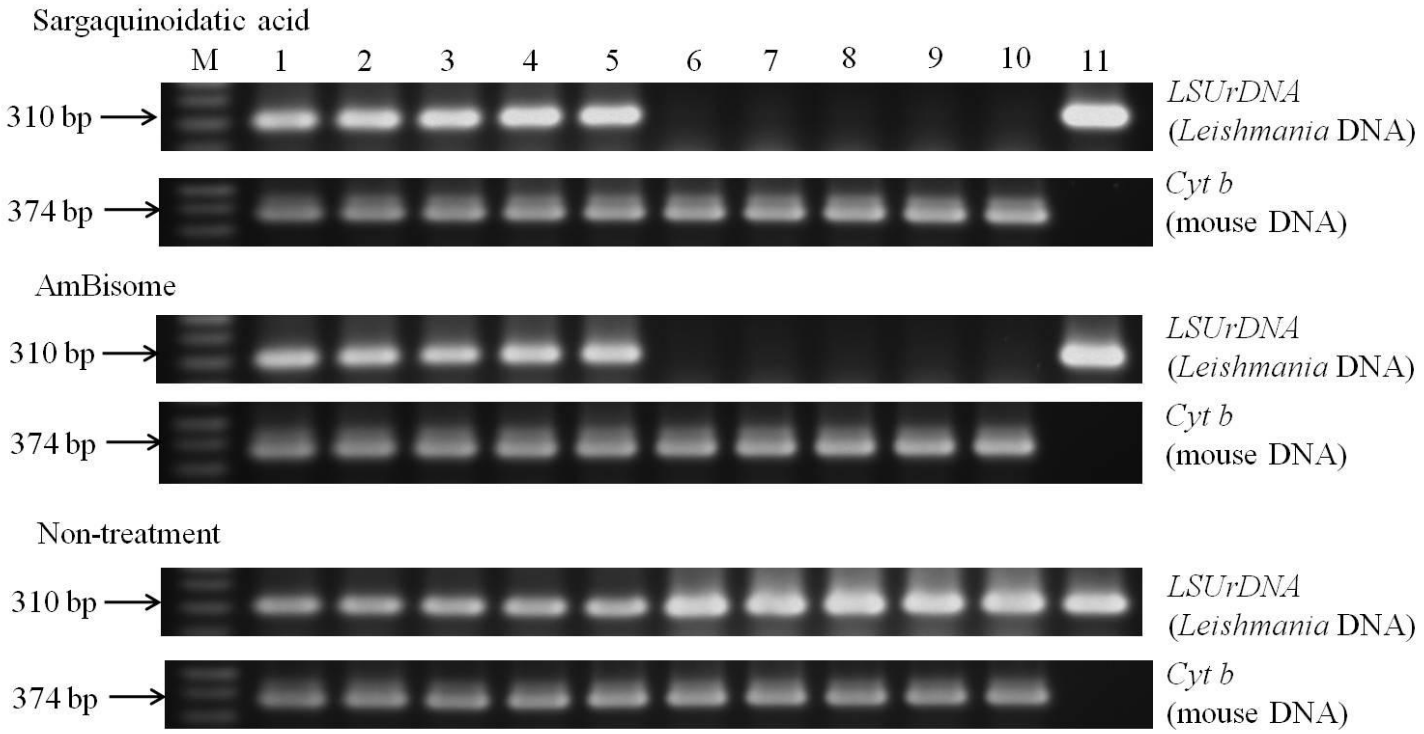

A
Total parasite number/organ
Log10
Sargaquinoidatic acid
AmBisome
Non-treatment
Detection limit
0
1
2
3
4
5
6
7
8
9
10
0
49
79
Treatment duration
Days post infection
B
Total parasite number/organ
Log10
Sargaquinoidatic acid
AmBisome
Non-treatment
Detection limit
0
1
2
3
4
5
6
7
8
9
10
11
0
49
79
Treatment duration
Days post infection

Fig. 25.



