Studies on experimental visceral leishmaniasis in immunodeficient mice

(免疫不全マウスにおける実験的内臓型リーシュマニア症の研究)

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Contents

	Page
General introduction	**3**
Chapter 1. Pathological characterization of *L. donovani* infection in RAG2-/- mice	
Abstract	**8**
Introduction	**9**
Materials and Methods	**11**
Results	**14**
Discussion	**16**
Chapter 2. Characterization of immune cells during experimental VL in RAG2-/- mice	
Abstract	**19**
Introduction	**20**
Materials and Methods	**22**
Results	**24**
Discussion	**26**

Chapter 3. Cytokine expression patterns during experimental VL in RAG2-/- mice

Abstract	**28**
Introduction	**29**
Materials and Methods	**32**
Results	**35**
Discussion	**36**
Conclusion	**38**
Acknowledgments	**39**
References	**40**
Figure legends	**44**
Tables	**49**
Figures	**53**
Abstract	**75**

GENERAL INTRODUCTION

Leishmaniasis is a disease caused by parasitic protozoa of the genus *Leishmania.* The disease has been reported in 88 countries in five continent -Africa, Asia, Europe, North America and South America (72 are classed as developing countries, including 13 of least developed countries) (11, 12). Over 20 *Leishmania* species had been reported to cause leishmaniasis in humans. There are three main forms of the disease in human: visceral leishmaniasis (VL also known as kala-azar); cutaneous leishmaniasis (CL); mucocutaneous leishmaniasis (MCL) (Fig. 1). Three hundred fifty million individuals are at risk of this disease and an estimated 2.0 million new cases occur each year (58), with an incidence of 1.5 million cases per annum of the disfiguring CL and half million cases per annum of the potentially fatal VL (2).

The *Leishmania* parasite is a protozoan belonging to the order Kinetoplastida and the family of Trypanosomatidae. The parasite contains two prominent organelles, the nucleus and the kinetoplast. The kinetoplast is found in all protozoa of the order Kinetoplastidae such as *Leishmania*, *Trypanosoma*, *Crithida*. The parasite exists in two developmental forms: the non-flagellated amastigote living in macrophages of the mammalian host, and the flagellated promastigotes living in the intestinal tract of the insect. The amastigotes in mammalian hosts consist of an ovoidal body containing a nucleus and a kinetoplast; in the vertebrate hosts, the body becomes spindle-shaped and has a single flagellum arising from the axoneme at the anterior end. After multiplication in the host cell the amastigotes are released. Subsequently other macrophages are infected and the infection spreads.

Leishmaniasis is a vector borne disease and *Leishmania* parasite transmitted by insect, a tiny 2 to 3 millimeter-long insect vector, the phlebotomine sandflies that feeds on blood and

breeds in forest areas, caves or the burrows of small rodents. A large number of wild and domestic animals, including dogs, can serve as a reservoir of infection. The disease is highly local, with a distribution restricted to tropical and temperate regions hospitable to sandfly populations. Of 500 known phlebotomine species, about 30 of them have been positively identified as vectors of the disease (11). Only the female sandfly transmits the *Leishmania* parasites. The flagellated metacyclic promastigote form of the parasite is injected into the vertebrate host by the sandfly, where it rapidly and vigorously invades its host cell, the macrophage. Inside macrophage phagolysosomes the promastigote converts into the non-flagellated amastigote form of the parasite (Fig. 2). In endemic foci of anthropozoonotic VL, dogs, foxes, jackals, wolves, and rodents have been recognized as the reservoirs, and the disease in humans is limited to areas where these reservoir animals inhabit (12). VL caused by *L. donovani* in India, Nepal and Bangladesh has been considered to be anthroponotic, that is, transmission occurs mainly between human and sandfly vectors and does not need reservoir animals (Fig. 2). Leishmaniasis can be transmitted by shared syringes among intravenous drug users, by blood transfusion and congenitally from mother to infant, but these modes of transmission are rarer than vector-borne transmission (47).

Clinical symptoms of VL include a grossly enlarged abdomen due to associated hepatosplenomegaly, and more general symptoms such as fever, weight loss, anemia and leukopenia. VL is responsible for significant morbidity and mortality in the developing countries and patients with active disease exhibit marked immunosuppression, and often succumb to secondary infections. At present, there is no antileishmanial vaccine, but there are drugs available to effectively treat VL, and among the most commonly used are pentavalent

antimonials (Glucantime, Pentostam), Amphotericin B, Pentamidine and Miltefosine. However, all of these drugs have now been associated with drug resistance and toxicity. VL is prevalent in 70 countries, with the largest endemic in the Indian subcontinent and in East Africa. An estimated 200,000 to 400,000 new cases of VL occur worldwide each year (1, 57). Over 90% of new cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan (Fig. 3) (56).

The relevance of this disease is further stressed out by the rise of *Leishmania*/HIV co-infection in many parts of the world including European countries such as Spain, Italy, France and Portugal where up to 9% of the AIDS patients suffer from VL (6). The first case of leishmaniasis associated with human immunodeficiency virus (HIV)-1 infection was reported in 1985 (10), and the number of reported cases in southern Europe subsequently increased rapidly. To date, as many as 35 countries throughout the world have reported cases of VL/HIV co-infection (Fig. 4), although most of the published literature concerns the countries of southern Europe. In areas endemic for VL, many people have asymptomatic infection. A concomitant HIV infection increases the risk of developing active VL by between 100 and 2320 times (1). In southern Europe, up to 70% of cases of VL in adults are associated with HIV infection. They characteristically have very high parasite loads and thus contribute to increased spread of the infection, which may include the spread of drug-resistant strains.

VL/HIV co-infection seems to present features different from VL in HIV-free patients. High incidence of disseminated leishmaniasis in AIDS patients has been reported, and atypical symptoms, for example gastrointestinal leishmaniasis representing diarrhea and gastrointestinal hemorrhage, are found in those patients (28). A recent study demonstrated

lower incidence of fever and hepatosplenomegaly in HIV-positive VL patients than HIV-negative VL (9). This indicates the influence of $CD4^+$ T cells to pathology of VL.

