博士論文

# Involvement of BAFF in development of splenomegaly during visceral leishmaniasis

（内臓型リーシュマニア症における脾腫形成機序への BAFF の関与）

Satoko Omachi

大間知　聡子

Contents

## Abbreviations

| | |
|---|---|
| BAFF | B-cell activating factor belonging to the TNF family |
| CL | cutaneous leishmaniasis |
| ELISA | enzyme-linked immunosorbent assay |
| HC | healthy control |
| HE | hematoxylin and eosin |
| KO | knockout |
| LDU | Leishman-Donovan Units |
| ND | not detected |
| NS | not significant |
| PBS | phosphate-buffered saline |
| p.i. | post infection |
| RA | rheumatoid arthritis |
| RP | red pulp |
| SD | standard deviation |
| SLE | systemic lupus erythematosus |
| VL | visceral leishmaniasis |
| WP | white pulp |
| WT | wild-type |

## General introduction

Leishmaniasis is a spectrum of diseases caused by protozoan parasites of the genus *Leishmania*. The diseases are classified roughly into three forms based on clinical manifestations: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis and visceral leishmaniasis (VL). VL, also known as kala-azar, is the most severe form generally caused by *Leishmania donovani* in East-Africa, India, and parts of the Middle East and by *L. infantum* in Europe, North Africa, South and Central America [1]. The annual incidence of VL is estimated as 200,000 to 400,000 cases, with about 20,000 to 40,000 associated deaths [2]. Clinical manifestations of VL include fever, hepatosplenomegaly, anemia and weight loss, and it can be fatal if left untreated. *Leishmania* parasites develop as promastigotes in sand flies. Once transmitted to mammalian hosts through blood feeding by sand flies, these parasites proliferate as amastigotes in macrophages of the hosts.

Studies on host immune response during leishmaniasis have been mainly done using CL mouse model. According to the studies, the roles of host immune response in pathology during *Leishmania* infection can be divided into two aspects, whereas these aspects are related to each other. One of the aspects is associated with host resistance and susceptibility to parasite infection. For example, based on the studies using mice infected with *L. major*, which is causative species of CL in human, resistance and susceptibility to the parasite infection depend on the balance of the subsets of helper T cells [3]. The other

aspect is associated with symptoms. A previous report using mice showed that cytotoxic T cells kill parasite-infected target cells, but the cytolytic response was not protective and rather promoted development of lesions during infection of *L. braziliensis*, which is causative species of CL in human [4].

Since establishment of VL mouse model representing progressive disease was more recent, knowledge about host immune response during VL is limited. Human VL is a chronic disease with the time from infection to disease onset between 2 to 6 months [5], when acquired immunity is involved. Generally speaking, T cells and B cells play important roles in acquired immunity. About relationships between these cells and resistance/susceptibility to infection of parasites, it was reported that T-cell depleted mice and nude mice, which lack mature T cells, showed resistance against *L. donovani* infection [6, 7]. Studies have also shown that mice lacking B cells are resistant to infection of *L. donovani* [8, 9]. However, the roles of these cells in development of symptoms during VL have been poorly evaluated.

Splenomegaly is one of the major symptoms in VL. Splenomegaly can cause abdominal pain and give the patients physical discomfort. While mechanisms underlying splenomegaly are various among the diseases, it seems that increase in the number of the cells which form spleen is one of major causes of splenomegaly. In VL, spleen is one of the main places for proliferation of *Leishmania* parasites. Although it is speculated that immune response to *Leishmania* causes splenomegaly during VL, it is not clear which cells and which signals are involved.

In the present study, involvement of B cells and plasma cells, which are collectively called B-cell lineage cells in this thesis, in development of splenomegaly during VL and *L. donovani*-infected mice was examined. In chapter 1, infection-induced strong activation of B-cell lineage cells during VL is shown using serum immunoglobulins as indicators for activation of the cells. In chapter 2, contribution of B-cell lineage cells to development of splenomegaly during infection of *L. donovani* was evaluated using mice. In chapter 3, the roles of a molecule designated BAFF (for B-cell activating factor belonging to the TNF family) in activation of B-cell lineage cells, consequently in development of splenomegaly in *L. donovani*-infected mice are focused. BAFF was found as a costimulator of B cell proliferation and function [10]. Serum BAFF in VL patients was also examined. Together the aim of this study was to demonstrate the involvement of BAFF via activation of B-cell lineage cells in development of splenomegaly during infection of *L. donovani* in mice and human VL.

# Chapter 1

# IgG-dominant hyperimmunoglobulinemia is a hallmark of VL

# Introduction

Hypergammaglobulinemia has been reported as one of the symptoms of VL patients [11-14]. Hypergammaglobulinemia is a clinical manifestation that abnormally increased levels of gamma globulins are present in the blood, which is judged from the result of serum protein electrophoresis. Other than VL, this manifestation can often be found in autoimmune diseases including systemic lupus erythematosus (SLE), Sjögren's syndrome, rheumatoid arthritis (RA) and polymyositis as well as tumors of immunoglobulin-producing cells such as multiple myeloma, Waldenström's macroglobulinemia, chronic lymphocytic leukemia, and other non-Hodgkin's lymphoma [15, 16].

Proteins belonging to gamma-globulin fraction are mostly immunoglobulins. Main immunoglobulin classes in amount are IgM, IgG and IgA in blood. Immunoglobulin class which is dominantly elevated is different by diseases. For example, patients with Waldenström's macroglobulinemia are characterized by elevation of serum monoclonal IgM [17]. Serum IgA levels were elevated in patients with IgA nephropathy, which resulted from IgA-specific B-cell hyperactivity in the patients [18]. Tumors of immunoglobulin-producing cells also contribute to elevation of certain class of immunoglobulins, and sometimes malignant monoclonal proliferations are associated with a deficient production of all other immunoglobulin classes [19].

In general, following activation by antigen, B cells can develop into plasma cells and plasma cells secrete immunoglobulins. Therefore, knowing what classes

of immunoglobulins are responsible for the hypergammaglobulinemia is an important process to understand mechanisms of B-cell activation during VL. However, such quantitative and systematic analyses on immunoglobulin classes are limited. In this chapter, serum or plasma levels of immunoglobulins in VL patients were quantified.

## Materials and methods

### Patients

Plasma samples of Bangladeshi VL patients (n = 22) and Bangladeshi healthy controls (HCs, n = 28) were collected. All the patients were parasite-positive by microscopy of the smear of aspirated spleen. Plasma samples were collected at S.K. hospital with informed consent and obtained from Mymensingh Medical College, Mymensingh, Bangladesh. All the samples were coded and archived at the hospital, and personal information of the donors were not available. Collection of the samples was approved by the ethical committee at Mymensingh Medical College, and usage of the samples was approved by Institutional Review Board of The University of Tokyo (No. 14-70).

Serum samples from Brazilian VL patients (n = 20), Brazilian Chagas' disease patients (n = 10) and Brazilian HCs (n = 9) were collected with informed consent from each donor at Prof. Edgard Santos University Hospital, Universidade Federal da Bahia, Salvador, Bahia, Brazil. All the samples were coded and archived at the hospital, and personal information of the donors including the name, sex and age were not available with the exception of the sample ID describing the classification of the donor group (i.e., VL, Chagas). This study was approved by the Institutional Review Board of The University of Tokyo (No. 11-63).

### Quantification of immunoglobulin levels

Quantification of plasma or serum levels of IgG, IgM and IgA in human was

performed by using Human total IgG ELISA Ready-Set-Go! ELISA kit, Human total IgM ELISA Ready-Set-Go! and Human total IgA ELISA Ready-Set-Go! (eBioscience, Inc., San Diego, CA), respectively, according to the manufacture's instruction.

### Statistical analysis

Differences in the levels of serum immunoglobulins among groups were analyzed by Mann-Whitney test, Kruskal-Wallis test followed by Dunn's multiple comparisons test or one-way ANOVA followed by Bonferroni multiple comparisons test, and $P$ values less than 0.05 were considered significantly different.

## Results

### IgG-dominant hyperimmunoglobulinemia in VL patients

Bangladeshi VL patients showed high levels of IgG in plasma (Figure 1). Plasma IgG levels of Bangladeshi VL patients ranged from 21.13 mg/ml to 66.69 mg/ml with the mean ± standard deviation (SD) value of 42.36 ± 14.63 mg/ml. Plasma IgG levels in Bangladeshi HCs ranged from 9.19 mg/ml to 23.09 mg/ml with the mean ± SD of 15.05 ± 3.25 mg/ml. The levels in VL patients were statistically higher than those in HCs ($P < 0.0001$) and 91% of the patients showed higher levels than mean + 3SD of HCs (24.79 mg/ml). The means ± SD values of plasma IgM in Bangladeshi VL patients and HCs were 2.19 ± 1.79 mg/ml and 2.04 ± 0.96 mg/ml, respectively, and those of IgA were 0.65 ± 0.40 mg/ml and 1.09 ± 0.45 mg/ml, respectively. While there was not a significant difference between the IgM levels of VL patients and those of HCs, the levels of IgA in VL patients were significantly lower than those in HCs ($P < 0.0001$).

Brazilian VL patients also showed high levels of IgG (Figure 2). The serum IgG levels in the VL patients ranged from 3.34 mg/ml to 64.13 mg/ml with the mean ± SD values of 24.97 ± 18.34 mg/ml. The serum IgG levels in the patients with Chagas' disease ranged from 3.15 mg/ml to 17.25 mg/ml with the mean ± SD value of 10.26 ± 4.66 mg/ml, and the levels in HCs ranged from 1.45 mg/ml to 8.03 mg/ml with the mean ± SD value of 4.68 ± 2.61 mg/ml. The levels in VL patients were statistically higher than those in Chagas' disease patients ($P < 0.05$) and HCs ($P < 0.01$), whereas no difference was found between Chagas' disease

patients and HCs ($P > 0.05$). When the mean + 3SD of HCs was set as a cutoff (12.5 mg/ml), 75% of VL patients showed elevated serum IgG, whereas 30% of Chagas' diseases patients did. The means ± SD values of serum IgM in Brazilian VL patients, the patients with Chagas' disease and HCs were 4.92 ± 8.14 mg/ml, 1.83 ± 0.75 mg/ml and 1.39 ± 0.82 mg/ml, respectively, and those of IgA were 0.48 ± 0.60 mg/ml, 1.34 ± 1.37 mg/ml and 0.77 ± 0.25 mg/ml, respectively. There was no significant difference between the IgM levels in VL patients and HCs, VL patients and Chagas' disease patients, or Chagas' disease patients and HCs. The IgA levels in VL patients were statistically lower than those in Chagas' disease patients ($P < 0.01$) and HC ($P < 0.05$), whereas no difference was found between Chagas' disease patients and HC ($P > 0.05$).

## Discussion

From the results, IgG-dominant hyperimmunoglobulinemia was shown in both Bangladeshi and Brazilian VL patients. The majority of the previous reports on hypergammaglobulinemia in VL are case reports, and there have been only a few reports about quantitative data of immunoglobulins which target more than ten patients. Especially about Bangladeshi VL patients, this is the first report of quantitative data of immunoglobulins. Increase of the mean value of IgG was 2.8-fold in Bangladeshi VL patients and 5.3-fold in Brazilian VL patients (comparison to HCs of each region). Previous reports from other region also showed marked increase in the level of IgG (4.4-fold in India [12]; 4.3-fold in India [20]; 4.4-fold in Somalia (comparison of median value)[21]). In both Bangladesh and Brazil, there was no significant difference in IgM levels between patients and HCs, and IgA levels were significantly lower in patients than HCs. Previous reports showed lesser increase than IgG or no increase of IgM and IgA in VL patients[12, 20, 21]. Together with other reports, IgG-dominant hyperimmunoglobulinemia is a hallmark of VL, which is not restricted by the geographical backgrounds or the infecting *Leishmania* species.

Clinically there is a reference value of serum IgG, 8.7-17 mg/ml (from website of Sysmex Corporation, Hyogo, Japan). In infectious diseases, detection of antigen-specific antibodies is common way for diagnosis. However, not all infectious diseases are supposed to be associated with elevation of immunoglobulin levels. In this study, the patients with Chagas' disease did not

show elevation of serum IgG whereas high titer of parasite antigen-specific IgG was reported [22].

Hypergammaglobulinemia can be classified into monoclonal or polyclonal hypergammaglobulinemia. Monoclonal hypergammaglobulinemia, which is characterized by the presence of sharp and strong band in the result of serum protein electrophoresis, can be found in patients with B-cell malignancy including multiple myeloma and Waldenström's macroglobulinemia as well as in people without any apparent symptoms called monoclonal gammopathy of undetermined significance [16]. In contrast, SLE and RA, where autoantibodies play important roles in pathology, are characterized by polyclonal hypergammaglobulinemia [15]. Hypergammaglobulinemia during VL also has been considered as polyclonal hypergammaglobulinemia because serum electrophoresis of VL patients represents not a single sharp peak but rather broad [23].

However, it is not that B-cell activation during VL is antigen-non-specific. First, most of the autoantibody reported is not IgG but IgM. Second, positive correlation between IgG levels and *Leishmania*-specific IgG antibody titer was found in VL patients (in-house data). Moreover, VL patients have selective IgG responses to *Leishmania*-specific antigens. For example, serum from VL patients showed no reactivity to species-specific antigen of *Trypanosoma cruzi*, which is causative species of Chagas' disease [22, 24, 25]. Because there are no reports of IgG autoantibody to be detected as high titers in VL patients as those to major antigens, the majority of serum IgG in VL patients seems to be antigen-specific rather than from non-specific B-cell activation.

A marked increase in the level of IgG in serum or plasma of VL patients was shown in this chapter. It is suggested that B-cell lineage cells are strongly activated during VL. Moreover, involvement of some factors which promote immunoglobulin class switching to IgG was indicated.

# Chapter 2

# Increase in the number of B cell-lineage cells contributes to splenomegaly in *L. donovani*-infected mice

# Introduction

Splenomegaly is one of the main symptoms in VL (from previous reports, 71% of 18,501 patients [26]; 77% of 2,130 patients [27]; 97% of 120 patients [28]). Splenomegaly is also seen in hematological diseases such as myelofibrosis, non-Hodgkin lymphoma and leukemia, infectious diseases such as viral hepatitis, tuberculosis and malaria and autoimmune diseases such as SLE and RA [29]. Splenomegaly can cause sense of distension and abdominal pain, which give the patients physical discomfort.

From previous reports increase in the number of the cells which form spleen is one of the main causes of splenomegaly though there is a variety of cell populations among the diseases. For example, in the patients with myelofibrosis, erythroblasts and megakaryocytes were significantly increased in spleen, which indicated splenic hematopoiesis [30]. Another report showed that accumulation of macrophages is one of the main causes of splenomegaly in the mice infected with *Plasmodium berghei*, which causes rodent malaria [31]. However, it is not clear which cells are involved in the development of splenomegaly during VL.

Spleen is the largest secondary lymphoid organ in the body. The structure of spleen is divided into the white pulp and the red pulp. The white pulp is organized as lymphoid sheaths, with T- and B-cell compartments, around the branching arterial vessels and functions as a secondary lymphoid tissue [32]. The red pulp consists of fibroblasts and reticular fibres and is a macrophage-rich region [32]. The region has capacity to filter blood and is responsible for the

removal by phagocytosis of aged erythrocytes, platelets and some blood-borne pathogens [32]. In VL, spleen is one of the main places for proliferation of *Leishmania* parasites and it is speculated that immune response to *Leishmania* causes splenomegaly during VL.

Because it was indicated that B-cell lineage cells were strongly activated during VL from the results of chapter 1, involvement of B-cell lineage cells in splenomegaly was focused. Spleen is one of the places where B cells proliferate. Antigen-binding B cells grow as plasmablasts and proliferate to form germinal centers, and then differentiate into plasma cells, which secrete immunoglobulins [33-35]. Elucidation of pathological features in the spleen of VL patients remains difficult due to ethical limitations, so experimentally infected mice were used. In this chapter, contribution of B-cell lineage cells to splenomegaly in *L. donovani*-infected mice is shown.