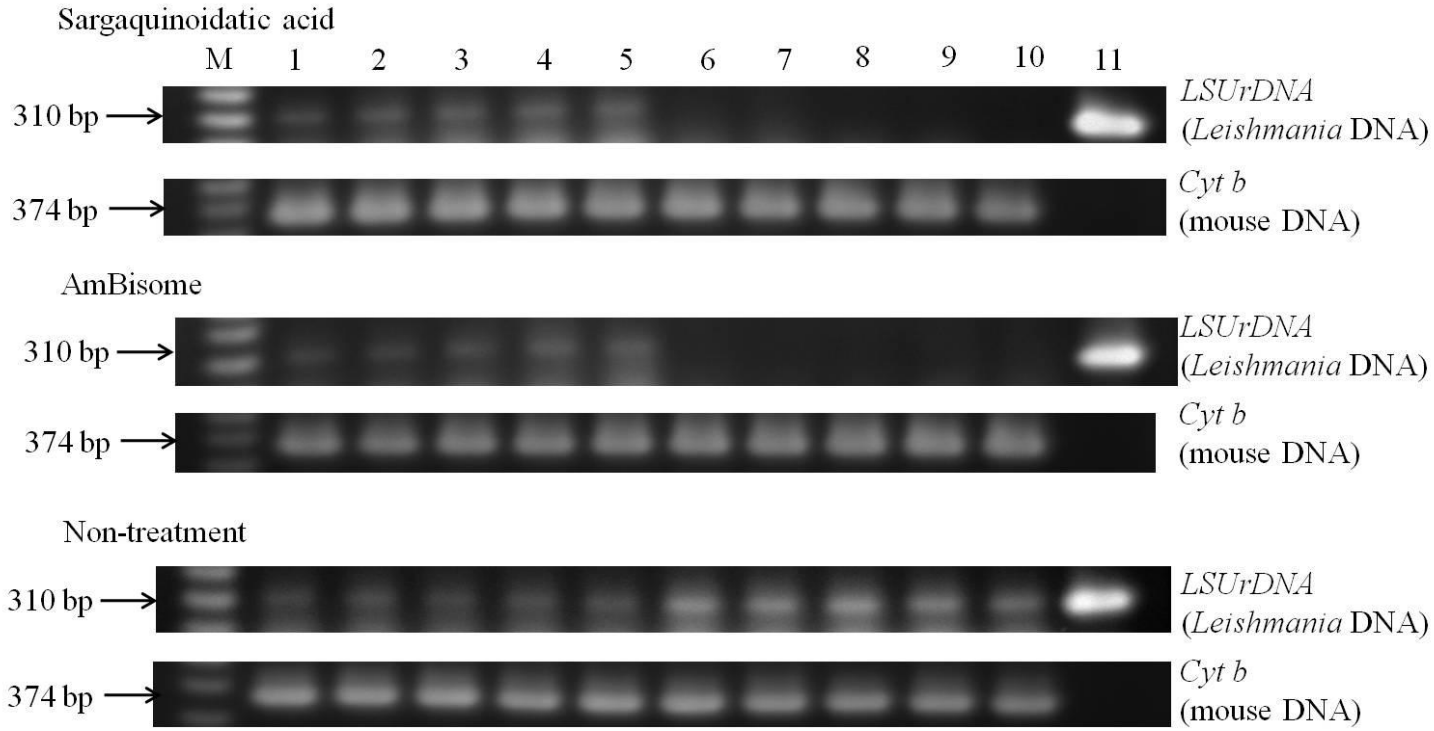
M. Marker
1. Before treatment mice spleen No. 1 (Day49)
2. Before treatment mice spleen No. 2 (Day49)
3. Before treatment mice spleen No. 3 (Day49)
4. Before treatment mice spleen No. 4 (Day49)
5. Before treatment mice spleen No. 5 (Day49)
6. After treatment mice spleen No. 1 (Day79)
7. After treatment mice spleen No. 2 (Day79)
8. After treatment mice spleen No. 3 (Day79)
9. After treatment mice spleen No. 4 (Day79)
10. After treatment mice spleen No. 5 (Day79)
11. *L. donovani* promastigotes (Positive control)

Fig. 26.