There are studies on immune responses during VL in humans and mice. Protective immunity against VL is associated with antigen-specific cell-mediated responses represented by lymphoproliferation and delayed type hypersensitivity (21) and production of Th1 cytokines like IFN-γ and IL-2 upon antigen recall (8). Also, elevation of serum TNF-α was reported in VL patients (5, 44). In contrast, IL-10, which is associated with T-cell hyporesponsiveness, is the predominant cytokine during active VL (18). Studies on experimental VL have further revealed the roles of those cytokines in protection against infection. IFN-γ is an important cytokine to activate macrophages, and IFN-γ knockout mice show higher parasite burden upon *L. donovani* infection (50). TNF-α and IL-12 are also reported to play a protective role during experimental VL (34, 43), whereas IL-10 exacerbates the infection (35). In contrast, studies on immunological mechanisms for pathology of VL are lagging behind.

Some studies have focused on the roles of acquired immunity in pathological changes during leishmaniasis. Previous studies in our laboratory have demonstrated that $CD4^+$ cells are indispensable for ulcer development in murine CL (51-53). In contrast, development of skin lesion associated with macrophage accumulation during murine CL does not require T cell or B cell (19, 51, 52). These works suggest that the roles of acquired immunity to developing the pathology vary for each manifestation during CL.

Taken together, this thesis was designed to explore the host immune response during experimental VL. The recombination activating gene 2 knockout BALB/c ($RAG2^{-/-}$) mice were used as a model. $RAG2^{-/-}$ mutants are viable that fail to produce mature B or T lymphocytes.

Very immature lymphoid cells were present in primary lymphoid the organs of the mutant animals as defined (45). This thesis consists of the comparison of immunodeficient RAG2$^{-/-}$ mice and immunocompetent BALB/c mice during experimental VL at tissue, cellular and molecular scale. In Chapter 1, RAG2$^{-/-}$ mice were infected with *L. donovani*, and pathological changes were examined. In Chapter 2, cell population in the spleen and liver were examined from the infected RAG2$^{-/-}$ and BALB/c mice. Chapter 3, cytokine expression in the spleen and liver during experimental VL was examined in RAG2$^{-/-}$ and BALB/c mice. The results suggested that differences in cellular components and cytokine expression patterns are basis for different outcomes in pathology caused by *L. donovani* infection between RAG2$^{-/-}$ mice and BALB/c mice.

Chapter.1. Pathological characterization of *L. donovani* infection in RAG2$^{-/-}$ mice

Abstract

A recent study demonstrated lower incidence of fever and hepatosplenomegaly in HIV-positive VL patients than HIV-negative VL patients, indicating that acquired immunity have important roles in development of hepatosplenomegaly also in human VL. To explore the roles of acquired immunity in pathology of VL, pathological characterization of *L. donovani* infection in RAG2$^{-/-}$ mice was performed. One hundred million promastigotes were inoculated intraperitoneal to RAG2$^{-/-}$ mice as well as BALB/c mice, and organs were harvested from the animals at 2, 4, 8 and 12 weeks after infection and examined for parasite burdens and pathological changes. *L. donovani* infection induced splenomegaly in BALB/c mice; the length of the spleen after 12 weeks of infected mice were as twice as that of naive mice. In contrast, such the enlargement was not observed in RAG2$^{-/-}$ mice. Tissue enlargement was also observed in the liver, lung and kidney of BALB/c. There was not a huge difference in parasite burdens between BALB/c mice and RAG2$^{-/-}$ mice. Accumulations of mononuclear cells were found in the liver of infected BALB/c mice, whereas such the formations were less found in RAG2$^{-/-}$ mice. These results suggest that development of hepatosplenomegaly during experimental VL is dependent on the T cell or B cell rather than the presence of parasites.

Introduction

Leishmaniasis is endemic in areas of the tropics, subtropics, and southern Europe, in settings ranging from rain forests in the Americas to deserts in western Asia, and from rural to peri urban areas (22). Annual incidence of VL is estimated at 500,000 cases in the world and mortality is officially estimated at 59,000 but it is clearly a severe underestimation (11).

The amastigote forms of *L. infantum* and *L. donovani* are found inside neutrophils and mononuclear phagocytes, or freely, mostly in the spleen, which may harbor the largest parasite burden. Bone marrow also is highly parasitized. The liver and lymph nodes are also important sites of infection (46).

Protracted fever, anemia, wasting, hepatosplenomegaly, hemorrhages, and bacterial co-infections are typical features. Patients who lost more weight had a higher parasite burden, and patients with epistaxis, abdominal pain, edema, and jaundice. This study suggests that higher parasite load is influenced by wasting, which may lead to more severe disease (46). Disseminated intravascular coagulation and thrombocytopenia are the main causes of bleeding. However, profound immunosuppression is demonstrable in acutely ill patients, which is manifested in the form of lack of T cell responsiveness to leishmanial antigen or even to mitogens (23, 41, 42). Recently, some comparative studies about the clinical presentation and outcome of VL in HIV-infected and immunocompetent patients have been reported. Gastrointestinal leishmaniasis representing diarrhea, dysphagia, odynophagia, abdominal pain, epigastric pain, gastrointestinal hemorrhage, and rectal discomfort (27). HIV-infected patients had a greater frequency and degree of leukopenia, lymphocytopenia, and thrombocytopenia (40). A recent study demonstrated lower incidence of fever and hepatosplenomegaly in HIV-

positive VL patients than HIV-negative VL (9). This indicates the influence of $CD4^+$ T cells to pathology of VL. However, immunological mechanisms underlying for pathology of VL remain largely unclear.

Some studies have focused on the roles of acquired immunity in pathological changes during leishmaniasis. Previous studies in our laboratory have demonstrated that $CD4^+$ cells are indispensable for ulcer development in murine CL (51-53). In contrast, development of skin lesion associated with macrophage accumulation during murine CL does not require T cell or B cell (19, 51, 52). These works suggest that the roles of acquired immunity in pathology vary for each manifestation during CL.

Taken together, this thesis was designed to explore the roles of acquired immunity in the development of pathology during VL. $RAG2^{-/-}$ mice were used in this study as a model of mice defective in acquired immunity. $RAG2^{-/-}$ mutants are viable that fail to produce mature B or T lymphocytes. Very immature lymphoid cells were present in primary lymphoid organs of mutant animals as defined (45). Here, $RAG2^{-/-}$ mice were infected with *L. donovani*, and pathological changes were examined by comparing with those in infected BALB/c mice.