## Materials and methods

### Mice and parasites

Male BALB/cA mice were purchased from Japan Clea, Tokyo, Japan. All mice were maintained under specific pathogen-free conditions. The mice were used for experiments at the age of 6-9 weeks. This animal experiment was reviewed and approved by an institutional animal research committee at the Graduate School of Agricultural and Life Sciences, The University of Tokyo (No. P14-930). Promastigotes of *L. donovani* (MHOM/NP/03/D10; gifted from National BioResource Project at Nagasaki University) were cultured in medium TC199 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Scientific, USA) and 25 mM HEPES buffer (MD Biomedicals, France) at 25ºC.

### Experimental infection and parasitological examination

*L. donovani* promastigotes in a late log or stationary phase were washed three times with phosphate buffered saline (PBS; Nissui Pharm) by centrifugation at 1,600 *g* for 10 minutes, and were resuspended with PBS at the concentration of $1 \times 10^8$ cells/ml. Mice were infected with $1 \times 10^7$ *L. donovani* promastigotes by intravenous injection to the tail vein, and sacrificed at 12 or 24 weeks post infection.

Stamp smears of the spleen and liver were fixed for 10 minutes in methanol (WAKO, Osaka, Japan) and stained for 30 minutes with 5% Giemsa solution

(Merck KGaA, Parmstadt, Germany). Amastigotes were counted by microscopic observation of the stained smear at 1,000× magnification, and Leishman-Donovan Units (LDU) was enumerated as the number of amastigotes per 1,000 host nuclei × the tissue weight in grams according to a previous study [36].

## Quantification of serum immunoglobulin levels

Mouse blood was centrifuged at 5,000 *g* for 10 minutes and the supernatant was collected as serum. Quantification of serum levels of IgG, IgM and IgA in mice was performed by using Mouse total IgG ELISA Ready-Set-Go! ELISA kit, Mouse total IgM ELISA Ready-Set-Go! ELISA kit and Mouse total IgA ELISA Ready-Set-Go! ELISA kit (eBioscience, Inc., San Diego, CA), respectively, according to the manufacture's instruction.

## Pathological examination of sections of paraffin-embedded spleen

Pieces of the spleens were fixed with 20% neutral buffered formalin and then embedded in paraffin. Paraffin-embedded spleens of *L. donovani*-infected mice and naive mice were cut at 4-6 μm in thickness using a microtome. Sections were dewaxed and then boiled in antigen retrieval buffer (Citrate buffer, pH 6 for IgG; Tris-EDTA buffer, pH 9 for B220 and CD3). The sections were incubated in methanol containing 0.3 % $H_2O_2$ followed by blocking buffer. Then the sections were probed with anti-B220 (B cell marker) antibody (BD Biosciences, USA) followed by peroxidase-conjugated anti-rat IgG (Nichirei Bioscience, Japan),

anti-CD3 (T cell marker) antibody (Santa Cruz, USA) followed by peroxidase-conjugated antibody anti goat IgG (Nichirei Bioscience, Japan) and HRP-conjugated anti-IgG antibody (GE Healthcare, USA). Probed sections were stained by DAB (Nichirei-Histofine Simple Stain, Nichirei Bioscience, Japan) and counterstained with hematoxylin (WAKO, Osaka, Japan).

Hematoxylin and eosin staining was also performed for histological analyses. The sections were stained with Mayer's hematoxylin solution for 2 minutes, and rinsed in running tap water for 1 hour. Next, the tissues were stained with eosin solution (MUTO PURE CHEMICALS CO., LTD., Tokyo, Japan) for 3 minutes.

### Flow cytometric analysis of splenocytes

Splenocytes of naive and infected mice were isolated through cell strainer (BD Pharmingen, USA) and red blood cells were lysed by lysing solution (Sigma-Aldrich, USA). The cells were washed three times with PBS + 1% heat-inactivated fetal bovine serum, and incubated with FITC-conjugated anti-CD3 monoclonal antibody and PE-conjugated anti-CD19 monoclonal antibody (BD Pharmingen, USA). After washing, the cells were fixed by BD Cytofix/Cytoperm Fixation/Permeabilization solution (BD Pharmingen, USA), and then washed again. At least 10,000 cells per sample were analyzed on the BD FACSVerse™, and data analysis was performed using BD FACSuite™ software. The lymphocytes were gated in the FSC-SSC dot plots, and the number of lymphocyte-gated $CD19^+$ cells and $CD3^+$ cells were analyzed by quadrant analysis of 2-dimentional dot plots.

## Statistical analysis

Differences in body, spleen and liver weight among groups were analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test, and $P$ values less than 0.05 were considered significantly different.

## Results

### IgG-dominant hyperimmunoglobulinemia in *L. donovani*-infected mice

Serum levels of IgG and IgM increased continuously in the course of infection (Figure 3). The mean ± SD of serum IgG level in naive mice was 0.13 ± 0.03 mg/ml, and at 12 and 24 weeks post infection, 5.99 ± 1.48 mg/ml and 24.00 ± 1.63 mg/ml, respectively. The means ± SD of serum IgM levels at the indicated time were 0.25 ± 0.02 mg/ml, 0.59 ± 0.10 mg/ml and 1.05 ± 0.15 mg/ml, respectively. The means ± SD of serum IgA levels at the indicated time were 0.013 ± 0.002 mg/ml, 0.282 ± 0.226 mg/ml and 0.250 ± 0.087 mg/ml, respectively. Ratio of serum levels of IgG, IgM and IgA was 10:19:1 at naive state, 21:2:1 at 12 weeks post infection and 96:4:1 at 24 weeks post infection.

The means ± SD of LDU for the spleen were 182 ± 10 at 12 weeks post infection and 2,219 ± 440 at 24 weeks post infection (Figure 4). The means ± SD of LDU for the liver were 1,114 ± 620 at 12 weeks post infection and 2,291 ± 1,802 at 24 weeks post infection.

### Splenomegaly in *L. donovani*-infected mice

*L. donovani*-infected mice showed splenomegaly (Figure 5). The mean ± SD of spleen weight was 0.079 ± 0.054 g in naive mice, and at 12 and 24 weeks post infection, the value was 0.352 ± 0.151 g and 1.639 ± 0.789 g, respectively. The infected mice also showed hepatomegaly (graph not shown). The means ± SD of

liver weight at indicated time were 1.553 ± 0.093 g, 2.057 ± 0.034 g and 2.642 ± 0.212 g, respectively.

Microscopic observation of HE-stained spleen sections demonstrated that both red pulp region (red staining) and white pulp region (dark blue staining) expanded after infection (Figure 6). In the red pulp of infected mice, other than red blood cells, macrophages which were infected with amastigotes were observed. In the white pulp of infected mice, lymphocytes which are characterized by round nucleus and thin rim of cytoplasm were increased.

## Increase in the number of B-cell lineage cells in the spleen of infected mice

In infected mouse spleen, there were much more cells stained by anti-IgG antibody compared to naive mouse spleen (Figure 7). Because those cells had round-shaped nucleus with large cytoplasm and cytoplasm was stained by anti-IgG antibody, the cells were judged as IgG-expressing plasma cells. The cells were observed beside white pulp region. By observing serial sections stained by anti-CD3 antibody and anti-B220 antibody, IgG-expressing cells were observed beside the region of CD3-positive cells, opposite site of the region of B220-positive cells.

From the flow cytometric analyses, average ratio of $CD19^+$ lymphocytes in total lymphocytes was increased after infection (from 23% to 38% at 12 weeks post infection and 30% at 24 weeks post infection) (Figure 8). The number of total splenic cells was also increased after infection. The calculated average number of

$CD19^+$ lymphocytes in naive mice was $1.3 \times 10^7$ cells, and in infected mice (24 weeks post infection) $1.4 \times 10^8$ cells, about 11-fold increased. In contrast, the calculated average number of $CD3^+$ lymphocytes in naive mice was $3.5 \times 10^7$ cells, and at 24 weeks post infection $1.0 \times 10^8$ cells, about 3-fold increased. The number of the lymphocytes other than $CD3^+$ or $CD19^+$ lymphocytes also increased. From the FSC-SSC dot plot analysis, the cells with higher FSC value than lymphocytes were increased after infection.

## Discussion

Spleen not only removes aged erythrocytes from the circulation but also works as a secondary lymphoid organ involved in the surveillance against blood-borne pathogens [32]. The protection provided by the spleen is dependent on compartmentalized microenvironments suitable for antigen trapping, in addition to cell interactions leading to the development and maintenance of immune response against circulating pathogens [32]. Immune response against pathogen infection can change spleen structure. For example, mouse cytomegalovirus infection induces significant remodeling of splenic microarchitecture, including loss of marginal zone macrophage populations and dissolution of T and B cell compartmentalization [37]. Another report showed that white pulp in the spleens of patients dying from malaria showed a marked architectural disorganization [38].

From the results of HE-stained spleen sections, both red pulp and white pulp expansion were prominent after infection. The mechanisms underlying splenomegaly during infection of *L. donovani* may be different from that in other diseases. It was reported that enlargement of red pulp was more prominent than that of white pulp in *Plasmodium berghei*-infected mice [31]. Thus the mechanisms in development of splenomegaly vary according to difference in pathogens and host immune response.

At 24 weeks post infection, boundary between white pulp and red pulp became complicated, when IgG-expressing plasma cells were present at marginal

zone extending to the red pulp. This disruption of the splenic histological structure in *L. donovani*-infected mice was somewhat similar to and somewhat different from that reported in *L. infantum*-infected dogs [39, 40]. The reports showed that plasma cells replace normal cell populations in the spleen and the changes in splenic structure are associated with the patterns of response to the infection of *L. infantum* in dogs. From those results, the numbers of B cells were reduced in both the follicles and marginal zones, which is different from the result of this study. They also showed splenic plasmacytosis is strongly associated with high serum globulin concentrations.

IgG-dominant hyperimmunoglobulinemia, which was seen in VL patients, was also seen in *L. donovani*-infected mice, and these mice are supposed to be useful for better understanding of B-cell activation during VL. The number of $CD19^+$ cells was increased significantly after infection. At 24 weeks post infection, the number of splenocytes other than $CD3^+$ or $CD19^+$ lymphocytes was more than $CD19^+$ lymphocytes. However, those splenocytes include plasma cells, which do not express CD19 on the cell surface. There have been only a few studies examining the number of B cells in splenomegaly during VL. In one previous study, ratio of T cells, B cells and macrophages in splenic nucleated cells in *L. donovani*-infected BALB/c mice was examined at 2 and 8 weeks post infection [41]. The report showed that the percentage of macrophages increased significantly and to a lesser extent that of B cells. In that study, the time points were set earlier and the degree of splenomegaly was much more moderate than this study. From the present study, it is indicated that infection-induced increase in B-cell

lineage cells is one of the main causes of splenomegaly in late phase of infection.

# Chapter 3

# BAFF is involved in development of splenomegaly via activation of B cell-lineage cells in *L. donovani*-infected mice

## Introduction

The results in chapter 2 showed that *L. donovani* infection-induced increase in B-cell lineage cells is one of the main causes of splenomegaly in mice. Then which factors are involved in hyper-activation of B-cell lineage cells?

For example, IL-4 is the representative Th2 cytokine that is involved in induction of humoral immunity, and there is a study that serum levels of IL-4 are high in VL patients in India [42]. VL patients also have elevated levels of total and antigen-specific IgE [43, 44], indicating the involvement of IL-4 in humoral responses during the disease. However, it should be noted that the role of IL-4 in VL animal model is controversial [45-47] and there is a report that spleen weights of IL-4 knockout mice and wild-type mice were not significantly different during infection of *L. donovani* [48].

In this study, the roles of one molecule designated BAFF (for B-cell activating factor belonging to the TNF family) in development of symptom was focused. BAFF, also known as B lymphocyte stimulator (BLyS), tumor necrosis factor- and ApoL-related leukocyte expressed ligand 1 (TALL-1) and tumor necrosis factor ligand superfamily 13B (TNFSF13B), is a critical regulator of B-cell development and differentiation [49]. Although the molecule is indispensable in maintaining B cell functions, aberrant expression of BAFF is associated with some diseases representing hypergammaglobulinemia. While few studies have been done about BAFF in infectious diseases, those diseases include SLE, RA and Sjögren's syndrome where inhibitors of BAFF signaling such as belimumab and blisibimod

are approved and/or being evaluated for treatment.

In the first half of this chapter, it was shown that BAFF levels in blood of VL patients and *L. donovani*-infected mice were increased. In the latter half, effects of BAFF deficiency in the development of splenomegaly during infection of *L. donovani* were examined in mice. Together the aim of this chapter is to show that BAFF is involved in splenomegaly via activation of B-cell lineage cells during infection of *L. donovani*.

## Materials and methods

### Production of BAFF-deficient mouse

BAFF-deficient mouse was produced by offset-nicking method of CRISPR/Cas system according to previous report [50] by Dr. Wataru Fujii at Laboratory of Applied Genetics, Graduate school of Agricultural and Life Sciences, the University of Tokyo. Briefly, exon 1 to exon 2 of BAFF locus, which include transmembrane region and proteolytic cleavage site [51] (Figure 9), was deleted by using the four gRNAs designed at the following loci; 5'-GGAGTAAGTGACCACAGGGGTGGG, 5'-GACCTTCAAAGTGCTCCTCGTGG, 5'-GTCGTCTTGTCACCTCCCACAGGG, and 5'-GACAAGACGACTAGGGTGCACGG. Approximately 4 pl of RNA solution that contain 100 μg/ml of $Cas9^{D10A}$ mRNA and 10 μg/ml of each gRNA was injected into the cytoplasm of each zygote obtained from naturally mated BALB/cA female mice. Ten injected-zygotes were transferred into the oviductal ampulla of a 0.5 dpc pseudo-pregnant recipient mouse, then 2 pups were obtained by natural birth. The genotypes of the pups were determined by the genomic-PCR using the primers (forward primer, 5'-CAAACAGGACTGTATTGAGATAGAATGAAAC, and reverse primer, 5'-ACAGTCTTTGCCATTTCTGTGTGTTTTGC) and sequencing of the PCR amplicons, indicated that both of the pups had bi-allelic region deletion. The mice were made specific pathogen-free at Central Institute for Experimental Animals, Kawasaki.

## Mice and parasites

Male wild-type BALB/cA mice were purchased from Japan Clea, Tokyo, Japan. Male BAFF-deficient BALB/cA mice were obtained by home breeding. All mice were maintained under specific pathogen-free conditions. The mice were used for experiments at the age of 6-9 weeks. This animal experiment was reviewed and approved by an institutional animal research committee (No. P15-80 and No. P14-930) and an institutional committee on genetically modified organisms (No. 830-2630) at the Graduate School of Agricultural and Life Sciences, The University of Tokyo. Promastigotes of *L. donovani* (MHOM/NP/03/D10; gifted from National BioResource Project at Nagasaki University) were cultured in medium TC199 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Scientific, USA) and 25 mM HEPES buffer (MD Biomedicals, France) at 25ºC.

## Experimental infection and parasitological examination

*L. donovani* promastigotes in a late log or stationary phase were washed three times with phosphate buffered saline (PBS; Nissui Pharm) by centrifugation at 1,600 *g* for 10 minutes, and were resuspended with PBS at the concentration of $1 \times 10^8$ cells/ml. Mice were infected with $1 \times 10^7$ *L. donovani* promastigotes by intravenous injection to the tail vein, and sacrificed at 12 or 24 weeks post infection.

## Patients

Plasma samples of Bangladeshi VL patients (n = 10) and Bangladeshi healthy controls (HCs, n = 30) were collected. All the patients were parasite-positive by microscopy of the smear of aspirated spleen. Plasma samples were collected at S.K. hospital with informed consent and obtained from Mymensingh Medical College, Mymensingh, Bangladesh. All the samples were coded and archived at the hospital, and personal information of the donors were not available. Collection of the samples was approved by the ethical committee at Mymensingh Medical College, and usage of the samples was approved by Institutional Review Board of The University of Tokyo (No. 14-70).