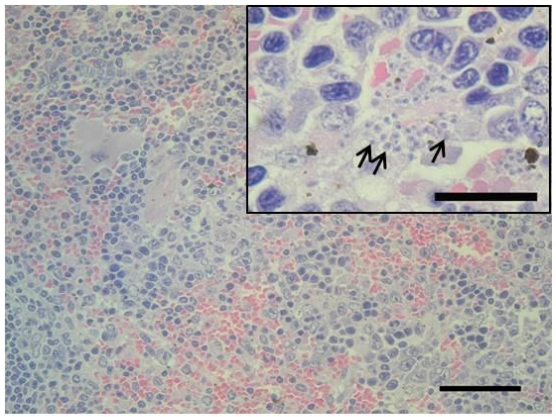
M. Marker
1. Before treatment mice liver No. 1 (Day49)
2. Before treatment mice liver No. 2 (Day49)
3. Before treatment mice liver No. 3 (Day49)
4. Before treatment mice liver No. 4 (Day49)
5. Before treatment mice liver No. 5 (Day49)
6. After treatment mice liver No. 1 (Day79)
7. After treatment mice liver No. 2 (Day79)
8. After treatment mice liver No. 3 (Day79)
9. After treatment mice liver No. 4 (Day79)
10. After treatment mice liver No. 5 (Day79)
11. *L. donovani* promastigotes (Positive control)

Fig. 27.

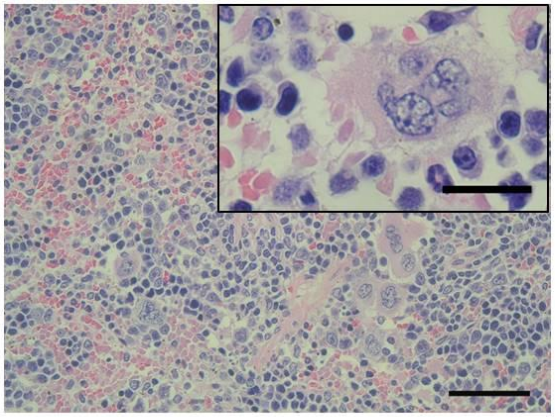


M. Marker
1. Before treatment mice blood No. 1 (Day49)
2. Before treatment mice blood No. 2 (Day49)
3. Before treatment mice blood No. 3 (Day49)
4. Before treatment mice blood No. 4 (Day49)
5. Before treatment mice blood No. 5 (Day49)
6. After treatment mice blood No. 1 (Day79)
7. After treatment mice blood No. 2 (Day79)
8. After treatment mice blood No. 3 (Day79)
9. After treatment mice blood No. 4 (Day79)
10. After treatment mice blood No. 5 (Day79)
11. *L. donovani* promastigotes (Positive control)

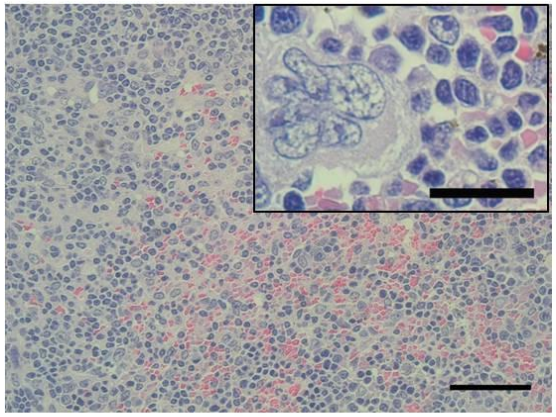
Fig. 28.