Materials and Methods

Parasites

Leishmania donovani (MHOM/NP/03/D10) was obtained from National BioResource Project at Nagasaki University. The parasites isolated from the Nepalese VL patient were used in this study (38). Promastigotes of *L. donovani* D10 were cultured in TC 199 medium at pH 7.2 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), supplemented with 10% heat-activated fetal bovine serum (Thermo Electron Corporation, Australia) and 25 mM HEPES (MP Biomedicals, LLC, France) at 25°C.

Mice and inoculation

Male BALB/c mice of 8 weeks of age were purchased from the CLEA Japan, Inc. (Tokyo, Japan). Male recombination activating gene 2 knockout BALB/c mice ($RAG2^{-/-}$) were obtained from the Central Institute of Experimental Animals, Kawasaki, Japan. All mice were maintained under specific pathogen-free conditions. $RAG2^{-/-}$ mice were used for experiments at the age of 7-8 weeks.

For infection, promastigotes in a stationary phase were washed three times with and resuspended in Hanks' balanced salt solution (HBSS, Gibso Life Technologies). The mice were inoculated intraperitoneal with 1×10^8 promastigotes and were sacrificed at 0, 2, 4, 8 and 12 weeks post inoculation (PI).

All experimental and animal care procedures were approved by the guiding principles of The University of Tokyo and were conducted in accordance with the institution's guidelines for the care and use of laboratory animals under the SPF condition during the experiment. The

protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Tokyo.

Detection of the parasite burden

The spleen, liver, kidney, brain and lung were taken for evaluation of organ weight and determination of parasite burden. The organ impression smears were made by lightly touching a small cut piece of organs to a glass slide. The slides were stained with Giemsa (Merck, Germany), and the numbers of amastigotes per 1,000 host nuclei were enumerated by microscopic observation.

Isolation of mouse bone marrow cells

The bone marrow cells were collected from the femoral bone of mice by inserting a 26-gauge needle attached to the 1 mL syringe filled with TC 199 (10% HI-FBS and 25mM HEPES) at bone cavity. The smears were made from the bone marrow cells then slides were stained with Giemsa and the numbers of amastigotes per 1,000 host nuclei were enumerated by microscopic observation.

Histopathological and immunohistochemistry analysis

Pieces of the spleen and liver were fixed with 10% buffered formalin (Sumitani Shoten Co., Ltd, Japan) and embedded in paraffin wax. Paraffin-embedded tissues were cut at 4 µm. For histology, sections were dewaxed with xylene, and then 100%, 90%, 80%, and 70% ethanol. The rehydrated sections were stained with hematoxylin and eosin (Wako, Japan) for subsequent light microscopy.

For immunohistochemistry, serial sections were deparaffinized, dehydrated, washed in distilled water for 1 min. The sections were treated with methanol containing 0.3% H_2O_2 for 30 min. The sections were washed with PBS (-) for 2 min each time and with PBS-Tween (PBS-T) for 1 min. The sections were incubated with 10% BlockAce (DS Pharma Biomedical Co., Ltd, Japan) for 10 min prior to incubation with each primary antibody which was anti-*Leishmania* monoclonal C11C antibody (2.5 µg/ml). The antibody C11C was developed against *Leishmania* amastigotes and has been shown to react with TSA (Thiol specific antioxidant) protein. After 1 hour 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 Biosciences, Tokyo, Japan). After 1 hour, sections were washed three times with PBS (-) for 2 min each time. Sections were developed for peroxidase activity in 3,3-diaminobenzidine tetrahydrochloride-DAB (Nichirei Biosciences, Tokyo, Japan). Sections were counterstained for 1 min with Mayer's hematoxylin (Wako, Japan), dehydrated and mounted in Mount quick (Daido Sangyo Co., Ltd, Japan).

Results

Measurement of visceral organ weight

At 2, 4, 8 and 12 weeks after infection, tissues were harvested from the animals and examined for tissue weights. Significant splenomegaly was observed in infected BALB/c mice after 12 weeks of infection (Fig. 5). In contrast, such the enlargement of the spleen was not observed in infected RAG2$^{-/-}$ mice (Fig. 5). Tissue enlargement was also observed in the liver, lung and kidney of the infected BALB/c mice (Fig. 6). Again, the enlargement was not observed in the infected RAG2$^{-/-}$ mice. Brain or lung did not show any changes in weight in both strains of mice (Fig. 6).

Quantitation of parasites in visceral organs

Parasitological examination of the spleen, liver and bone marrow smears was performed after necropsy of the infected mice. The smears were stained with Giemsa and examined by optical microscopy for identification of amastigotes. The amastigotes were observed in the spleen, liver and bone marrow stamp smear of *L. donovani* infected RAG2$^{-/-}$ and BALB/c mice after 4 weeks (Fig. 7). The degree of infection was determined by amastigotes number in the spleen, liver and bone marrow quantitated as LDU (Leishman Donovani Units) in smears. Two weeks after the inoculation, the amastigotes were observed in the spleen, liver and bone marrow of RAG2$^{-/-}$ and BALB/c mice, respectively. In the bone marrow, RAG2$^{-/-}$ mice showed higher parasite burden than BALB/c mice. In the liver and spleen, both mice showed similar levels of parasite burdens (Fig. 8).

Histopathological and immunohistochemical analysis

The liver and spleen sections were stained with hematoxylin and eosin (HE). After 4 weeks of infection, accumulations of mononuclear cells were found in the liver of BALB/c mice. In contrast, such the mononuclear cells were less in RAG2$^{-/-}$ mice (Fig. 9). Histological analyses revealed that both red pulp and white pulp were enlarged in BALB/c mice. As the spleen weight was unchanged by *L. donovani* infection in RAG2$^{-/-}$ mice, there was no significant change in the cross-sectional area of the spleen in these mice (Fig. 10).

The parasites were also confirmed by immunohistochemistry using anti-*Leishmania* monoclonal antibody, C11C. By staining with C11C, positive signals were found in the tissues from infected mice. C11C staining also showed the similar results as analysis on Giemsa-stained samples, in other words, increased numbers of positive staining were observed over the course of infection in RAG2$^{-/-}$ mice and BALB/c mice (Fig. 11). The spleen was also analyzed by immunohistochemistry using C11C, and again, increased numbers of positive staining were observed over the course of infection in RAG2$^{-/-}$ mice and BALB/c mice (Fig. 12).