Serum samples from Brazilian VL patients (n = 20), Brazilian Chagas' disease patient (n = 10) and Brazilian HCs (n = 9) were collected with informed consent from each donor at Prof. Edgard Santos University Hospital, Universidade Federal da Bahia, Salvador, Bahia, Brazil. All the samples were coded and archived at the hospital, and personal information of the donors including the name, sex and age were not available with the exception of the sample ID describing the classification of the donor group (i.e., VL, Chagas). This study was approved by the Institutional Review Board of The University of Tokyo (No. 11-63).

## Quantification of serum BAFF levels and immunoglobulins

Mouse blood was centrifuged at 5,000 *g* for 10 minutes and the supernatant was collected as serum. Quantification of serum BAFF levels in mice was performed by using commercial ELISA kits (R&D Systems, USA) according to the

manufacture's instruction. Quantification of serum or plasma BAFF levels in human was performed by using commercial ELISA kits (R&D Systems, USA) according to the manufacture's instruction.

### Immunohistochemistry of spleen of infected mice

Pieces of the spleens were fixed with 20% neutral buffered formalin and then embedded in paraffin. Paraffin-embedded spleens of *L. donovani*-infected mice and naive mice were cut at 4-6 μm in thickness. Sections were dewaxed and then boiled in antigen retrieval buffer (Tris-EDTA buffer, pH 9). The sections were incubated in methanol containing 0.3 % $H_2O_2$ and blocking buffer. Then the sections were probed with anti-B220 (B cell marker) antibody (BD Biosciences, USA) or anti-CD138 antibody (BD Biosciences, USA) followed by peroxidase-conjugated antibody anti rat IgG (Nichirei Bioscience, Japan). Probed sections were stained by DAB (Nichirei-Histofine Simple Stain, Nichirei Bioscience, Japan) and counterstained with Mayer's hematoxylin solution (WAKO, Osaka, Japan).

The sections were also stained with hematoxylin for 2 minutes, and rinsed in running tap water for 1 hour. Next, the tissues were stained with eosin solution (MUTO PURE CHEMICALS CO., LTD., Tokyo, Japan) for 3 minutes.

### Flow cytometric analysis of splenocytes

Splenocytes of naive and infected mice were isolated through cell strainer (BD Pharmingen, USA) and red blood cells were lysed by lysing solution

(Sigma-Aldrich, USA). The cells were washed three times with PBS + 1% heat-inactivated fetal bovine serum, and incubated with FITC-conjugated anti-CD3 monoclonal antibody and PE-conjugated anti-CD19 monoclonal antibody (BD Pharmingen, USA). After washing, the cells were fixed by BD Cytofix/Cytoperm Fixation/Permeabilization solution (BD Pharmingen, USA), and then washed again. At least 10,000 cells per sample were analyzed on the BD FACSVerse™, and data analysis was performed using BD FACSuite™ software. The lymphocytes were gated in the FSC-SSC dot plots, and the number of lymphocyte-gated $CD19^+$ cells and $CD3^+$ cells were analyzed by quadrant analysis of 2-dimentional dot plots.

## Statistical analysis

Differences in BAFF levels among groups were analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test and differences between wild-type mice and knockout mice were analyzed by two-way ANOVA followed by Bonferroni multiple comparisons test. *P* values less than 0.05 were considered significantly different.

## Results

### Increase in the BAFF levels in serum or plasma of VL patients

Plasma BAFF levels of Bangladeshi HCs ranged from 0.689 to 1.37 ng/ml with the mean ± SD of 0.954 ± 0.189 ng/ml (Figure 10A). Those of Bangladeshi VL patients ranged from 0.795 to 3.64 ng/ml with the mean ± SD of 1.87 ± 0.865 ng/ml. The levels of VL patients were statistically higher than those of HCs ($P < 0.0001$). When the mean + 3SD of HCs was set as a cutoff (1.52 ng/ml), 60% of VL patients showed elevated plasma BAFF.

Serum BAFF levels of Brazilian HCs ranged from 0.504 to 1.48 ng/ml with the mean ± SD of 1.08 ± 0.34 ng/ml (Figure 10B). Those of VL patients ranged from 2.01 to 8.33 ng/ml with the mean ± SD of 4.65 ± 2.16 ng/ml. Those of Chagas' disease patients ranged from 0.519 to 2.88 ng/ml with the mean ± SD of 1.19 ± 0.672 ng/ml. The levels of VL patients were statistically higher than those of HCs and Chagas' disease patients ($P < 0.0001$), whereas there was no statistical difference between HCs and Chagas' disease patients ($P > 0.05$). When the mean + 3SD of HCs was set as a cutoff (2.089 ng/ml), 90% of VL patients showed elevated serum BAFF, whereas 10% of Chagas' diseases patients did.

### Increase in the serum BAFF levels in *L. donovani*-infected mice

Means ± SD of serum BAFF levels in mice at 12 and 24 weeks post infection were 8.59 ± 0.90 ng/ml and 11.65 ± 1.43 ng/ml, respectively, in contrast to the levels in naive mice being 5.34 ± 1.19 ng/ml (Figure 11). The levels in infected

mice at 12 and 24 weeks post infection were statistically higher than those in naive mice ($P < 0.05$ and $P < 0.05$, respectively) and the levels at 24 weeks post infection were statistically higher than those at 12 weeks post infection ($P < 0.05$).

### Deletion of BAFF gene and protein in BAFF knockout mice

Expected size of PCR products from complete gene was 1,212 bp and that from gene lacking targeted region was 480 bp. The bands corresponding with the expected size of PCR products were detected from DNA of homozygous knockout mice (about 480 bp), heterozygous knockout mice (about 480 bp and 1,212 bp) and wild-type mice (about 1,212 bp)(Figure 12).

Serum BAFF levels in homozygous knockout mice were under detection limit (< 0.625 ng/ml) while means ± SD of the levels in heterozygous knockout mice were 2.334 ± 0.255 ng/ml and wild-type mice 4.787 ± 0.331 ng/ml.

### Moderate splenomegaly in *L. donovani*-infected BAFF knockout mice

Means ± SD of spleen weight of naive wild-type mice and naive BAFF knockout mice were 0.079 ± 0.005 g and 0.054 ± 0.006 g, respectively (Figure 13). At 12 weeks post infection, those of wild-type mice and knockout mice were 0.352 ± 0.031 g and 0.151 ± 0.013 g, respectively, and at 24 weeks post infection, 1.639 ± 0.165 g and 0.789 ± 0.058 g, respectively. The spleen weight of knockout mice at 12 and 24 weeks post infection was statistically lower than those of wild-type mice ($P < 0.05$ and $P < 0.05$, respectively) whereas there was no statistical difference between the spleen weight of naive wild-type mice and that of naive

BAFF knockout mice. On the other hand, though both wild-type and knockout mice showed hepatomegaly after infection, there were no statistical differences between the liver weight of both groups at each time point.

## Increase in the number of B cell-lineage cells and IgG-dominant hyperimmunoglobulinemia were not seen in *L. donovani*-infected BAFF knockout mice

While the ratio of CD19-positive lymphocytes in total splenocytes in infected wild-type mice was increased (the average ratio was 38% at 12 weeks post infection and 30% at 24 weeks post infection) compared to naive wild-type mice (23%), the ratio was decreased in infected knockout mice (7% at 12 weeks post infection and 1% at 24 weeks post infection) compared to naive knockout mice (9%) (Figure 14). While the number of CD19-positive lymphocytes in wild type mice increased after infection (from $1.3 \times 10^7$ cells in naive mouse to $1.4 \times 10^8$ cells at 24 weeks post infection), that in knockout mice did not increase (from $1.6 \times 10^6$ cells in naive mouse to $1.0 \times 10^6$ cells at 24 weeks post infection). While increase ratio of CD3-positive cells was 2.98 and that of the splenocytes other than CD19-positive or CD3-positive lymphocytes in wild-type mice was 24.46, those in knockout mice were 2.07 and 19.07, respectively.

Immunohistochemical staining of spleen sections showed that proportion of B220-positive cells and CD138-positive cells in spleen was significantly low in BAFF deficient mice compared to wild-type mice at each time point (Figure 15).

Means ± SD of serum IgG levels in naive wild-type mice and knockout mice

were 0.131 ± 0.025 mg/ml and 0.026 ± 0.003 mg/ml, respectively (Figure 16). Those in infected wild-type mice and knockout mice were 5.994 ± 1.480 mg/ml and 0.304 ± 0.191 mg/ml at 12 weeks post infection and 24.004 ± 1.627 mg/ml and 1.201 ± 0.757 mg/ml at 24 weeks post infection, respectively.

## Parasite burden of spleen and liver in BAFF knockout mice were equivalent to or more than that in wild-type mice

Parasites were detected from spleen and liver of infected mice of both strains. At 12 weeks post infection, the means ± SD of LDU for the spleen from wild-type mice and knockout mice were 182 ± 10 and 62 ± 20, respectively, and at 24 weeks post infection, 2,219 ± 440 and 1,980 ± 255, respectively (Figure 17). The means ± SD of LDU for the liver from wild-type and knockout were 1,114 ± 620 and 1,535 ± 751, respectively, at 12 weeks post infection and 2,291 ± 1,802 and 9,791 ± 1,166, respectively, at 24 weeks post infection. There were no statistical differences between spleen LDU of wild-type and knockout at 12 or 24 weeks post infection. While there was no statistical differences between liver LDU of wild-type and knockout at 12 weeks post infection, that of knockout was rather higher than that of wild-type at 24 weeks post infection.

## Discussion

Both Bangladeshi and Brazilian VL patients showed higher serum or plasma BAFF levels than HCs of each country. In Bangladesh causative species is *L. donovani* and in Brazil *L. infantum*, so the elevation of BAFF may not be restricted by the geographical backgrounds or the infecting *Leishmania* species. The magnitude of BAFF elevation in the VL patients was 2.0 (ratio comparing mean value) in Bangladesh and 4.3 in Brazil. Though the magnitude of elevation is different in Bangladesh and in Brazil, both were equivalent to or higher than that previously reported for other diseases (in SLE patients 2.4 [52]; in RA patients 1.5 [52]; in the patients with Sjögren's syndrome 5.1 [53]; in malaria patients 2.2 [54]). In SLE, RA and Sjögren's syndrome, inhibitors of BAFF signaling such as belimumab and blisibimod are approved and/or being evaluated for treatment. Elevation of BAFF in blood was reported in some infectious diseases other than leishmaniasis such as malaria [54], hepatitis C [55] and AIDS [56]. However, not all infectious diseases are supposed to be associated with BAFF elevation because the patients with Chagas' disease did not show elevation of serum BAFF in this study.

Elevation of serum BAFF was also shown in *L. donovani*-infected wild-type mice. From the results of chapter 2, splenomegaly with increase in the number of B-cell lineage cells and IgG-dominant hyperimmunoglobulinemia were shown in the infected mice. On the other hand, the degree of spleen enlargement was more moderate in BAFF knockout mice compared to wild-type mice and the number of

B cell-lineage cells did not increase in the knockout mice. IgG-dominant hyperimmunoglobulinemia were not also seen in the infected knockout mice. A previous report showed that transgenic mice overexpressing BAFF have excessive numbers of mature B cells, high plasma cell numbers in secondary lymphoid organ and high levels of immunoglobulins as well as splenomegaly [57]. So it was indicated that infection of *L. donovani* promoted expression of BAFF and induced phenomena which are comparable to the mice overexpressing BAFF.

It was reported that BAFF costimulates proliferation of blood-derived B cells concomitantly with cross-linking of the B-cell receptors using anti-IgM antibodies [10]. Hence, it is speculated that BAFF promotes proliferation of B cells and secretion of immunoglobulins with stimulation of B-cell receptors by antigen of *Leishmania*. Moreover, since there has been a report that shows the role of BAFF in survival of plasma cells via BCMA (for B cell maturation antigen), one of the receptors for BAFF [58], it is possible that involvement of BAFF in survival of plasma cells also leads to splenomegaly during VL.

Although significant increase in the number of B-cell lineage cells and IgG-dominant hyperimmunoglobulinemia were not seen in BAFF knockout mice, there existed some B-cell lineage cells and some immunoglobulins in the mice. Therefore it is indicated that there are BAFF-dependent pathway and BAFF-independent pathway to maintain B-cell lineage cells and to promote the cells to secrete immunoglobulins. In that way it is reasonable to say that BAFF-dependent B-cell activation is significant in increase in the number of B-cell lineage cells in spleen and IgG-dominant hyperimmunoglobulinemia

during VL.

Among the diseases where serum BAFF elevation is seen, some diseases are related to splenomegaly but others are not. For example, splenomegaly is a major symptom in patients with SLE, but it is not a major symptom in the patients with Sjögren's syndrome. Some reports showed that local BAFF levels are more related to symptoms than serum BAFF in some diseases. It was shown that the levels of BAFF in saliva of the patients with Sjögren's syndrome did not correlate with the levels in sera but correlate with the status of symptoms of the disease [59]. It was also shown that BAFF levels in clinically inflamed synovial fluids were greater than those in sera of the patients with RA [60]. It seems that involvement of BAFF in symptoms varies depending on the production sites of BAFF and blood levels of BAFF are not necessarily related to the degree of the symptoms.

While there is a report that spleen weights of IL-4 knockout mice and wild-type mice were not significantly different during infection of *L. donovani* [48], the present study showed that *L. donovani*-infected BAFF knockout mice showed milder splenomegaly than wild-type. Therefore it is supposed that BAFF is more influential factor in development of splenomegaly during VL than IL-4.

In the previous report using VL mouse model, enhanced resistance was shown in mutant mice that lack mature B cells [9]. Also in CL mouse models, resistance was observed with depletion of B cells using anti-IgM antibody [61] or in BALB xid mice, lacking B-1 B cells [8], and susceptibility was increased by transfer of B cells [62] or administration of B-cell hematopoietic factor, IL-7 [63].

Moreover, BALB/c mice lacking IgG were more resistant to *L. major* [64]. Internalization of immunoglobulin-coated amastigotes by macrophages was shown to lead to IL-10 production and consequent enhancement of intracellular parasite growth [65]. However, while BAFF knockout mice used in this study have much fewer B cells and lower immunoglobulin levels, *L. donovani* parasites were not eliminated in the course of infection. Recently one report showed that deficiency of TACI, which is one of the receptors for BAFF, leads to alternatively activated macrophage phenotype and susceptibility to *Leishmania* infection [66]. Conversely it was suggested that BAFF has the role to induce M1 polarization of macrophages, which leads macrophages to be resistant to *Leishmania* infection. Taken together, BAFF may have dual function in resistance/susceptibility to infection of *L. donovani* via polarization of macrophage or via production of immunoglobulins.

## General discussion

In chapter 1, infection-induced strong activation of B-cell lineage cells during VL was shown using serum immunoglobulins as indicators for B-cell activation. A marked increase in the level of IgG shown in the chapter indicated that B-cell lineage cells are highly activated during VL. In chapter 2, contribution of B-cell lineage cells to development of splenomegaly in *L. donovani*-infected mice was evaluated. From the results, it was shown that infection-induced increase in B-cell lineage cells is one of the main causes of splenomegaly. In chapter 3, the roles of BAFF in development of splenomegaly during VL are focused. From elevation of serum BAFF in VL patients and *L. donovani*-infected mice, it was suggested that BAFF-mediated pathway was activated during VL and infection of *L. donovani*. Since the degree of splenomegaly became moderate by deficiency of BAFF in mice, it was indicated that BAFF promotes development of splenomegaly during *L. donovani* infection in mice.

It is suggested that strong activation of B-cell lineage cells is one of the main causes of splenomegaly during infection of *L. donovani*, and BAFF is a key molecule in that mechanisms (Figure 18). BAFF-mediated host immune response may have dual function in resistance and susceptibility to infection of *L. donovani*, but it may promote splenomegaly via activation of B-cell lineage cells. In the experiments using mice, *L. donovani* was used as infecting parasites in this study. From the results of human VL, serum BAFF elevation and IgG elevation were commonly observed in Bangladeshi patients and Brazilian patients. Since *L.*

*infantum* is the causative species in Brazil, BAFF-mediated activation of B-cell lineage cells may also be applicable to infection of *L. infantum*. Taken together, it is indicated that BAFF is involved in development of splenomegaly during VL via activation of B-cell lineage cells.