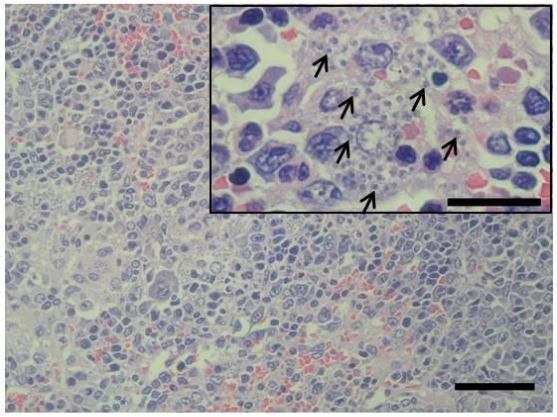
Before treatment (Day49)



Sargaquinoidatic acid (Day79)



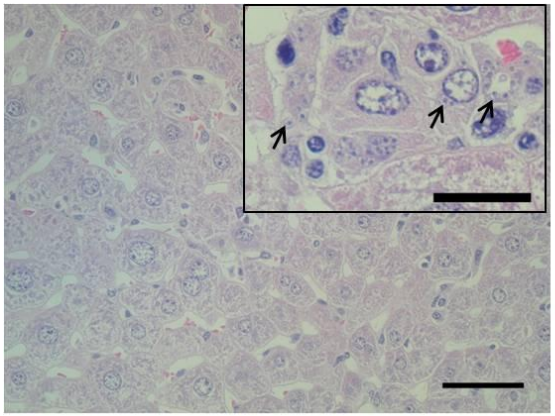
AmBisome (Day79)



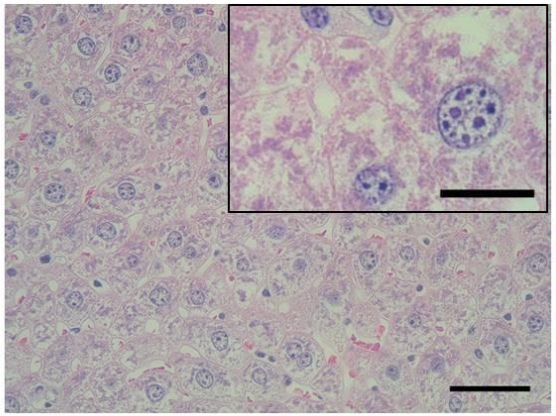
Non-treatment (Day79)

Fig. 29.

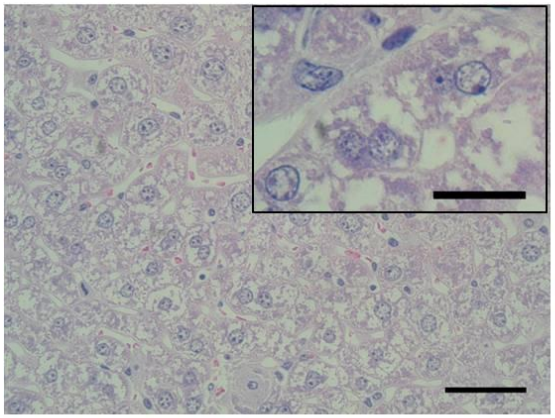
Bar : 50 μm (inset : 20 μm)



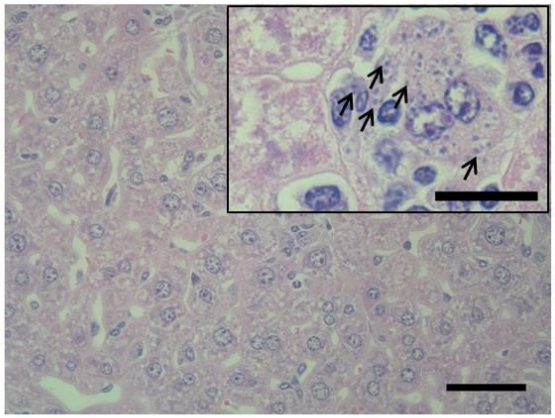
Before treatment (Day49)



Sargaquinoidatic acid (Day79)



AmBisome (Day79)



Non-treatment (Day79)

Fig. 30.