Discussion

Intraperitoneal inoculation of RAG2$^{-/-}$ and BALB/c mice with *L. donovani* promastigotes was shown to induce progressive infection accompanied with increasing parasite load in the spleen, liver and bone marrow during the study period up to 12 weeks following the infection. The numbers of amastigotes per 1000 host nuclei were enumerated by microscopic observation. In the liver, spleen and bone marrow, RAG2$^{-/-}$ mice and BALB/c mice showed increasing number of parasites over the course of infection. These results suggested that BALB/c and RAG2$^{-/-}$ mice were showed susceptibility to *L. donovani* infection. Other groups also reported the infectivity of *L. donovani* to immunodeficient mice. Parasite burdens in SCID mice were found to be lower up to day 14 of infection, whereas the mice had between two and threefold higher parasite burdens than BALB/c mice by 28 days (16). Taken together, it is suggested that immunodeficient mice are susceptible to *L. donovani*.

When it comes to pathology, however, the outcome was completely different between BALB/c mice and RAG2$^{-/-}$ mice. Hepatosplenomegaly is a hallmark of VL symptoms in humans, and it was reproduced in the present experimental VL. However, only BALB/c mice manifested hepatosplenomegaly by *L. donovani* infection, but the manifestation was not apparent in RAG2$^{-/-}$ mice. This result suggests that the pathology is dependent on acquired immunity, rather than the sole parasite burden. The dependence on acquired immunity varies at individual manifestations during leishmaniasis. For example, *L. major* infection causes the development of skin lesion in RAG2$^{-/-}$ mice comparable to BALB/c mice, demonstrating T and B cell-independent mechanism for lesion development (19). In contrast, ulceration of the skin lesion in

L. amazonensis infection is $CD4^+$ T cell-dependent (51-53). Together, it is considered that the pathological mechanisms under various manifestations in leishmaniasis are not uniform, and understanding the mechanisms is important for the control of individual symptoms to develop new interventions.

Histological examinations revealed the accumulations mononuclear cells in the liver of BALB/c mice after 4 weeks of infection. In contrast, such the accumulation mononuclear cells were less intense in $RAG2^{-/-}$ mice. Intense cellular accumulation was also observed in the spleen of infected BALB/c mice but not in that of $RAG2^{-/-}$ mice. These results suggest that cellular accumulation is associated with hepatosplenomegaly and is largely dependent on the presence of T cells or B cells. A recent study demonstrated lower incidence of hepatosplenomegaly in HIV-positive VL patients than HIV-negative VL patients (9), indicating that acquired immunity has important roles in development of hepatosplenomegaly also in human VL. It is reported that in BALB/c and C57BL/6 mice, the inflammatory mononuclear cells reaction around infected kupffer cells is developed and the infection is resolved by 4–8 weeks after infection (33). Cellular and molecular interactions mediated by kupffer cells, monocytes, $CD4^+$ and $CD8^+$ T cells and a number of cytokines and chemokines are required for effective hepatic granuloma formation (15, 24, 33, 37, 49). Although previous studies have identified B cells in hepatic cell infiltrations and functional studies in B cell-deficient mice have suggested a role for B cells in the control of experimental visceral leishmaniasis, little is known about the behavior of B cells in the mononuclear cells microenvironment. The hepatic B cell population in infected mice, where ≈60% of B cells are located within mononuclear cells, with that of naïve mice (25). Taken

together, characterization of cells accumulated in the enlarged spleen and liver of infected BALB/c mice may help understand the mechanisms of hepatosplenomegaly during VL.

Chapter 2. Characterization of immune cells during experimental VL in RAG2$^{-/-}$ mice

Abstract

Because cell accumulation was observed in the enlarged tissues of infected BALB/c mice in Chapter 1, those cells may be related to the different pathology observed between BALB/c mice and RAG2$^{-/-}$ mice. In Chapter 2, characterization of immune cells during experimental VL in BALB/c mice and RAG2$^{-/-}$ mice was performed. For cell population analyses, immunohistochemical staining using antibodies to mouse cell surface antigens was performed. In both the spleen and liver of infected BALB/c mice, increase of CD3ε^{+} and B220^{+} cells was observed. In contrast, increase of those cells was not observed in infected RAG2$^{-/-}$ mice. In the case of MRP8 and MRP14, increase of the positive cells over the course of infection was observed in BALB/c mice and RAG2$^{-/-}$ mice, whereas the intensity of macrophage accumulation was higher in BALB/c mice. These results suggest that *L. donovani* infection causes increase of T cells and B cells in BALB/c mice, and that macrophage accumulation to the spleen and liver is also affected by T cells or B cells. Because hepatosplenomegaly was observed in BALB/c mice but not in RAG2$^{-/-}$ mice, those increased T cells or B cells themselves as well as macrophages under the control of acquired immunity seem to be responsible for development of such the pathology.

Introduction

B and T lymphocytes recognize foreign antigen through specialized receptors: the immunoglobulins and the T cell receptor (TCR), respectively. The highly polymorphic antigen-recognition regions of these receptors are composed of variable (V), diversity (D), and joining (J) gene segments that undergo somatic rearrangement prior to their expression by a mechanism known as V(D)J recombination (54). V(D)J recombination represents a critical checkpoint in the development of the immune system. Indeed, all the animal models carrying a defective gene of either one of the known V(D)J recombination factors, either natural (murine SCID) or exhibit a profound defect in the lymphoid developmental program owing to an arrest of the B and T cell maturation at early stages (31, 45). The phenotype consists of a virtually complete absence of both circulating T and B lymphocytes, associated with a defect in the V(D)J recombination process, while natural killer (NK) cells are normally present and functional (32).

Immune responses are classified into two stages, namely innate and acquired immunity. In each stage, various types of cells contribute to the host defense against infectious agents (17, 29). Innate immunity involves macrophages, natural killer (NK) cells, and neutrophils, which limit the growth of pathogens; and acquired immunity requires the pathogen-specific B and T cells to sterilize foreign pathogens. Despite the fact that acquired immunity is essential for eradication of pathogens in severe infection, innate immunity decreasing the mass of pathogen is also important for establishing effective acquired immune responses. In addition, studies using various immunodeficient mouse lines such as severe combined immunodeficient (SCID) mice have demonstrated that innate immune responses are often sufficient to control infection with foreign pathogens (4).