Currently chemotherapy remains favored option for control of VL. However, there are many problems in chemotherapy for leishmaniasis such as emergence of resistant strains and toxicities [67]. While it is not still clear how infection of *Leishmania* parasite leads to BAFF elevation, this study showed possibility that targeting of BAFF may effective for alleviation of splenomegaly.

## Acknowledgements

I would like to express my gratitude to Assistant Professor Wataru Fujii (Laboratory of Applied Genetics, Graduate school of Agricultural and Life Sciences, the University of Tokyo) for producing and providing BAFF knockout mice used in this study.

I'm also very grateful to Associate Professor Yasuyuki Goto (Laboratory of Molecular Immunology, Graduate school of Agricultural and Life Sciences, the University of Tokyo) for giving me the opportunity to work in his research project and constitutive advice and support during this study.

I also want to express my sincere thanks to all the member of Laboratory of Molecular Immunology, for the warm and kind friendship.

Finally I would like to express my gratitude to my supervisor, Professor Yoshitsugu Matsumoto (Laboratory of Molecular Immunology, Graduate school of Agricultural and Life Sciences, the University of Tokyo) for providing invaluable discussions.

## References


1. Lukes, J., et al., *Evolutionary and geographical history of the Leishmania donovani complex with a revision of current taxonomy.* Proc Natl Acad Sci U S A, 2007. **104**(22): p. 9375-80.
2. Alvar, J., et al., *Leishmaniasis worldwide and global estimates of its incidence.* PLoS One, 2012. **7**(5): p. e35671.
3. Scott, P., et al., *Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens.* J Exp Med, 1988. **168**(5): p. 1675-84.
4. Novais, F.O., et al., *Cytotoxic T cells mediate pathology and metastasis in cutaneous leishmaniasis.* PLoS Pathog, 2013. **9**(7): p. e1003504.
5. Chappuis, F., et al., *Visceral leishmaniasis: what are the needs for diagnosis, treatment and control?* Nat Rev Microbiol, 2007. **5**(11): p. 873-82.
6. Murray, H.W., et al., *Experimental visceral leishmaniasis: production of interleukin 2 and interferon-gamma, tissue immune reaction, and response to treatment with interleukin 2 and interferon-gamma.* J Immunol, 1987. **138**(7): p. 2290-7.
7. Skov, C.B. and D.W. Twohy, *Cellular immunity to Leishmania donovani. I. The effect of T cell depletion on resistance to L. donovani in mice.* J Immunol, 1974. **113**(6): p. 2004-11.
8. Hoerauf, A., et al., *The Xid defect determines an improved clinical course of murine leishmaniasis in susceptible mice.* Int Immunol, 1994. **6**(8): p. 1117-24.
9. Smelt, S.C., et al., *B cell-deficient mice are highly resistant to Leishmania donovani infection, but develop neutrophil-mediated tissue pathology.* J Immunol, 2000. **164**(7): p. 3681-8.
10. Schneider, P., et al., *BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth.* J Exp Med, 1999. **189**(11): p. 1747-56.
11. Evans, T., et al., *American visceral leishmaniasis (kala-azar).* West J Med, 1985. **142**(6): p. 777-81.
12. Ghose, A.C., et al., *Serological investigations on Indian kala-azar.* Clin Exp Immunol, 1980. **40**(2): p. 318-26.

13. Jeannel, D., et al., *Imported and autochthonous kala-azar in France.* BMJ, 1991. **303**(6798): p. 336-8.
14. Kafetzis, D.A., *An overview of paediatric leishmaniasis.* J Postgrad Med, 2003. **49**(1): p. 31-8.
15. Ehrenstein, M.R. and D.A. Isenberg, *Hypergammaglobulinaemia and autoimmune rheumatic diseases.* Ann Rheum Dis, 1992. **51**(11): p. 1185-7.
16. Benbassat, J., N. Fluman, and A. Zlotnick, *Monoclonal immunoglobulin disorders: a report of 154 cases.* Am J Med Sci, 1976. **271**(3): p. 325-34.
17. Kyrtsonis, M.C., et al., *Waldenstrom's macroglobulinemia: clinical course and prognostic factors in 60 patients. Experience from a single hematology unit.* Ann Hematol, 2001. **80**(12): p. 722-7.
18. Hale, G.M., et al., *Evidence for IgA-specific B cell hyperactivity in patients with IgA nephropathy.* Kidney Int, 1986. **29**(3): p. 718-24.
19. Heremans, J.F. and P.L. Masson, *Specific analysis of immunoglobulins. Techniques and clinical value.* Clin Chem, 1973. **19**(3): p. 294-300.
20. Neogy, A.B., et al., *B lymphocyte population and immunoglobulins in Indian kala-azar in response to chemotherapy.* Acta Trop, 1986. **43**(3): p. 237-44.
21. Shiddo, S.A., et al., *Reference ranges for IgG, IgM and IgA in the serum of urban and rural Somalis.* Trop Geogr Med, 1994. **46**(1): p. 27-31.
22. Goto, Y., D. Carter, and S.G. Reed, *Immunological dominance of Trypanosoma cruzi tandem repeat proteins.* Infect Immun, 2008. **76**(9): p. 3967-74.
23. Cooper, G.R., C.R. Rein, and J.W. Beard, *Electrophoretic analysis of kala-azar human serum; hypergammaglobulinemia associated with seronegative reactions for syphilis.* Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine, 1946. **61**: p. 179-83.
24. Braz, R.F., et al., *The sensitivity and specificity of Leishmania chagasi recombinant K39 antigen in the diagnosis of American visceral leishmaniasis and in differentiating active from subclinical infection.* Am J Trop Med Hyg, 2002. **67**(4): p. 344-8.
25. Houghton, R.L., et al., *A multi-epitope synthetic peptide and recombinant protein for the detection of antibodies to Trypanosoma cruzi in radioimmunoprecipitation-confirmed and consensus-positive sera.* J Infect

Dis, 1999. **179**(5): p. 1226-34.
26. Coura-Vital, W., et al., *Prognostic factors and scoring system for death from visceral leishmaniasis: an historical cohort study in Brazil.* PLoS Negl Trop Dis, 2014. **8**(12): p. e3374.
27. Mueller, Y.K., et al., *Clinical epidemiology, diagnosis and treatment of visceral leishmaniasis in the Pokot endemic area of Uganda and Kenya.* Am J Trop Med Hyg, 2014. **90**(1): p. 33-9.
28. Sundar, S., et al., *Noninvasive management of Indian visceral leishmaniasis: clinical application of diagnosis by K39 antigen strip testing at a kala-azar referral unit.* Clin Infect Dis, 2002. **35**(5): p. 581-6.
29. Pozo, A.L., E.M. Godfrey, and K.M. Bowles, *Splenomegaly: investigation, diagnosis and management.* Blood Rev, 2009. **23**(3): p. 105-11.
30. Thiele, J., et al., *Splenic haematopoiesis in primary (idiopathic) osteomyelofibrosis: immunohistochemical and morphometric evaluation of proliferative activity of erytro- and endoreduplicative capacity of megakaryopoiesis (PCNA- and Ki-67 staining).* Virchows Arch B Cell Pathol Incl Mol Pathol, 1993. **64**(5): p. 281-6.
31. Mizobuchi, H., et al., *The accumulation of macrophages expressing myeloid-related protein 8 (MRP8) and MRP14 in the spleen of BALB/cA mice during infection with Plasmodium berghei.* Exp Parasitol, 2014. **138**: p. 1-8.
32. Mebius, R.E. and G. Kraal, *Structure and function of the spleen.* Nat Rev Immunol, 2005. **5**(8): p. 606-16.
33. Toellner, K.M., et al., *Immunoglobulin switch transcript production in vivo related to the site and time of antigen-specific B cell activation.* J Exp Med, 1996. **183**(5): p. 2303-12.
34. Jacob, J. and G. Kelsoe, *In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers.* J Exp Med, 1992. **176**(3): p. 679-87.
35. Liu, Y.J., et al., *Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens.* Eur J Immunol, 1991. **21**(12): p. 2951-62.
36. Huff, C.G., et al., *An approach toward a course in the principles of parasitism.* J Parasitol, 1958. **44**(1): p. 28-45.

37. Benedict, C.A., et al., *Specific remodeling of splenic architecture by cytomegalovirus.* PLoS Pathog, 2006. **2**(3): p. e16.
38. Urban, B.C., et al., *Fatal Plasmodium falciparum malaria causes specific patterns of splenic architectural disorganization.* Infect Immun, 2005. **73**(4): p. 1986-94.
39. Silva-O'Hare, J., et al., *Disruption of Splenic Lymphoid Tissue and Plasmacytosis in Canine Visceral Leishmaniasis: Changes in Homing and Survival of Plasma Cells.* PLoS One, 2016. **11**(5): p. e0156733.
40. Silva, J.S., et al., *Low CXCL13 expression, splenic lymphoid tissue atrophy and germinal center disruption in severe canine visceral leishmaniasis.* PLoS One, 2012. **7**(1): p. e29103.
41. Reiner, N.E., *Host-parasite relationship in murine leishmaniasis: pathophysiological and immunological changes.* Infect Immun, 1982. **38**(3): p. 1223-30.
42. Sundar, S., et al., *Circulating T helper 1 (Th1) cell- and Th2 cell-associated cytokines in Indian patients with visceral leishmaniasis.* Am J Trop Med Hyg, 1997. **56**(5): p. 522-5.
43. Atta, A.M., et al., *Anti-leishmanial IgE antibodies: a marker of active disease in visceral leishmaniasis.* Am J Trop Med Hyg, 1998. **59**(3): p. 426-30.
44. Saha, S., et al., *Leishmania promastigote membrane antigen-based enzyme-linked immunosorbent assay and immunoblotting for differential diagnosis of Indian post-kala-azar dermal leishmaniasis.* J Clin Microbiol, 2005. **43**(3): p. 1269-77.
45. Kaye, P.M., A.J. Curry, and J.M. Blackwell, *Differential production of Th1- and Th2-derived cytokines does not determine the genetically controlled or vaccine-induced rate of cure in murine visceral leishmaniasis.* Journal of immunology, 1991. **146**(8): p. 2763-70.
46. Melby, P.C., et al., *The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like cytokine response.* Journal of immunology, 2001. **166**(3): p. 1912-20.
47. Melby, P.C., et al., *Cloning of Syrian hamster (Mesocricetus auratus) cytokine cDNAs and analysis of cytokine mRNA expression in experimental visceral leishmaniasis.* Infect Immun, 1998. **66**(5): p.

2135-42.
48. Stager, S., et al., *Both interleukin-4 (IL-4) and IL-4 receptor alpha signaling contribute to the development of hepatic granulomas with optimal antileishmanial activity.* Infect Immun, 2003. **71**(8): p. 4804-7.
49. Tangye, S.G., et al., *BAFF, APRIL and human B cell disorders.* Semin Immunol, 2006. **18**(5): p. 305-17.
50. Fujii, W., et al., *Efficient generation of genome-modified mice via offset-nicking by CRISPR/Cas system.* Biochem Biophys Res Commun, 2014. **445**(4): p. 791-4.
51. Mackay, F. and P. Schneider, *Cracking the BAFF code.* Nat Rev Immunol, 2009. **9**(7): p. 491-502.
52. Zhang, J., et al., *Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus.* J Immunol, 2001. **166**(1): p. 6-10.
53. Groom, J., et al., *Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome.* J Clin Invest, 2002. **109**(1): p. 59-68.
54. Nduati, E., et al., *The plasma concentration of the B cell activating factor is increased in children with acute malaria.* J Infect Dis, 2011. **204**(6): p. 962-70.
55. Tarantino, G., et al., *Serum BLyS/BAFF predicts the outcome of acute hepatitis C virus infection.* J Viral Hepat, 2009. **16**(6): p. 397-405.
56. Carbone, J., et al., *Potential role of serum BAFF as a biomarker in HIV infection.* Infect Dis (Lond), 2015. **47**(4): p. 260-2.
57. Mackay, F., et al., *Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations.* J Exp Med, 1999. **190**(11): p. 1697-710.
58. O'Connor, B.P., et al., *BCMA is essential for the survival of long-lived bone marrow plasma cells.* J Exp Med, 2004. **199**(1): p. 91-8.
59. Pers, J.O., et al., *Is periodontal disease mediated by salivary BAFF in Sjogren's syndrome?* Arthritis Rheum, 2005. **52**(8): p. 2411-4.
60. Cheema, G.S., et al., *Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases.* Arthritis Rheum, 2001. **44**(6): p. 1313-9.
61. Sacks, D.L., et al., *Cutaneous leishmaniasis in anti-IgM-treated mice: enhanced resistance due to functional depletion of a B cell-dependent T cell*

*involved in the suppressor pathway.* J Immunol, 1984. **132**(4): p. 2072-7.
62. Hoerauf, A., M. Rollinghoff, and W. Solbach, *Co-transfer of B cells converts resistance into susceptibility in T cell-reconstituted, Leishmania major-resistant C.B-17 scid mice by a non-cognate mechanism.* Int Immunol, 1996. **8**(10): p. 1569-75.
63. Hoerauf, A., et al., *Effect of IL-7 treatment on Leishmania major-infected BALB.Xid mice: enhanced lymphopoiesis with sustained lack of B1 cells and clinical aggravation of disease.* Int Immunol, 1995. **7**(11): p. 1879-84.
64. Miles, S.A., et al., *A role for IgG immune complexes during infection with the intracellular pathogen Leishmania.* J Exp Med, 2005. **201**(5): p. 747-54.
65. Kane, M.M. and D.M. Mosser, *The role of IL-10 in promoting disease progression in leishmaniasis.* J Immunol, 2001. **166**(2): p. 1141-7.
66. Allman, W.R., et al., *TACI deficiency leads to alternatively activated macrophage phenotype and susceptibility to Leishmania infection.* Proc Natl Acad Sci U S A, 2015. **112**(30): p. E4094-103.
67. Sundar, S. and A. Singh, *Recent developments and future prospects in the treatment of visceral leishmaniasis.* Ther Adv Infect Dis, 2016. **3**(3-4): p. 98-109.

Figure legends

**Figure 1　Bangladeshi VL patients showed high levels of IgG in plasma.**

The plasma levels of IgG, IgM and IgA of Bangladeshi VL patients and Bangladeshi HCs were measured by sandwich ELISA. Solid bars represent the mean value of individual groups. A dotted line represents the cutoff value calculated as the mean + 3SD of HCs. The differences between the groups were analyzed by Mann-Whitney test.

**Figure 2　Brazilian VL patients showed high levels of IgG in serum.**

Each column indicates serum levels of IgG, IgM and IgA in Brazilian VL patients, Chagas' disease patients (Chagas) and HCs. Solid lines represent the mean value of individual groups. A dotted line represents the cutoff value calculated as the mean + 3SD of HCs. The differences among the groups were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test for IgG, and Kruskal-Wallis test followed by Dunn's multiple comparisons test for IgM and IgA.