Bar : 50 μm (inset : 20 μm)

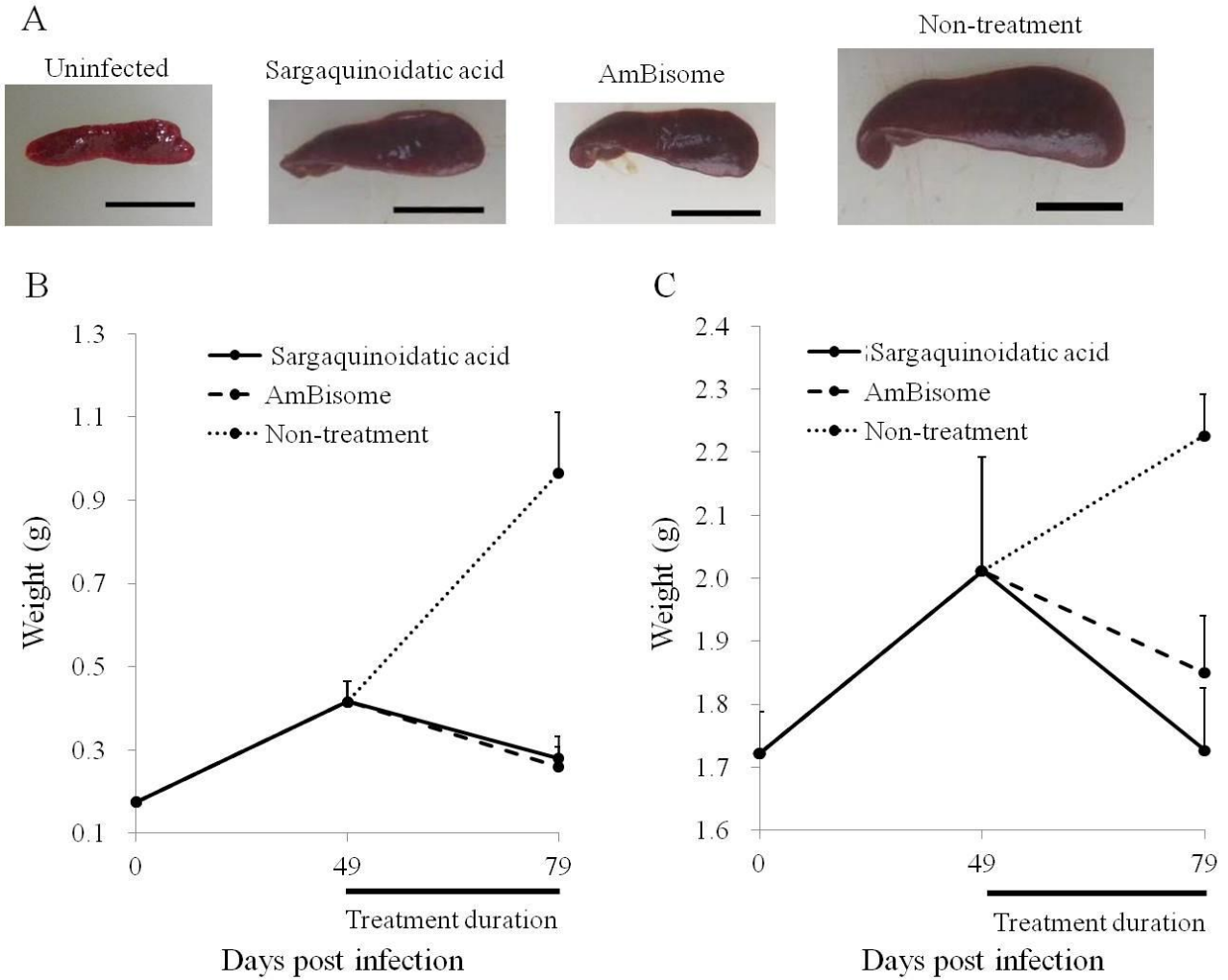

A
Uninfected
Sargaquinoidatic acid
AmBisome
Non-treatment
B
Weight (g)
1.3
1.1
0.9
0.7
0.5
0.3
0.1
Sargaquinoidatic acid
AmBisome
Non-treatment
0
49
79
Treatment duration
Days post infection
C
Weight (g)
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
Sargaquinoidatic acid
AmBisome
Non-treatment
0
49
79
Treatment duration
Days post infection

Fig. 31.