Organ-specific immunity has been described in various experimental VL studies in mouse models (14, 15). The liver is the site of an acute but resolving infection. In contrast, the spleen becomes a site of parasite persistence with associated immunopathological changes (49). The difference in pathology between BALB/c and RAG2$^{-/-}$ mice may result from distinct immunological players in the spleen and liver between those mice. A previous study demonstrated that *L. major* infection causes the development of skin lesion in RAG2$^{-/-}$ mice comparable to BALB/c mice, demonstrating T and B cell-independent mechanism for lesion development (19). The study also demonstrated that massive macrophage accumulation is associated with the lesion development and the degree of accumulation is comparable despite of the presence of T or B cells (19). However, it is still unclear whether macrophages accumulate in the enlarged tissues during VL, and if so, whether the accumulation is under control of acquired immunity.

The present study was aimed to characterize cell population in the spleen and liver of infected BALB/c and RAG2$^{-/-}$ mice in order to understand the mechanisms for hepatosplenomegaly during VL.

Materials and methods

Parasites

Leishmania donovani (MHOM/NP/03/D10) was obtained from National BioResource Project at Nagasaki University. The parasites which isolated from the Nepalese VL patient were used in this study (38). Promastigotes of *L. donovani* D10 were cultured in TC 199 medium at pH=7.2, supplemented with 10% heat-activated fetal bovine serum (Thermo Electron Corporation, Australia) and 25 mM HEPES (MP Biomedicals, LLC, France) at 25°C.

Mice and inoculation

Male BALB/c mice of 8 weeks of age were purchased from the CLEA Japan, Inc. (Tokyo, Japan). Male recombination activating gene 2 knockout BALB/c mice ($RAG2^{-/-}$) were obtained from the Central Institute of Experimental Animals, Kawasaki, Japan. All mice were maintained under specific pathogen-free conditions. $RAG2^{-/-}$ mice were used for experiments at the age of 7-8 weeks.

For infection, promastigotes in a stationary phase were washed three times with and resuspended in Hanks' balanced salt solution. The mice were inoculated intraperitoneal with 1×10^8 promastigotes and were sacrificed at 4 and 8 weeks post inoculation (p.i.).

All experimental and animal care procedures were approved by the guiding principles of The University of Tokyo and were conducted in accordance with the institution's guidelines for the care and use of laboratory animals under the SPF condition during the experiment. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Tokyo.

Immunohistochemistry

Pieces of the spleen and liver were fixed with 10% buffered formalin and embedded in paraffin wax. Paraffin-embedded tissues were cut at 4 μm in thickness. The sections were deparaffinized, rehydrated, washed in distilled water. Hydrated sections were treated with methanol containing 0.3% H_2O_2 for 30 min. The sections were washed with PBS (-) for 2 min each time and with PBS-Tween (PBS-T) for 1 min. The sections were incubated with 10% BlockAce for 10 min prior to incubation with each primary antibodies (Table.1). Used antibodies were CD3ε as a T-cell marker, B220 as a B-cell marker, MRP8/MRP14 as markers for macrophages, at 1:100 dilutions with 10 % BlockAce with PBS-Tween. For negative control sections, PBS was used instead of the primary antibody. Sections were developed for peroxidase activity in 3,3-diaminobenzidine tetrahydrochloride-DAB. Sections were counterstained for 1 min with Mayer's hematoxylin, dehydrated and mounted in Mount quick.

Results

Increase of CD3ε^{+} cells after infection in BALB/c mice not in RAG2$^{-/-}$ mice

The liver sections were stained with anti-CD3ε antibody. BALB/c mice had increased number of CD3ε-positive cells after infection (Fig. 13). The increase was observed as early as 4 weeks after infection, and the intensity was higher at 8 weeks. In contrast, no prominent increase was observed in *L. donovani* infected RAG2$^{-/-}$ mice (Fig. 13). The spleen showed a similar pattern in CD3ε staining to the liver. BALB/c mice had increased number of CD3ε-positive cells after *L. donovani* infection, from as early as 4 weeks, whereas no prominent increase was observed in RAG2 $^{-/-}$mice (Fig. 14).

Increase of B220^{+} cells after infection in BALB/c mice not in RAG2$^{-/-}$ mice

The liver sections of *L. donovani* infected BALB/c and RAG2-/- mice were stained with anti-B220 antibody. BALB/c mice had increased number of B220-positive cells after infection (Fig. 15). The increase was observed as early as 4 weeks after infection, and the intensity was higher at 8 weeks. In contrast, no prominent increase was observed in RAG2$^{-/-}$ mice (Fig. 15). The spleen showed a similar pattern in B220 staining to the liver. BALB/c mice had increased number of B220-positive cells after infection, from as early as 4 weeks, whereas no prominent increase was observed in RAG2$^{-/-}$ mice (Fig. 16).

Increase of MRP8^{+} and MRP14^{+}cells after infection in BALB/c mice not in RAG2$^{-/-}$mice

First, the liver sections of *L. donovani* infected BALB/c and RAG2$^{-/-}$ mice were stained with anti-MRP8 antibody. BALB/c mice had increased number of MRP8-positive cells after infection (Fig. 17). The increase was observed as early as 4 weeks after infection, and the

intensity was higher at 8 weeks. Such the increase was also observed in RAG2$^{-/-}$ mice (Fig. 17). The increase was observed as early as 4 weeks after infection, and the intensity was higher at 8 weeks. The spleen showed a similar pattern in MRP8 staining to the liver. BALB/c mice had increased number of MRP-positive cells after infection, from as early as 4 weeks, and such the increase was also observed in RAG2$^{-/-}$ mice (Fig. 18). Although accumulation of MRP8^{+} cells in the spleen and liver was observed in BALB/c and RAG2$^{-/-}$ mice, the intensity was higher in BALB/c mice.

Next, the liver sections of *L. donovani* infected BALB/c and RAG2$^{-/-}$ mice were stained with anti-MRP14 antibody. The staining pattern of MRP14 resembled that of MRP8. BALB/c mice had increased number of MRP14-positive cells after infection (Fig. 19). The increase was observed as early as 4 weeks after infection, and the intensity was higher at 8 weeks. Such the increase was also observed in RAG2$^{-/-}$ mice (Fig. 19). The increase was observed as early as 4 weeks after infection, and the intensity was higher at 8 weeks. The spleen showed a similar pattern in MRP14 staining to the liver. BALB/c mice had increased number of MRP14 positive cells after infection, from as early as 4 weeks, and such the increase was also observed in RAG2$^{-/-}$ mice (Fig. 20). Similar to MRP8, although accumulation of MRP14 positive cells in the spleen and liver was observed in BALB/c and RAG2$^{-/-}$ mice, the intensity was higher in BALB/c mice.