**Figure 3　IgG-dominant hyperimmunoglobulinemia in *L. donovani*-infected mice**

Each column indicates serum IgG, IgM and IgA levels of mice. Naive mice consist of six mice and infected mice consist of three mice for each time point. Means +

SD are shown. *$P < 0.05$ with one-way ANOVA with Bonferroni multiple comparisons

**Figure 4　Parasite burden of spleen and liver of *L. donovani*-infected mice**

Each column indicates parasite burdens of spleen and liver of the mice expressed as LDU. LDU was calculated as the number of amastigotes / host nucleated cell × organ weight (in mg). Each group consists of three mice. Means + SD are shown. *$P < 0.05$ with t test; ND: not detected

**Figure 5　*L. donovani*-infected mice showed splenomegaly.**

Each column indicates spleen weight of mice. Means + SD are shown. Naive mice consist of six mice and infected mice consist of three mice for each time point. Inset: bar = 1 cm

**Figure 6　Expansion of white pulp and red pulp in spleen of *L. donovani*-infected mice**

The spleen sections of naive and infected (24 weeks post infection) mice were stained by HE. bar = 2 mm

**Figure 7　The area of IgG-expressing plasma cells in the spleen of infected mice was significantly expanded in red pulp beside the T-cell area.**

The spleen sections of naive and infected (12 weeks post infection) mice were probed by anti-IgG antibody and stained by DAB, followed by counterstain with hematoxylin. Serial sections of infected mice spleen were stained using anti-CD3 and anti-B220 antibody.

**Figure 8　Increase in the number of CD19$^+$ lymphocytes in the spleen of infected mice**

Spleen nucleated cells were probed by FITC-linked anti-CD3 antibody and PE-linked anti-CD19 antibody and analyzed by flow cytometry. (A) The lymphocytes were gated in the FSC-SSC dot plots, and the number of lymphocyte-gated CD19$^+$ cells and CD3$^+$ cells were analyzed by quadrant analysis of 2-dimentional dot plots. (B) Each column indicates ratio of lymphocyte-gated CD19$^+$ cells and CD3$^+$ cells in total nucleated cells in spleens of naive mice (n = 3) and infected mice (12 and 24 weeks post infection, n = 3). Means are shown. (C) Each column indicates the number of CD19$^+$ or CD3$^+$ cells in spleen. Means are shown.

**Figure 9　Exon 1 and 2 of BAFF gene which include transmembrane region and furin cleavage site were deleted to produce BAFF knockout mice.**

Top: Genomic structure of BAFF gene.

Bottom: Schematic of BAFF and receptors.

**Figure 10　Increase in the BAFF levels in serum or plasma of VL patients**

(A) The plasma levels of BAFF in Bangladeshi VL patients and HCs were measured by sandwich ELISA. Solid bars represent the mean value of individual groups. Dotted lines represent cutoff values calculated as the mean + 3SD of HCs. The difference between the groups was analyzed by t test. (B) Each column indicates serum levels of BAFF in Brazilian VL patients, Chagas' disease patients (Chagas) and HCs. Solid lines represent the mean value of individual groups. A dotted line represents cutoff value calculated as the mean + 3SD of HCs. Differences among groups were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons test.

**Figure 11　Increase in the serum BAFF levels in *L. donovani*-infected mice**

Each column indicates serum BAFF level of mice. Each group consists of three mice. Means + SD are shown. $*P < 0.05$ with one-way ANOVA with Bonferroni multiple comparisons

**Figure 12　Deletion of BAFF gene and protein in BAFF knockout mice**

Left: Analysis of PCR products from total DNA of the tails for genotyping. Expected product sizes are 1,212 bp (wild-type) and 480 bp (target-deletion).

Right: Serum BAFF levels of homozygous knockout mice (-/-, n = 2), heterozygous knockout mice (-/+, n = 3) and wild-type mice (+/+, n = 3) were determined by ELISA. Means + SD are shown. ND: not detected (< 625 pg/ml)

**Figure 13　Moderate splenomegaly in *L. donovani*-infected BAFF knockout mice**

Each column indicates organ weight of wild-type mice and BAFF knockout mice. Means + SD are shown. Naive mice consist of six mice and infected mice consist of three mice for each time point. . $*P < 0.05$ with two-way ANOVA followed by Bonferroni's multiple comparisons test. NS: not significant. p.i.: post infection. WT: wild-type

**Figure14　Increase in the number of $CD19^+$ lymphocytes in spleen was not seen in *L. donovani*-infected BAFF knockout mice**

Spleen nucleated cells were probed by anti-CD3 and anti-CD19 antibody and analyzed by flow cytometry. Left: Each column indicates ratio of $CD19^+$ or $CD3^+$ lymphocytes in splenocytes. Right: Each column indicates the number of $CD19^+$ or $CD3^+$ cells in spleen. Means are shown. Naive mice consist of six mice and infected mice consist of three mice for each time point. WT: wild-type

**Figure 15　Proportion of B220-positive cells and CD138-positive cells in spleen was significantly low in BAFF-deficient mice.**

The spleen sections of wild type and BAFF knockout mice were probed by (A) anti-B220 antibody or (B) anti-CD138 antibody and stained by DAB, followed by counterstain with hematoxylin. bar = 200 μm

**Figure 16　IgG-dominant hyperimmunoglobulinemia was not seen in *L. donovani*-infected BAFF knockout mice.**

Serum IgG levels were measured by ELISA. Means + SD are shown. WT: wild-type BALB/cA mice (naive: n = 6, infected: n = 3), KO: BAFF knockout BALB/cA mice (naive: n = 6, infected: n = 3), $*P < 0.05$ with two-way ANOVA with Bonferroni multiple comparisons, wks: weeks post infection

**Figure 17　*L. donovani* parasites were not eliminated from spleen or liver of infected BAFF knockout mice.**

Each column indicates parasite burdens of spleen and liver of wild-type and BAFF-knockout BALB/cA mice expressed as LDU. LDU was calculated as the number of amastigotes / host nucleated cell × organ weight (in mg). Each groups consist of three mice. Means + SD are shown. $*P < 0.05$ with two-way ANOVA with Bonferroni multiple comparisons NS: not significant; ND: not detected

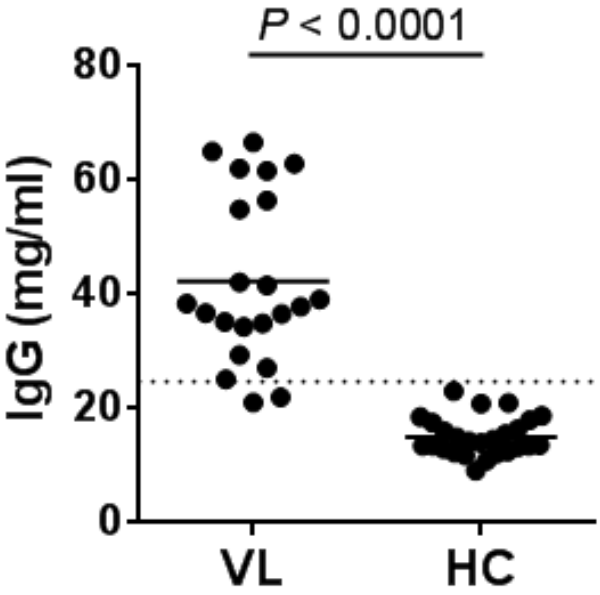
**Figure 18　Summary of this study**

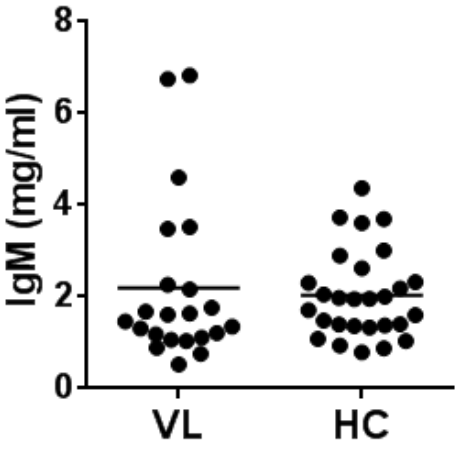
While it is not still clear how infection of *Leishmania* parasite leads to BAFF elevation, it is suggested that strong activation of B-cell lineage cells is one of the

main causes of splenomegaly during infection of *L. donovani*, and BAFF is a key molecule in that mechanisms.

Figures

**Figure 1 Bangladeshi VL patients showed high levels of IgG in plasma.**



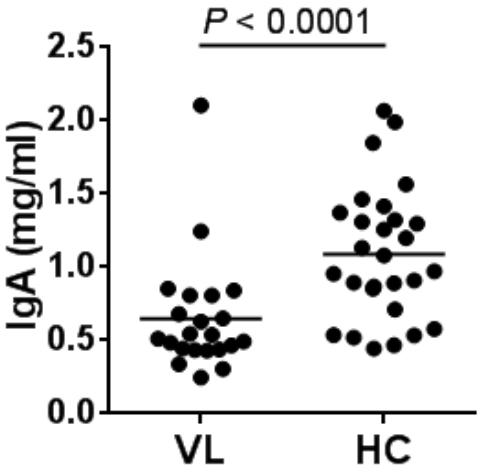



Figure 2 Brazilian VL patients showed high levels of IgG in serum.

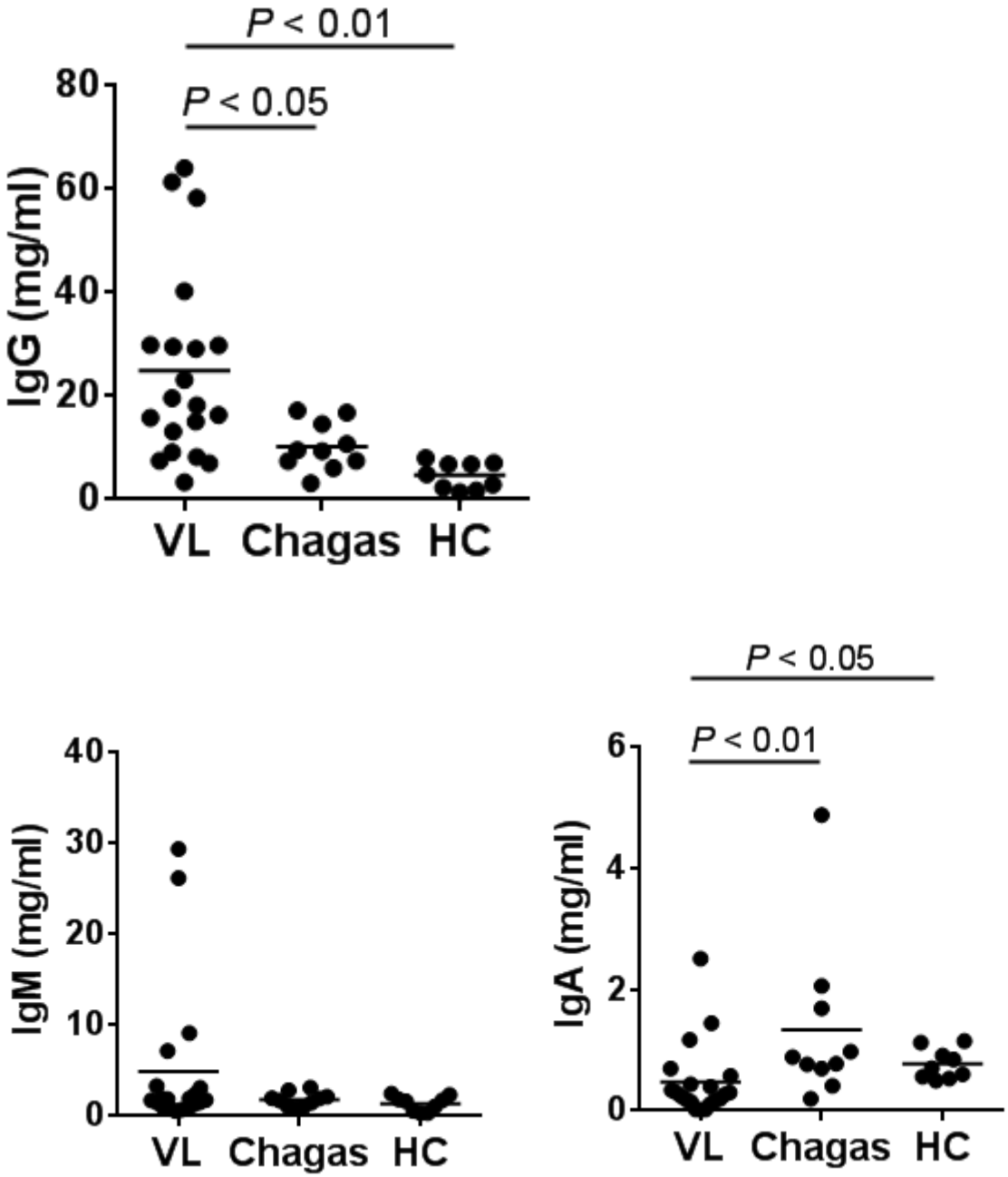


Figure 3 IgG-dominant hyperimmunoglobulinemia in *L. donovani*-infected mice

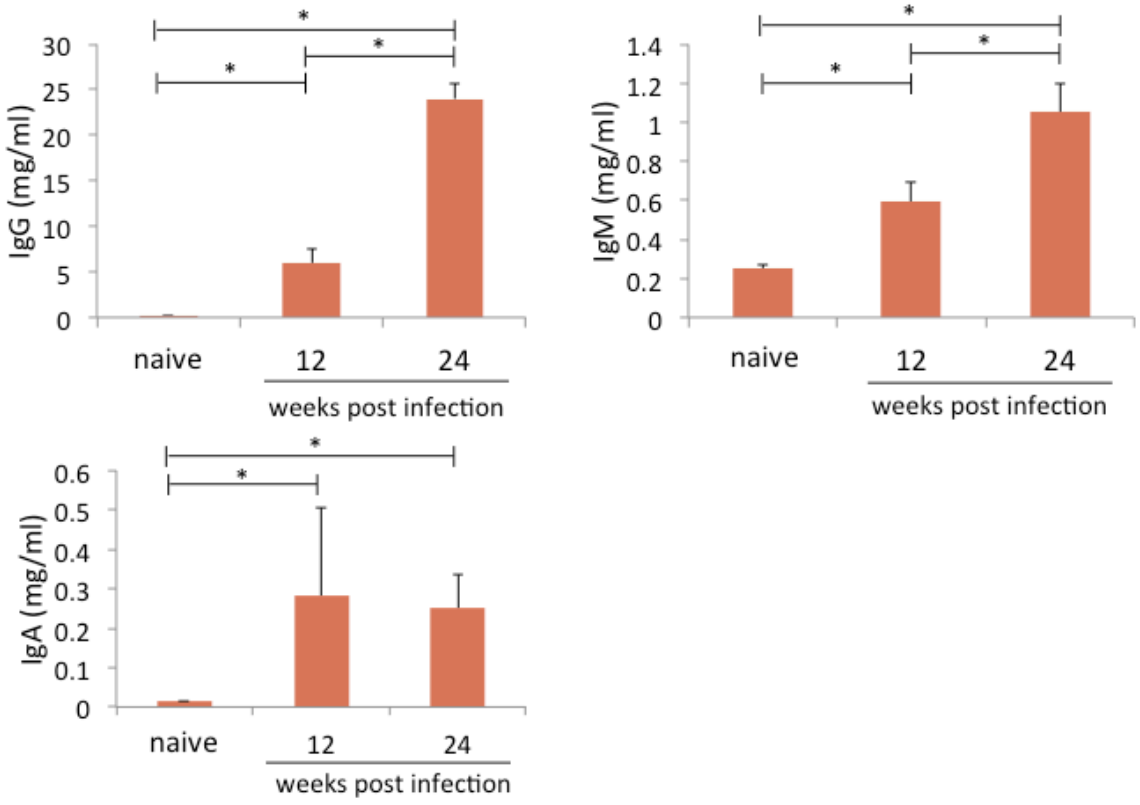


Figure 4 Parasite burden of spleen and liver of *L. donovani*-infected mice

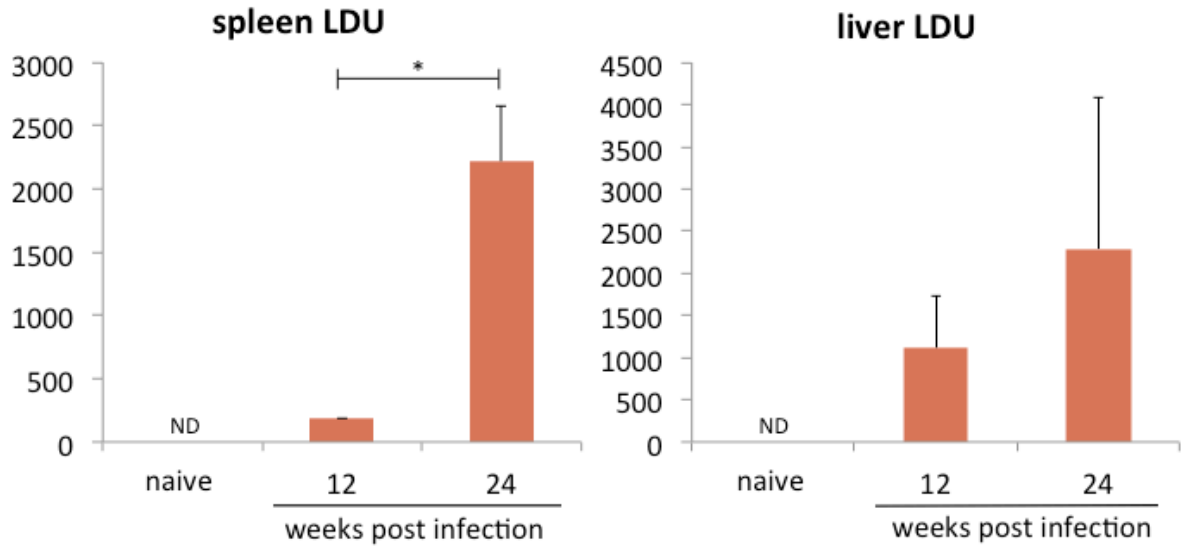


Figure 5 *L. donovani*-infected mice showed splenomegaly.