論 文 の 内 容 の 要 旨

応用動物科学
平成 23 年度博士課程進学
氏名　長田　康孝
指導教員名　松本　芳嗣

論文題目　Studies on experimental visceral leishmaniasis
(実験的内臓型リーシュマニア症に関する研究)

内臓型リーシュマニア症(Visceral leishmaniasis, VL)は *Leishmania donovani* complex に属する原虫によって引き起こされる原虫感染症で、世界で毎年 50 万人もの新規患者が発生し、WHO によれば早急にコントロールが必要な 6 大疾患の一つである。本症は脾臓、肝臓で原虫が増殖し、肝脾腫、発熱、貧血、削痩などを引き起こし、適切な治療が施されなければ死に至る重篤な感染症である。しかしながら、VL の病態解析において有用な病態マウスモデルが確立されていないこともあり、*L. donovani* がもたらす VL の病態機序に関する研究は立ち遅れている。本研究は重篤な病態を再現出来る VL マウスモデルを確立し、病態解析、免疫学的生体防御機構の一端を明らかにし、さらに治療薬評価への有用性を示すことを目的としている。

第一章では持続的に病態が進行し、重篤な病態を示す VL マウスモデルの確立を目的とし、世界各地の *L. donovani* complex 分離株のマウスへの感染実験を試みた。そのうちネパール人 VL 患者より分離した *L. donovani* D10 (MHOM/NP/03/D10, 長崎大学熱帯医学研究所より分与)が BALB/c マウスに対して最も強い病原性を示すことを明らかにした。脾臓、肝臓における感染原虫数を各臓器の押捺標本を用いて LDU(Leishman Donovan units; 宿主有核細胞あたりの原虫数×臓器重量 mg)を指標に評価した結果、両臓器の LDU は経時的に増加しており、感染 12 週では脾臓で 805±121、肝臓で 3,139±489 であった。また両臓器重量を測定した結果、両臓器とも経時的に増加し、臓器重量は感染 12 週では未感染マウスと比較し、脾臓が約 8 倍、肝臓が約 1.4 倍に腫大し顕著な肝脾腫を呈し、病理組織学的解析の結果、両臓器で原虫感染宿主細胞の著しい浸潤及び増加が観察された。*L. donovani* D10 株感染 BALB/c マウスが脾臓、肝臓における持続的な原

虫の増殖、進行性の肝脾腫を示し、重篤な VL の病態を再現するマウスモデルになると考えられた。更に、*L. donovani* D10 株感染 BALB/c マウス末梢血からの PCR 法による原虫遺伝子の検出及び培養法による原虫の分離を試みたところ、感染 4 週目には原虫遺伝子が検出され、8 週目には生きた原虫が分離された。これらの結果は PCR 法の末梢血を試料とした早期診断法の可能性を示すとともに、感染時の原虫血症の実験的証明といえる。