Discussion

Increase of CD3ε-positive cells and B220-positive cells over the course of infection was prominent in BALB/c mice but not in RAG2$^{-/-}$ mice. These results suggest that increase of T cells and B cells is responsible for hepatosplenomegaly which can be found in BALB/c mice but not in RAG2$^{-/-}$ mice. In contrast, increase of MRP8-positive cells and MRP14-positive cells was found in both of BALB/c mice and RAG2$^{-/-}$ mice. These results indicate that there is a T-cell and B-cell independent mechanism for macrophage accumulation to the spleen and liver caused by *L. donovani* infection. The existence of such an innate type mechanism for macrophage accumulation is comprehensible because macrophages are the host cells of *Leishmania* parasites; the parasites need their houses even if the host is in immunodeficient condition. This finding matches with the results in Chapter 1 that RAG2$^{-/-}$ and BALB/c mice are susceptible to *L. donovani* infection. However, it should be also noted that the intensity of macrophage accumulation was higher in BALB/c mice than in RAG2$^{-/-}$ mice. This suggests that more intense macrophage accumulation in BALB/c mice serves not as places for parasitization but as the cause of pathology. In fact, many of macrophages in infected BALB/c mice were not parasitized.

A previous study demonstrated that an accumulation of macrophages expressing MRP8 and MRP14 in skin lesions of *L. major* infected RAG2$^{-/-}$ mice (19). In the study, the intensity of MRP8^{+} and MRP14^{+} cell accumulation was comparable between BALB/c and RAG2$^{-/-}$ mice, indicating the cell accumulation was largely independent from T cells or B cells in the model. In contrast, hepatosplenomegaly occurred in BALB/c mice but not in RAG2$^{-/-}$ mice during *L. donovani* infection, and the accumulation of MRP8 and MRP14 positive cells to the spleen and liver was also more prominent in BALB/c mice than in RAG2$^{-/-}$ mice (Table.2). These indicate

that immunological mechanisms for macrophage accumulation are different between experimental CL and VL. It is still unclear whether such the difference is due to different parasite species or different target organs. Nonetheless, it can be hypothesized that expression patterns of cytokines and chemokines at the infection sites are different in the two models, affecting the accumulation of macrophages.

As a summary, results in this chapter suggest that *L. donovani* infection causes increase of T cells and B cells in BALB/c mice, and that macrophage accumulation to the spleen and liver is also affected by T cells or B cells. Because hepatosplenomegaly was observed in BALB/c mice but not in $RAG2^{-/-}$ mice, those increased T cells or B cells themselves as well as macrophages under the control of acquired immunity seem to be responsible for development of such the pathology. Exploring molecular mechanisms controlling the accumulation of these cells may help understand the pathology of hepatosplenomegaly during VL.

Chapter 3. Cytokine expression patterns during experimental VL in RAG2$^{-/-}$ mice

Abstract

Results from Chapters 1 and 2 demonstrated the differences in pathology and cellular kinetics during *L. donovani* infection between BALB/c mice and RAG2$^{-/-}$ mice. It is easily hypothesized that such the differences in pathology and cellular kinetics are caused by difference in molecules such as cytokines. In Chapter 3, therefore, cytokine expression in the spleen and liver during experimental VL was examined in BALB/c mice and RAG2$^{-/-}$ mice. Here, four cytokines, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 4 (IL-4) and interleukin 10 (IL-10) were examined for mRNA expression levels at 0, 4, 8 and 12 weeks of infection by RT-PCR. In BALB/c mice, mRNA expressions of all the cytokines examined were increased over the course of infection. Especially, interleukin-4 and interleukin-10 were significantly increased in BALB/c mice. In contrast, no change in mRNA expression level was observed in RAG2$^{-/-}$ mice for any cytokines. In the liver, some cytokines were detectable at certain time points. However, the expression levels in the liver were overall much lower than those in the spleen. The results that mRNA levels of IFN-γ, TNF-α, IL-4 and IL-10 in the spleen were increased over the course of *L. donovani* infection in BALB/c mice but not in RAG2$^{-/-}$ mice suggests that T cells or B cells are indispensable for such the increased expression of the cytokines in the spleen during experimental VL. Because these cytokines are known to relate with protection/exacerbation of *Leishmania* infection, further studies on mechanisms for expression of these cytokines by T cells or B cells may lead to better understanding of pathology during VL.

Introduction

Clinical symptoms of VL include a grossly enlarged abdomen due to associated hepatosplenomegaly, and more general symptoms such as fever, weight loss, anemia and leukopenia. VL is responsible for significant morbidity and mortality in the developing countries and patients with active disease exhibit marked immunosuppression, and often succumb to secondary infections. Although it is clear that understanding pathological mechanisms for VL is important for development of new interventions, such the studies are yet inadequate.

Recent studies indicate the influence of acquired immunity to pathology of VL. High incidence of disseminated leishmaniasis in AIDS patients, who has deficiency in acquired immunity, has been reported, and atypical symptoms, for example gastrointestinal leishmaniasis representing diarrhea and gastrointestinal hemorrhage, are found in those patients (28). A recent study demonstrated lower incidence of fever and hepatosplenomegaly in HIV-positive VL patients than HIV-negative VL (9). In Chapter 1, I demonstrated that hepatosplenomegaly during experimental VL is also dependent on acquired immunity by using $RAG2^{-/-}$ mice. Also, results in Chapter 2 demonstrated that accumulation of T cells, B cells and macrophages is associated with hepatosplenomegaly. Because acquired immunity was responsible even for macrophages accumulation to the spleen and liver during *L. donovani* infection, it is suggested that T cells or B cells are the master regulators for hepatosplenomegaly in VL. Therefore, characterization of effector molecules, which are produced by T cells or B cells and affect the cell accumulation, may lead to understating of pathological mechanisms for hepatosplenomegaly in VL.

There are studies on immune responses during VL in humans and mice. Protective immunity against VL is associated with antigen-specific cell-mediated responses represented by lymphoproliferation and delayed type hypersensitivity (21) and production of Th1 cytokines like IFN-γ and IL-2 upon antigen recall (8). Also, elevation of serum TNF-α were reported in VL patients (5, 44). In contrast, IL-10, which is associated with T-cell hyporesponsiveness, is the predominant cytokine during active VL (18). Studies on experimental VL have further revealed the roles of those cytokines in protection against infection. IFN-γ is an important cytokine to activate macrophages, and IFN-γ knockout mice show higher parasite burden upon *L. donovani* infection (50). TNF-α and IL-12 are also reported to play a protective role during experimental VL (34, 43), whereas IL-10 exacerbates the infection (35). However, studies on immunological mechanisms for pathology of VL are lagging behind.