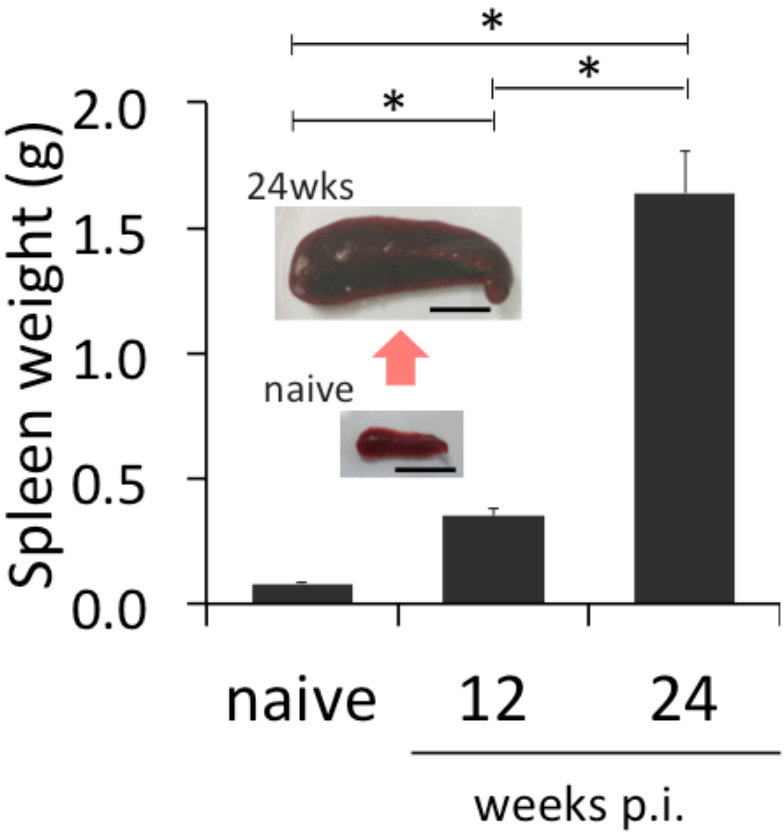


Figure 6　Expansion of white pulp and red pulp in spleen of *L. donovani*-infected mice

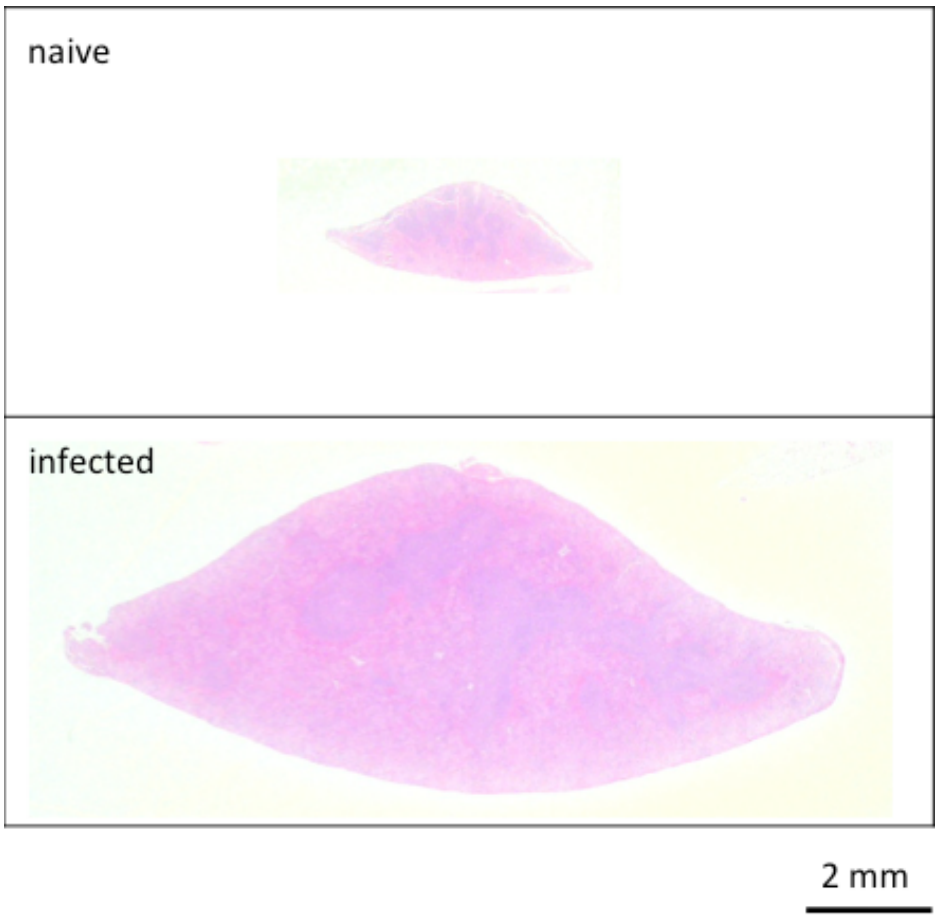


Figure 7　The area of IgG-expressing plasma cells in the spleen of infected mice was significantly expanded in red pulp beside the T-cell area.

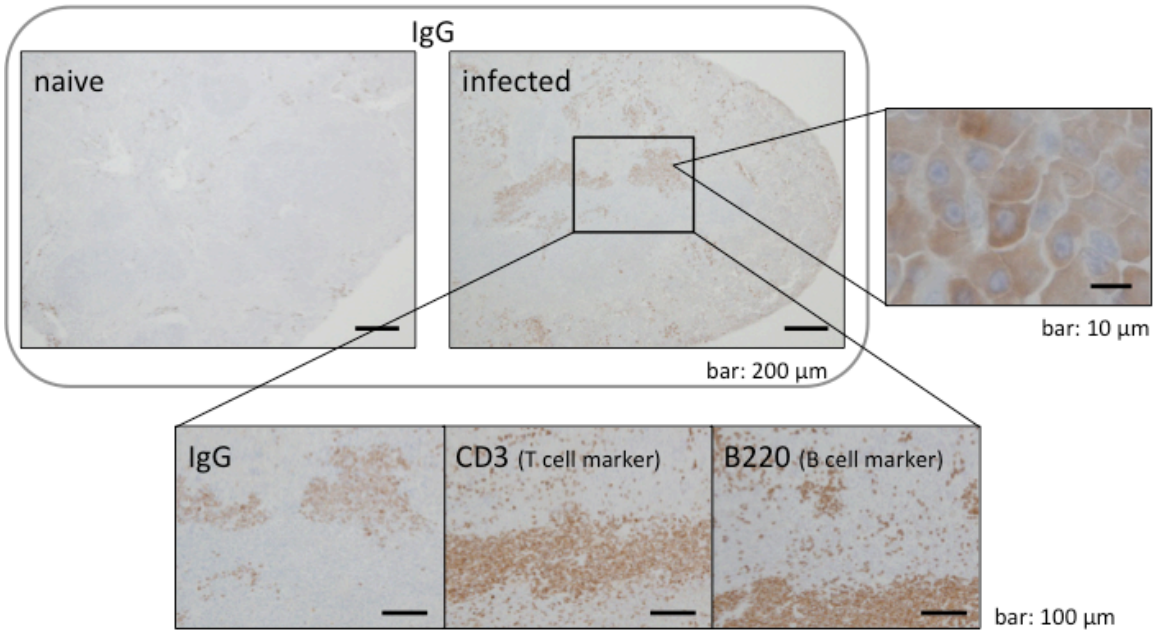


Figure 8　Increase in the number of CD19⁺ lymphocytes in the spleen of infected mice

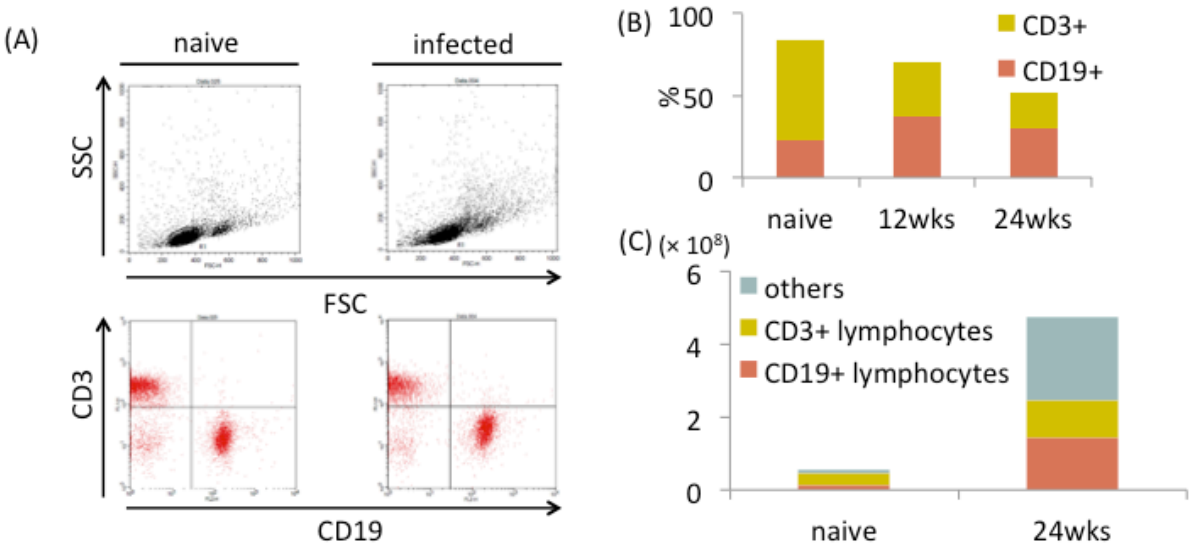
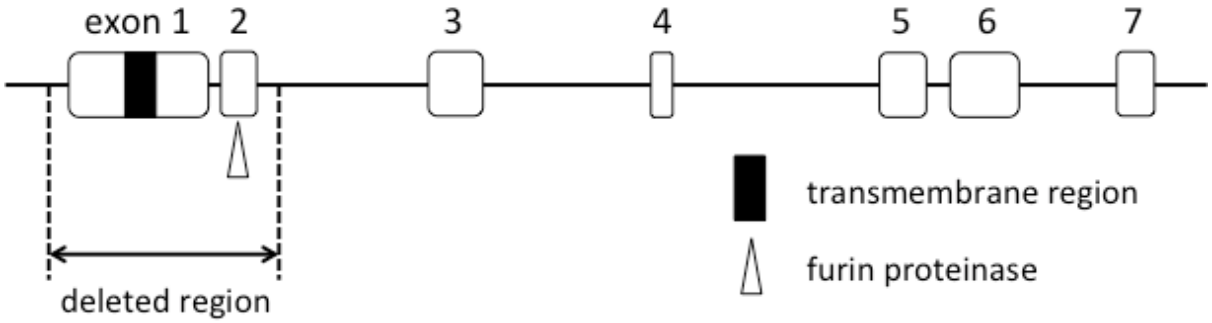
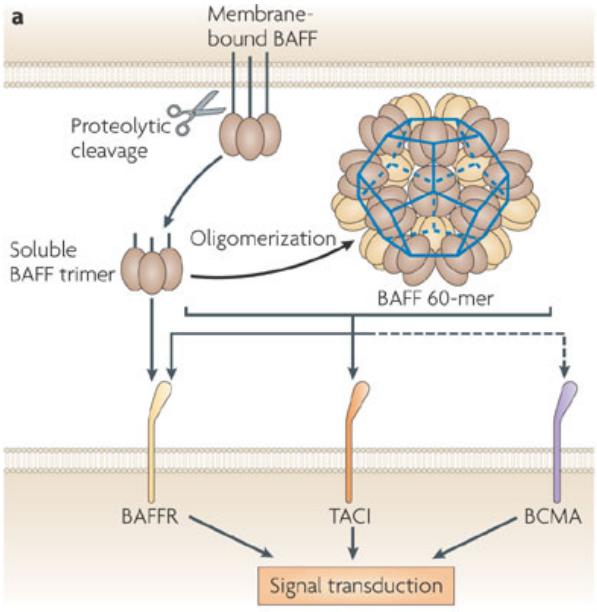


**Figure 9　Exon 1 and 2 of BAFF gene which include transmembrane region and furin cleavage site were deleted to produce BAFF knockout mice.**

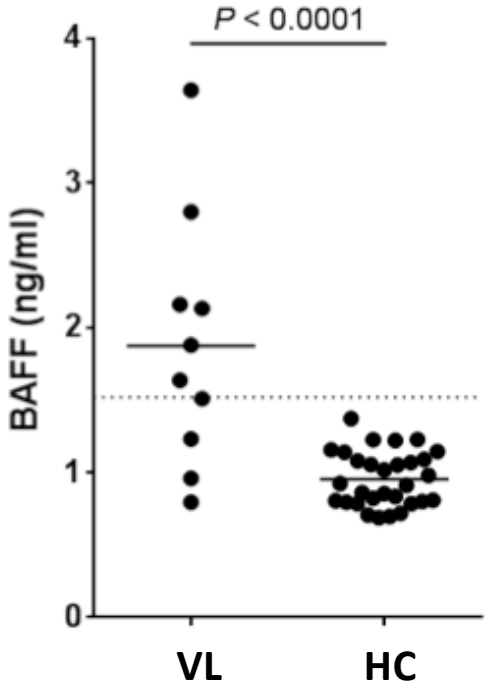



(Mackay and Schneider, 2009, Nat Rev Immunol [51])