本症の病態に関わるファクターの探索など免疫学的解析を行うためには、*L. donovani* 感染において病態の違いを示すマウスモデルの確立が必要である。第二章では近交系マウス 3 系統(BALB/c、C57BL/6、C3H)における感染動態を検討した。重篤な VL 病態を示す BALB/c マウスに比べ、C57BL/6 マウスでは LDU が感染 12 週後では脾臓で 96±67、肝臓で 329±122、感染 24 週後では脾臓で 82±66、肝臓で 0 と、両臓器において BALB/c マウスに比べ明らかに原虫数が低く、また臓器重量を測定した結果、感染 12 週において脾臓重量は約 4 倍に増加したものの、感染 24 週後においても約 4 倍と変化はなく、BALB/c マウスのような明らかな進行性の脾腫は観察されなかった。すなわち感染はするが、進行性の病態悪化は見られず自然治癒すると考えられた。一方、C3H マウスでは両臓器において原虫の感染が確認されなかったうえ、臓器重量も重篤な肝脾腫を呈した BALB/c マウス及び一過性の脾腫を呈した C57BL/6 マウスとは異なり、肝脾腫は認められず抵抗性であると考えられた。*L. donovani* 感染において、明瞭な病態の違いを示す BALB/c、C57BL/6、C3H マウスにおける原虫抗原特異的 Th1 反応及び Th2 反応びついて、感染 12 週マウス由来脾細胞を原虫粗抗原で刺激した際の IFN-γ 及び IL-4 産生量を測定して評価したところ、培養上清中の IFN-γ は BALB/c で 1.93±0.26 ng/ml、C57BL/6 で 3.93±2.18 ng/ml、C3H で 5.34±1.18 ng/ml であったのに対して、IL-4 は BALB/c で 47.31±18.84 pg/ml、C57BL/6 で 3.06±2.46 pg/ml、C3H で 11.55±5.65 pg/ml であり、*L. donovani* 感染に対して感受性を示した BALB/c マウスでは最も低い IFN-γ 産生、最も高い IL-4 産生を示し、Th2 型の免疫反応が誘導されたと考えられ、また自然治癒を示した C57BL/6 及び抵抗性を示した C3H は Th1 型の免疫反応が誘導されたことが考えられた。以上のことから、*L. donovani* 感染に対する感受性に Th2 反応、抵抗性に Th1 反応が関与していることが明らかになった。

現在本症の治療薬として 5 価のアンチモン製剤である sodium antimony gluconate が広く用いられているが、副作用が強く、薬剤耐性原虫の出現が問題となっており、新規治療薬の探索が緊急の課題である。本症の新規治療薬の探索には実験動物を用いた薬剤の in vivo 評価系が必要である。第三章では本病態マウスモデルを用いた治療薬 in vivo 評価系の有用性を示すことを目的とした。まず始めに褐藻類ヒバマタ目ホンダワラ科アズマネジモク(*Sargassum yamadae*)より単離したキノンテルペノイド 10 種について in vitro における原虫増殖抑制効果を評価した結果、

10 種のうち 6 種が 10 μg/ml の濃度で 50%以上の増殖抑制効果を示した。100%の増殖抑制効果を示したキノンテルペノイドについて、in vivo における原虫増殖抑制効果を検討した。対照として既知の治療薬である AmBisome (200 μg/head)を用いた。感染 49 日後に剖検し重篤な VL を発症していることを確認後、30 日間の腹腔内投与(始めの 14 日間:1 mg/head、残りの 16 日間:2 mg/head)を行ったところ、治療終了翌日には双方の治療群の脾臓、肝臓において押捺標本を用いた LDU 及びより検出感度の高い培養法のいずれの方法でも感染原虫は観察されなかった。また、治療群及び未治療群の脾臓、肝臓重量を測定した結果、双方の治療群の臓器重量は、治療開始時より減少し、肝脾腫が改善されていた。尚、このキノンテルペノイドを sargaquinoidatic acid と命名し、抗リーシュマニア症治療薬として特許を取得した。また、本マウスモデルが VL 治療薬の in vivo 評価に有用であることが示された。

本論文は、世界中の広い地域に蔓延し、猛威を振るう原虫感染症である内臓型リーシュマニア症に関して 1) *L. donovani* 感染において脾臓、肝臓で持続的な原虫の増殖、病態の悪化を伴い、重篤な VL 病態を再現出来るマウスモデルを作出し、その病態を解析し、2) 本症の重症化に Th2 型の免疫反応が、抵抗性に Th1 型の免疫反応が強く関わることを明らかにした。3) 本マウスモデルが VL 治療薬の評価に有用であることを示し、更に新規キノンテルペノイド sargaquinoidatic acid が VL の新規治療薬候補化合物として有望であるとことを示した。本論文で示した VL マウスモデルは本症の病態解析、免疫学的解析など基礎研究から、診断法、新規治療薬及びワクチンの開発など応用研究まで多岐に渡る VL の研究に有用であると考えられる。