The immunological mechanism involved in the infection and the relationship between the immune system and the clinical forms of the disease have been studied by evaluating cellular profiles and cytokine production in spleen and lymph node aspirates, as well as in peripheral blood mononuclear cells and in plasma samples from patients with different clinical forms of VL. Analysis of plasma samples is a suitable alternative to the other methods because it is simpler and less invasive (20, 26, 39). The plasma concentrations of both IFN-γ and IL-10 are higher in patients with the active disease relative to asymptomatic individuals and are also higher in asymptomatic individuals compared to uninfected individuals (26). The visceral *Leishmania* sp., the type 1 response is suppressed at least in part by TGF-β and IL-10 without type 2 cytokine production. This contrasts with the cutaneous species *L. major*, in which a type 2 response suppresses type 1 cytokine production and leads to murine disease progression. Population and

family studies are beginning to elucidate human genetic determinants predisposing to different outcomes of *Leishmania* infection. Additional types of $CD4^+$ cells expressing TGF-β and/or IL-10 have more recently been described (59).

For the purpose of this study, I examined cytokine expression in the spleen and liver during experimental VL in BALB/c mice and $RAG2^{-/-}$ mice.

Materials and Methods

Parasites

Leishmania donovani (MHOM/NP/03/D10) was obtained from National BioResource Project at Nagasaki University. The parasites which isolated from the Nepalese VL patient were used in this study (38). Promastigotes of *L. donovani* D10 were cultured in TC 199 medium at pH=7.2 supplemented with 10% heat-activated fetal bovine serum and 25 mM HEPES at 25°C.

Mice and inoculation

Male BALB/c mice of 8 weeks of age were purchased from the CLEA Japan, Inc. (Tokyo, Japan). Male recombination activating gene 2 knockout BALB/c mice ($RAG2^{-/-}$) were obtained from the Central Institute of Experimental Animals, Kawasaki, Japan. All mice were maintained under specific pathogen-free conditions. $RAG2^{-/-}$ mice were used for experiments at the age of 7-8 weeks.

For infection, promastigotes in a stationary phase were washed three times with and resuspended in Hanks' balanced salt solution. The mice were inoculated intraperitoneal with 1×10^8 promastigotes and were sacrificed at 0, 4, 8 and 12 weeks post inoculation (p.i.).

All experimental and animal care procedures were approved by the guiding principles of The University of Tokyo and were conducted in accordance with the institution's guidelines for the care and use of laboratory animals under the SPF condition during the experiment. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Tokyo.

Extraction of total RNA

The spleen and liver samples were homogenized with 1 ml of TRIZOL reagent (Invitrogen, USA) and a glass- bead (0.1 mm). After the homogenization the samples were added 0.2 ml of chloroform per 1 ml of Trizol reagent and vortexed the samples vigorously for 15 seconds. Then, the samples incubated at room temperature for 5 min. After that all samples were centrifuged at 12,000 x g for 15 min at 4 °C. Upper aqueous phase was transferred carefully without disturbing the interphase into a new tube.

The RNA was suspended in 50 µL of MilliQ water, and RNA quantity was determined spectrophotometrically at 260 nm using the spectrophotometer (DU730 Beckman Coulter, USA), then was stored at –80°C until use.

cDNA synthesis

To prepare the cDNA, 1 µl of RNA was added with 1 µl oligo (dT)12-18 (500 µg/ml) (Invitrogen by Life Technologies, USA) in a PCR tube (RIKAKEN Ltd., Japan) and made up to a final volume of 9 µl using nuclease-free water (Fisher Scientific UK, Ltd). Next, 1 µl of dNTP mix (25 mM; Invitrogen, Life Technologies Ltd) were added and the tube incubated at 65°C for 5 min. Then 4 µl of 5X first-strand buffer (Invitrogen, Life Technologies Ltd), 2 µl of DTT (0.1 M; Invitrogen, Life Technologies Ltd) and 1 µl of M-MLV Reverse Transcriptase (Invitrogen, Life Technologies Ltd, USA) enzyme were added to the reaction tube, which was incubated at 37°C for 50 min and then at 70°C for 15 min. The resulting cDNA was stored at -20 °C until use.

Reverse transcription PCR

Reverse transcription PCR was performed using 1 µg total RNA isolated from spleen and liver as a template. RT-PCR was conducted in a final volume of 25 µl. The RT-PCR was

performed using cDNA and a set of gene specific primers (Table. 3) with hot start at 94°C for 5 min, and 35 amplification cycles (each consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s), followed by final extension at 72°C for 10 min per cycle using the PCR thermal cycler (Takara, Japan). PCR products were separated by gel electrophoresis in 1.5% agarose gel (Lonza, USA). After staining the gel in ethidium bromide solution (Wako, Japan), the photograph was taken under ultraviolet illumination.

Results

Reverse transcription PCR

Targeted cytokines in this study were Interferon gamma, tumor necrosis factor alpha, interleukin-4 and interleukin-10. Beta-actin was included for RT-PCR analysis as a housekeeping gene. Sequences of primers used for these genes and the expected products were shown in the table (Table 3).

The cytokine expression was examined at 0, 4, 8 and 12 weeks of infection in the spleen and liver of BALB/c mice and $RAG2^{-/-}$ mice. In spleen of BALB/c mice, mRNA expressions of all the cytokines examined were increased over the course of infection (Fig. 21). Especially, IL-4 and IL-10 were significantly increased. Increase of IL-10 was observed as early as 4 weeks and the expression was higher at 12 weeks. Increase of IL-4 was observed as early as 8 weeks and the expression was higher at 12 weeks. Increase of IFN-γ was observed as early as 8 weeks and the expression was higher at 12 weeks. Increase of TNF-α was observed also, but the degree was smaller than the other cytokines. In contrast, no change in mRNA expression level was observed in $RAG2^{-/-}$ mice for any cytokines.

In the liver, some cytokines were detectable at certain time points. However, the expression levels in the liver were overall much lower than those in the spleen.