**Figure 10　Increase in the BAFF levels in serum or plasma of VL patients**

(A) Bangladeshi




(B) Brazilian

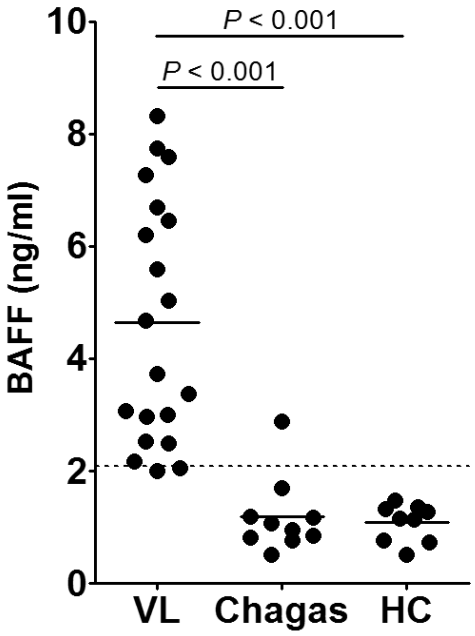


Figure 11 Increase in the serum BAFF levels in *L. donovani*-infected mice

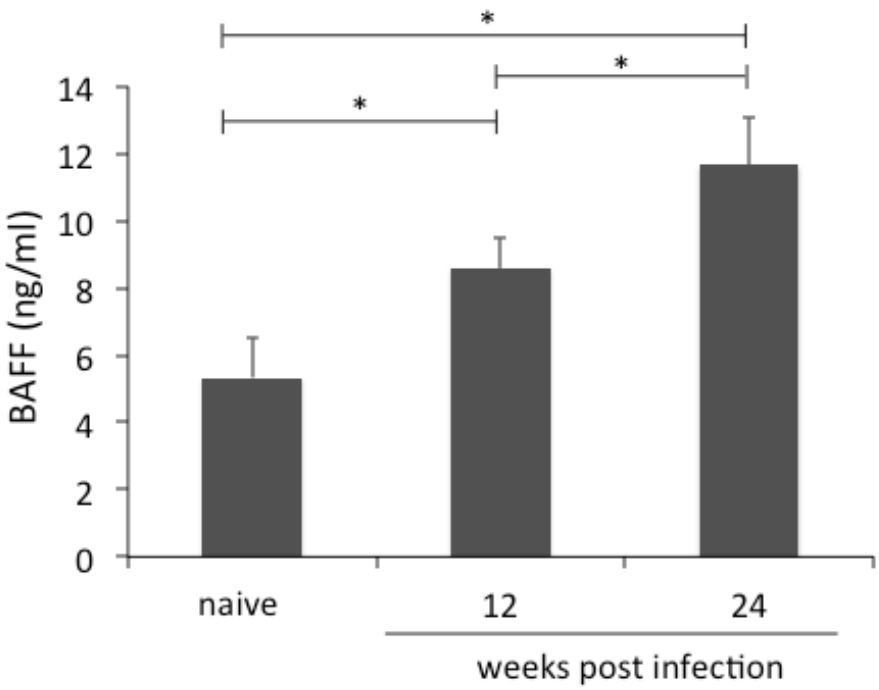


Figure 12　Deletion of BAFF gene and protein in BAFF knockout mice

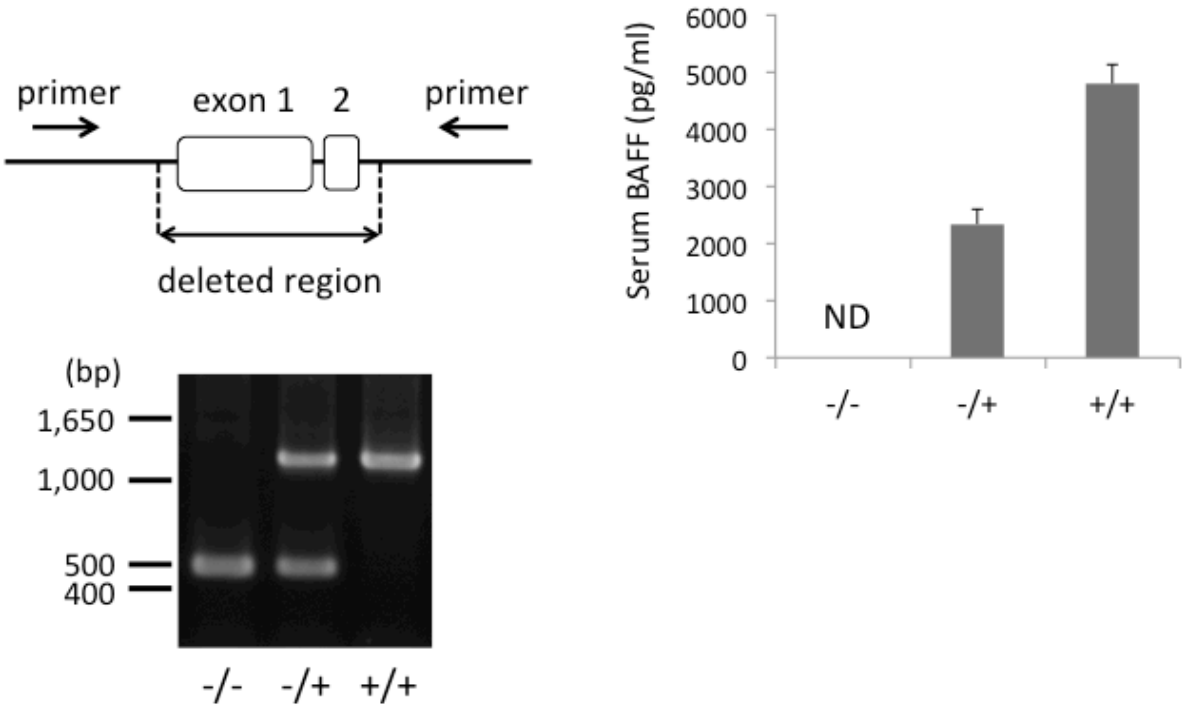


Figure 13 Moderate splenomegaly in *L. donovani*-infected BAFF knockout mice

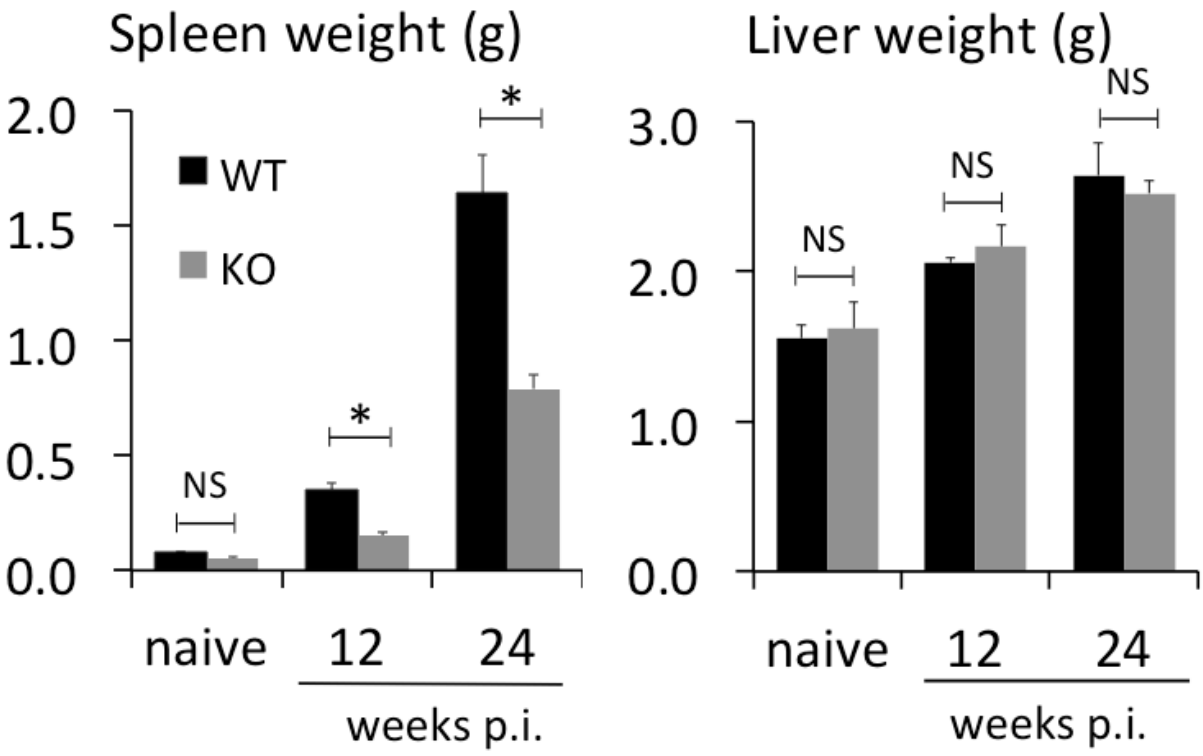


Figure 14　Increase in the number of CD19$^{+}$ lymphocytes in spleen was not seen in *L. donovani*-infected BAFF knockout mice

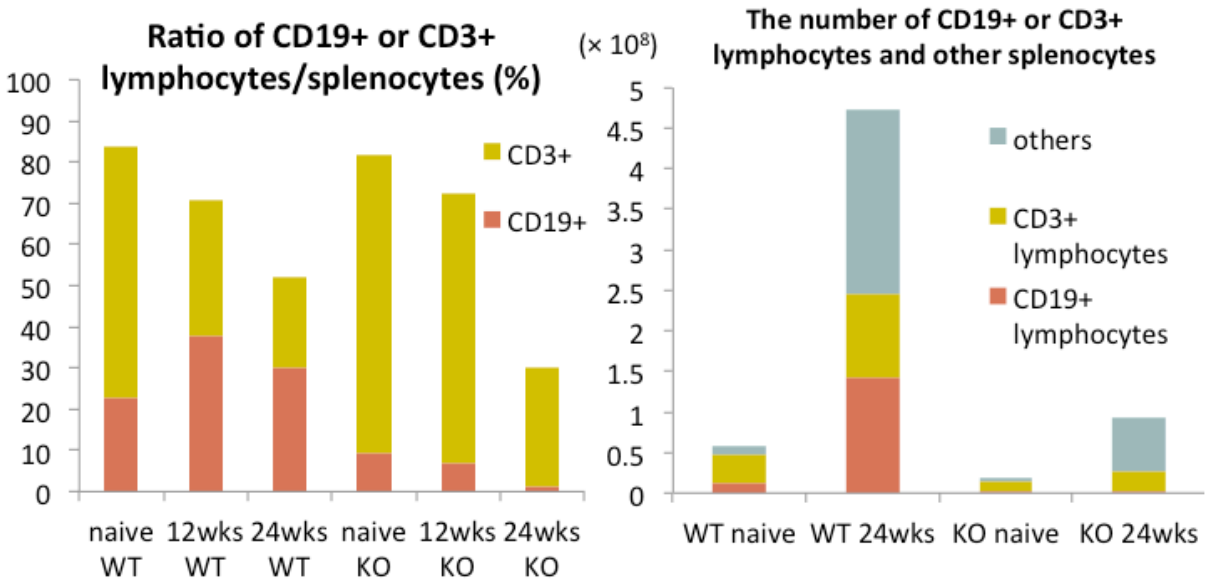
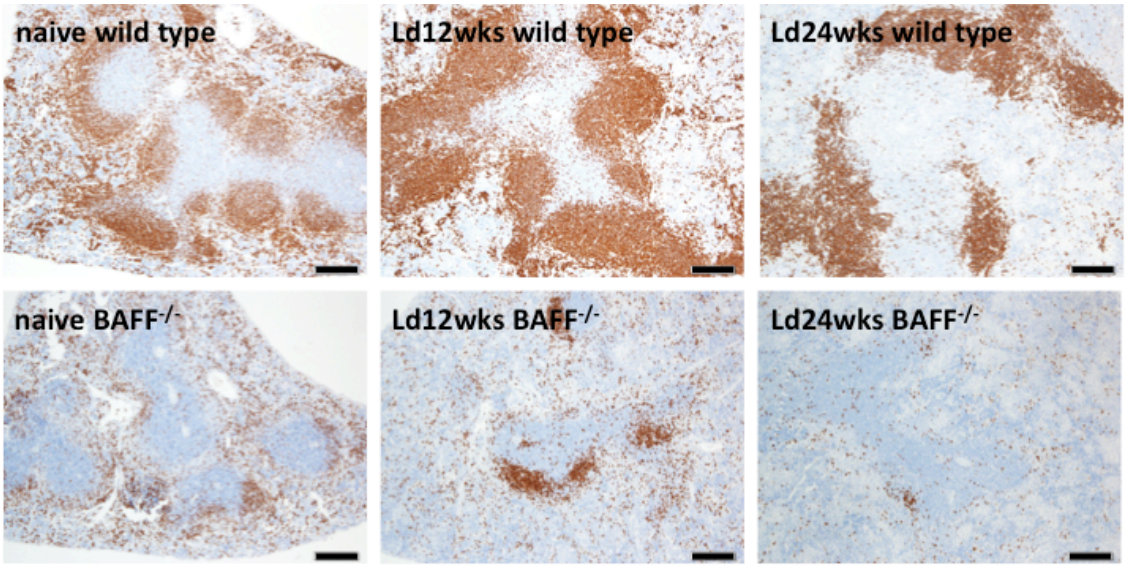


Figure 15　Proportion of B220-positive cells and CD138-positive cells in spleen was significantly low in BAFF-deficient mice.

(A) B220




(B) CD138

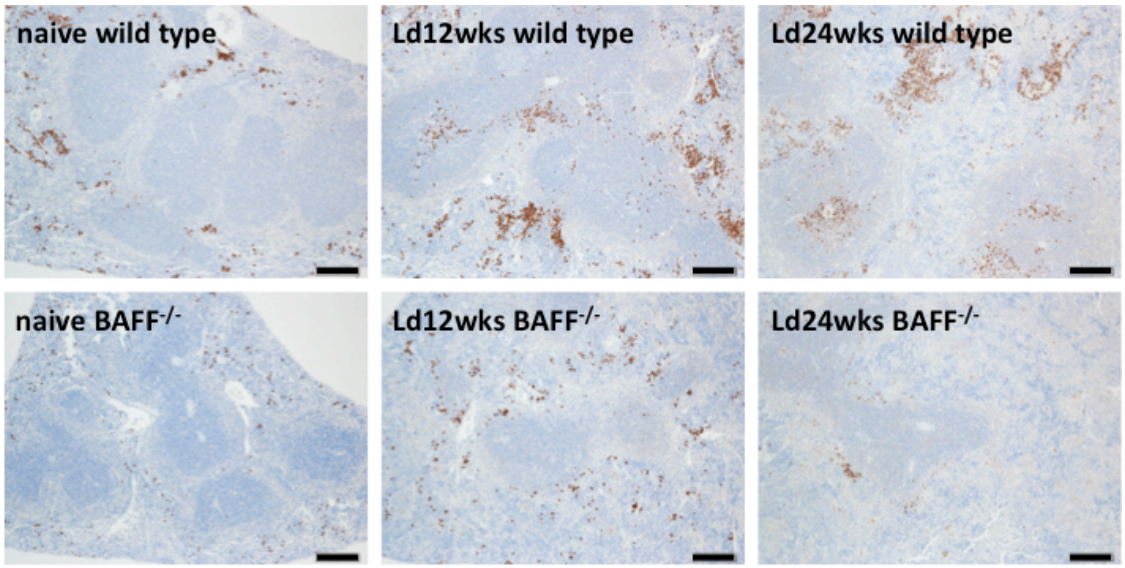


Figure 16 IgG-dominant hyperimmunoglobulinemia was not seen in *L. donovani*-infected BAFF knockout mice.

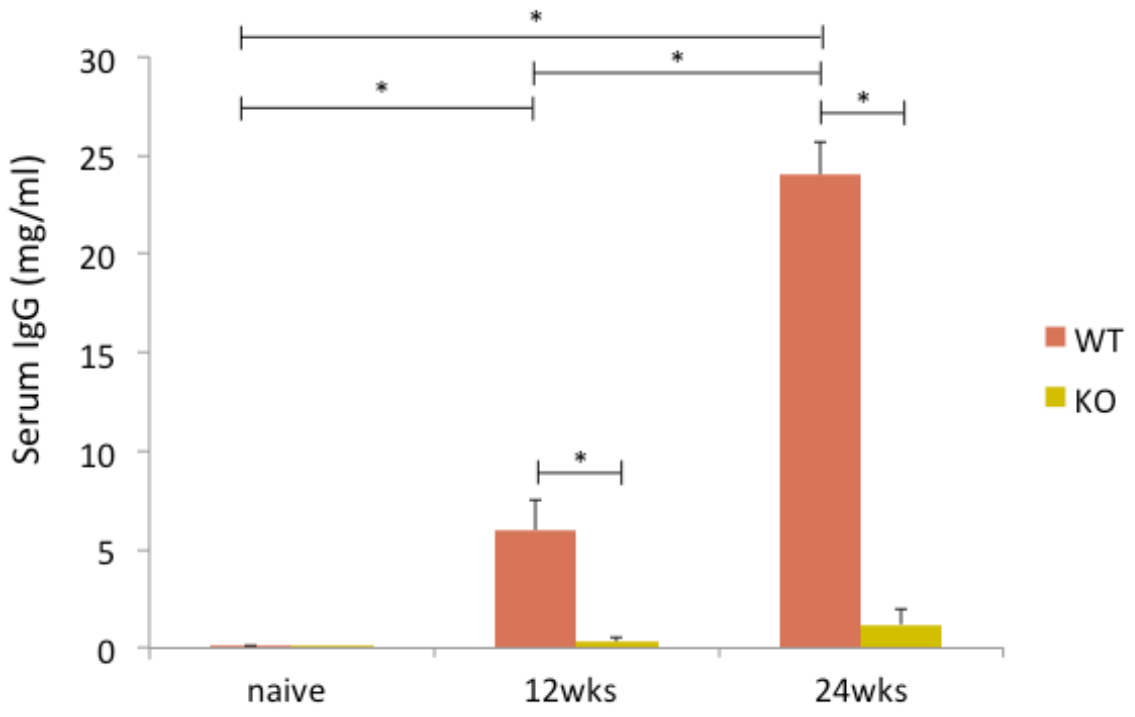


Figure 17 *L. donovani* parasites were not eliminated from spleen or liver of infected BAFF knockout mice.

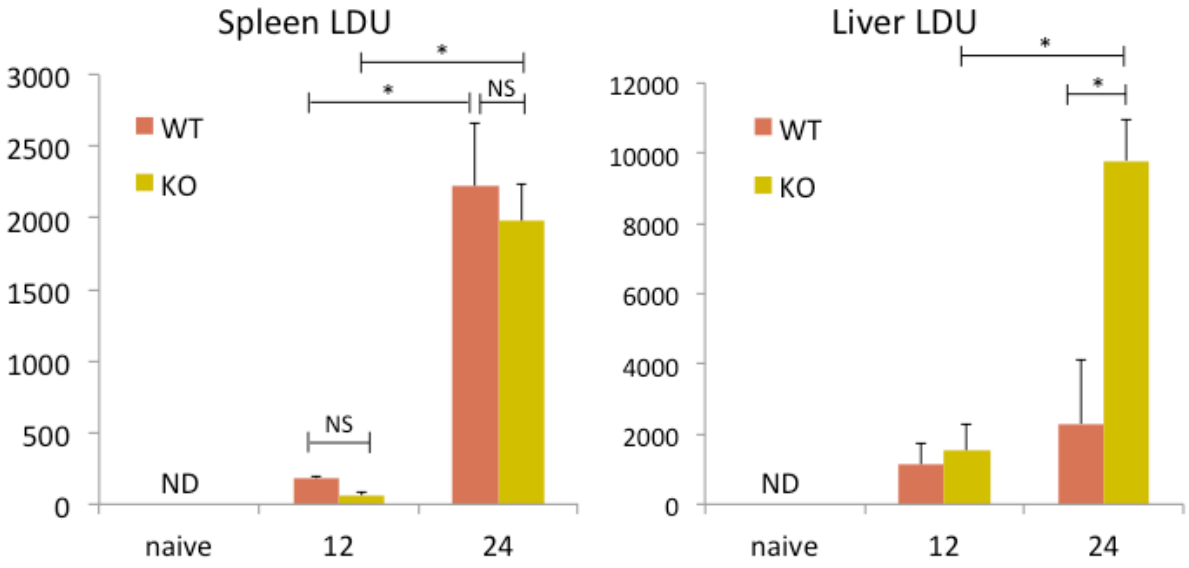
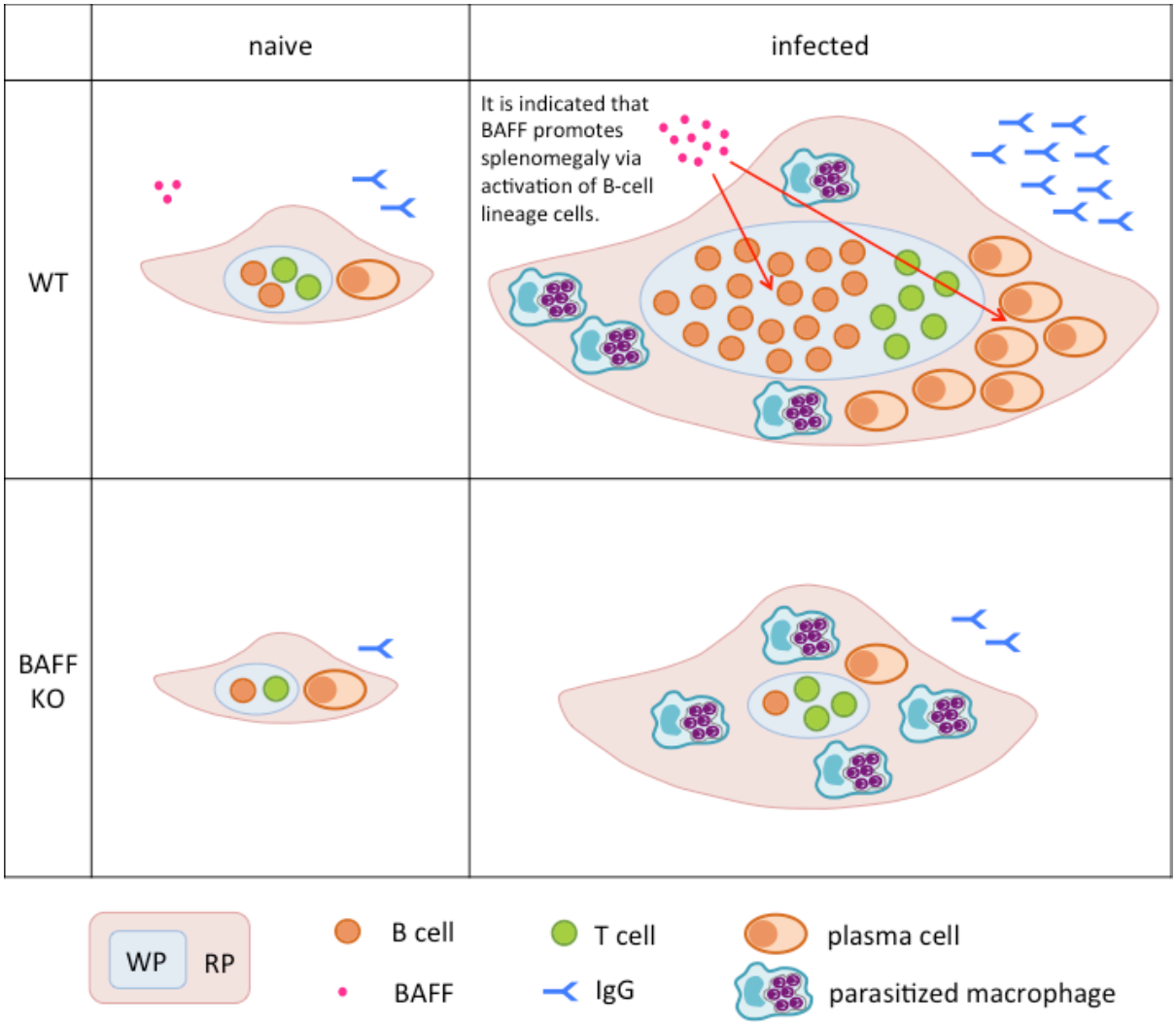


Figure 18　Summary of this study



Summary (in Japanese)

# 論文の内容の要旨

応用動物科学専攻
平成 26 年度　博士課程進学
氏名　　大間知 聡子
指導教員名　　松本　芳嗣

論文題目

Involvement of BAFF in development of splenomegaly
during visceral leishmaniasis
（内臓型リーシュマニア症における脾腫形成機序への BAFF の関与）

背景と目的：内臓型リーシュマニア症（VL）はリーシュマニア症の病型の一つで、*Leishmania donovani* や *L. infantum* によって引き起こされる原虫感染症である。VL の主な症状の一つに脾腫が挙げられる。脾腫は免疫応答の異常を示唆する他、膨満感や腹痛の原因にもなりうる症状であるが、VL における脾腫の形成機序は不明である。脾臓は免疫応答を担う細胞が多数存在する組織であるとともに、VL においてはリーシュマニア原虫の増殖の主要な場である。既存の研究から、原虫に対する宿主の免疫応答が原虫感染に対する抵抗性や感受性に関与することが明らかになってきている。宿主の免疫による原虫数の変化の結果として症状の改善・悪化にも影響が出ることはこれまでも報告されてきたが、宿主免疫と症状の直接的な関係についてはあまり研究されてこなかった。VL は慢性感染症であり、T 細胞や B 細胞といった獲得免疫系の細胞が免疫応答に関与すると考えられる。本研究では B 細胞に焦点を当て、脾腫への B 細胞の関与について明らかにすることにした。第 1 章では、血中免疫グロブリン濃度を指標として、VL 患者において B 細胞や抗体を産生する形質細胞（この 2 つを合わせて、以降、B 細胞系列の細胞と表現する）の強い活性化が起きていることを示す。第 2 章では、*L. donovani* 感染マウスにおいて B 細胞系列の細胞の増加が脾腫の主要な原因の一つであることを示す。第 3 章では、宿主に発現が見られる分子である BAFF（B-cell activating factor belonging to the TNF family）に着目する。BAFF は B 細胞の成熟や分化に関わる重要な因子として知られる一方で、B 細胞の活性化の異常に起因する疾患との関連が示唆されている分子である。まず、VL 患者血清中における BAFF 濃度を指

標として、VL における BAFF の関与を示し、さらに、BAFF の欠損が原虫感染に伴う脾腫に与える影響をマウスを用いて解析し、*L. donovani* 感染時の B 細胞系列の細胞の増加を伴う脾腫における BAFF の関与を示す。

第 1 章 : 免疫グロブリンは B 細胞が分化した形質細胞によって主に産生されることから、免疫グロブリン濃度の増加が B 細胞系列の細胞の活性化の指標となりうるが、VL における免疫グロブリンの定量的な解析の報告はほとんどない。本章では、VL 患者における血中免疫グロブリン濃度をクラス別に解析した。方法は、バングラデシュの VL 患者および健常人の血漿、ブラジルの VL 患者、シャーガス病患者および健常人の血清を用いて、IgG、IgM、IgA の濃度を ELISA 法によって測定した。両地域で VL 患者において IgG 濃度が健常人に比べて有意に高く、バングラデシュで 91%、ブラジルで 75%の患者が健常人の平均+3SD 以上の高い値を示した。IgM 濃度については、両地域とも患者と健常人で有意差がなく、IgA 濃度については、両地域とも患者より健常人の方が有意に高かった。また、免疫グロブリン濃度の顕著な増加はシャーガス病患者においては見られなかった。シャーガス病は VL と同じく原虫感染症であり、原虫抗原特異的な抗体の産生は見られることが過去に報告されているが、患者で免疫グロブリン濃度の有意な増加は見られなかった。一方で、VL 患者においては血中免疫グロブリン濃度の異常な高値が示されたことから、VL において B 細胞系列の細胞が強く活性化されることが示唆された。VL 患者における免疫グロブリンの産生は特に IgG の増加が顕著であることから、IgG へのクラススイッチを特に促進するようなシグナルの関与が考えられた。

第 2 章 : VL において脾腫は頻発の症状である。脾臓は最も大きい二次リンパ器官であり、VL においては原虫増殖の場でもある。脾腫の一因としては、脾臓を構成する細胞数の増加が考えられる。第 1 章の結果から、VL における B 細胞系列の細胞の強い活性化が示唆されたことから、脾臓におけるそれらの細胞の動態に着目した。本章では、*L. donovani* 感染マウスにおいて B 細胞系列の細胞の数の増加が脾腫に寄与することを示すことを目的とした。VL を引き起こす原虫種である *L. donovani* を感受性の BALB/cA マウスに $1\times10^7$ ずつ尾静脈より接種し、感染 12 週後と 24 週後に剖検した。血清中の IgM、IgG、IgA の濃度の ELISA 法による測定、脾臓重量の測定、切片の HE 染色・免疫染色、脾細胞のフローサイトメトリーによる解析を行った。免疫グロブリン濃度は、測定した全てのクラスで増加したが、増加率、増加量、濃度の値ともに IgG が最も顕著であり、VL 患者で見られた IgG を主成分とする高免疫グロブリン血症がマウスにおいても見られた。感染後、脾臓重量は顕著に増加した。脾臓切片の HE 染色像から、白脾髄領域・赤脾髄領域ともに顕著な拡大が観察された。白脾髄においてはリンパ球様の細胞の増加が見られ、赤脾髄においては原虫貪食マクロファージの増加が見られた。また、脾臓切片における免疫染色により IgG 産生形質細胞を検出したところ、非感染時にはほとんど見られないのに対し、感染時には T 細胞領域に隣接した赤脾髄領域にそれらの細胞の領域が観察された。さらに、フローサイ

トメトリーによる解析から、感染後に総脾細胞数が増加し、また、CD19（B 細胞マーカー）陽性細胞数が顕著に増加した。以上の結果から、*L. donovani* 感染マウスにおいて B 細胞系列の細胞の増加が脾腫に寄与することが明らかとなった。

第 3 章：BAFF は B 細胞の活性化の異常に起因する疾患との関連が示唆されているが、VL への関与については報告が無い。第 1 章、第 2 章から、VL における B 細胞系列の細胞の強い活性化と、*L. donovani* 感染マウスにおける B 細胞系列の細胞の増加を伴う脾腫が明らかになったことから、本章では BAFF がその機序に関与することを示すことを目的とした。まず、バングラデシュとブラジルの VL 患者と健常人における血清あるいは血漿中の BAFF 濃度を ELISA 法により測定したところ、両地域とも VL 患者において健常人と比べて有意に高い値を示したことから、VL への BAFF の関与が示唆された。次に、第 2 章に示した方法でマウスに感染実験を行い、血清 BAFF 濃度を測定したところ、血清 BAFF 濃度は感染後に顕著に増加した。そこで、BAFF の脾腫への影響を調べるため、BAFF ノックアウトマウス（KO マウス）を作製し、感染実験を行った。感染 24 週後の脾臓重量は野生型マウスに比べて KO マウスで有意に低かった。また、フローサイトメトリーによる解析から、野生型マウスで見られる脾細胞における CD19 陽性細胞数の増加が KO マウスでは見られなかった。また、脾臓切片の免疫染色像からも、B220（B 細胞マーカー）陽性細胞や CD138（形質細胞マーカー）陽性細胞の増加は KO マウスでは見られなかった。IgG 濃度の顕著な増加も KO マウスでは見られなかった。一方、原虫の主要な増殖の場である脾臓と肝臓について押捺標本における原虫数の測定を行ったところ、KO マウスでは野生型マウスと比べて脾臓では同程度、肝臓では有意に多い原虫数を示した。以上の結果から、BAFF が VL の脾腫形成における B 細胞系列の細胞の増加を引き起こす主要な因子であることが明らかになった。

考察：本研究により、*L. donovani* 感染マウスにおける脾腫において、B 細胞系列の細胞の増加が主要な原因であること、また、BAFF がその増加を引き起こす主要な因子であることが明らかになった。BAFF は原虫数の増加を介してではなく、脾腫を促進させていると考えられる。ヒトの VL においても BAFF が関与していることが示されたこと、また、B 細胞系列の細胞の強い活性化が示されたことから、同様の機序が VL 患者の脾腫形成においても起きている可能性がある。マウスを用いた実験では *L. donovani* を用いたが、VL 患者における血中 BAFF 濃度の増加や IgG 濃度の増加は *L. donovani* が主要な原虫種であるバングラデシュだけでなく *L. infantum* が主要な原虫種であるブラジルにおいても見られたことから、*L. infantum* の感染においても脾腫形成において同様の機序があることが示唆される。さらに、本研究の成果から、宿主免疫系の分子を標的にして症状を緩和することが可能であることが示唆された。