Discussion

The spleen is an initial site for the generation of cell-mediated immune responses, but ultimately becomes a site of parasite persistence with associated immunopathological changes. Parasite persistence in the spleen is associated with severe splenomegaly and an array of changes to the splenic lymphoid microenvironment (3, 13, 48), and decreased responsiveness to leishmanial antigens (36). These include splenomegaly and a breakdown in tissue architecture that is postulated to contribute to the immunocompromised status of the host. The progressive development of splenic pathology is largely associated with high levels of TNF-α and IL-10 (3). In SCID mice, TNF-α positive cells were rare and almost always either neutrophils or monocytes. In BALB/c mice, heavily infected TNF-α positive kupffer cells were readily observed, within granulomas at various stages of development (16).

In the present study, high levels of IL-4 and IL-10 were observed in BALB/c mice. In contrast, no change in mRNA expression level was observed in $RAG2^{-/-}$ mice for any cytokines. The concentration of IL-4, a cytokine that generally plays an immunosuppressive role (30), was detected the high level in patients with the active disease. However, in VL, the role of Th1 or Th2 cytokines in the resistance and susceptibility, respectively, is not very obvious because both Th1 and Th2 cytokines have been detected in VL patients showing different clinical disease manifestations (55).

In vitro, when IL-4 is added after cell stimulation, the production of IFN-γ is reduced (7), whereas the addition of IL-4 to cells before stimulation increases IFN-γ production (7, 39). A synergism between IL-4 and IL-10 has been reported in the literature. High concentrations of these cytokines have been shown to promote the deactivation of macrophage leishmanicidal

activity and, consequently, favor the multiplication of the parasite and the development of the disease and are accompanied by the expression of Th2 cytokines (39).

Conclusion

This study has demonstrated that the BALB/c and RAG2$^{-/-}$ mice show a progressive parasite burden without any signs of heal. In contrast, the hepatosplenomegaly occurs in BALB/c mice but not in RAG2$^{-/-}$ mice. Also, accumulation of T cells, B cells and macrophages was prominent in the spleen and liver of infected BALB/c mice. These results suggest that cellular accumulation is associated with hepatosplenomegaly and is dependent on acquired immunity. Together, pathological mechanisms during VL are not very simple; although parasite burden is important for pathology, it is not the sole factor affecting the severity of the disease. Rather, acquired immunity regulated by T or B cells may have a more significant role in development of hepatosplenomegaly during VL. The mechanisms by which T or B cells contribute to the pathology may include cytokine molecule. In fact, expression of cytokines such as IL-4 and IL-10 was upregulated over the course of infection in BALB/c mice, whereas such the increase was not found in RAG2$^{-/-}$ mice. How those cytokines induce hepatosplenomegaly remains to be studied. Immune responses are often 'a double-edged sword', the protective immunity against pathogen often serves as a pathological factor. This work demonstrated that pathology of VL, especially enlargement of the spleen and the liver is also under influence of acquired immunity. Because such acquired immunity is important for elimination of parasites, this study may serve as the important first step to understand the watershed of protection/pathology by acquired immunity and will lead to development of better interventions for VL. Finally, this mouse model can be useful to understand the influence of acquired immunity (T cell/ B cell) to pathology for VL.

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Figure legends

Figure 1. Clinical manifestations of leishmaniasis. **A.** A typical ulcer lesion with a markedly evaluated border of a cutaneous leishmaniasis patient observed in Ecuador (Picture taken by Dr. Matsumoto); **B.** Visceral leishmaniasis patient with splenomegaly observed in Turkey (Picture taken by Dr. Sanjoba).

Figure 2. Transmission cycles of leishmaniasis. *Leishmania* parasites exist as an extra cellular flagellated promastigotes in the midgut of the sandfly and intracellular amastigote of in the macrophage of the susceptible vertebrate hosts.

Figure 3. Status of endemicity of visceral leishmaniasis, worldwide, 2012. Highly endemic in the Indian subcontinent and in East Africa, Brazil.

Figure 4. The geographical distribution of *Leishmania*/HIV co-infection. 35 countries have reported cases of *Leishmania*/HIV co-infection worldwide, and the co-infection now accounts for 2-12% of all VL cases.

Figure 5. Prominent enlargement of the spleen in BALB/c mice. Representatives of the spleen from uninfected and infected mice.

Figure 6. Kinetics of organ weight during experimental VL. Kinetics of weights of the spleen, liver, lung, brain and kidney during the course of infection. Blue bars are for RAG2$^{-/-}$ mice and red bars are for BALB/c mice. Data represented the mean ± SD of 4 mice per group.

Figure 7. Giemsa-stained liver, spleen and bone marrow smears from RAG2$^{-/-}$ and BALB/c mice 4 weeks infected with *L. donovani*. Arrows indicate *Leishmania* amastigotes.

Figure 8. Parasite burden in the liver, spleen and bone marrow from RAG2$^{-/-}$ and BALB/c mice infected with *L. donovani*. The numbers of amastigotes per 1,000 host nuclei which were enumerated by microscopic observation. Data represented the mean ± SD of 4 mice per group.

Figure 9. Histological analysis of the liver of RAG2$^{-/-}$ and BALB/c mice infected with *L. donovani*. The liver sections were stained with hematoxylin and eosin. Arrows indicate the mononuclear cells. Scale bar: 50 μm (inset: 20μm).

Figure 10. Histological analysis of RAG2$^{-/-}$ and BALB/c mice infected with *L. donovani*. The spleen sections stained with hematoxylin and eosin. Scale bar: 50 μm.

Figure 11. Immunohistochemical detection of amastigotes in the liver of RAG2$^{-/-}$ and BALB/c mice infected with *L. donovani*. The liver sections stained with anti-*Leishmania* monoclonal antibody, C11C (2.5 µg/ml). Scale bar: 50 µm (inset: 20 µm).

Figure 12. Immunohistochemical detection of amastigotes in the spleen of RAG2$^{-/-}$ and BALB/c mice infected with *L. donovani*. The spleen sections stained with anti-*Leishmania* monoclonal antibody, C11C (2.5 µg/ml). Scale bar: 50 µm (inset: 20 µm).

Figure 13. Immunohistochemistry in the liver of *L. donovani* infected RAG2$^{-/-}$ and BALB/c mice. The liver sections were stained using the anti-CD3ε affinity purified goat polyclonal (SANTA CRUZ BIOTECHNOLOGY, INC) and counterstained with hematoxylin. Arrows indicate the CD3ε positive cells. Scale bar: 200 µm.

Figure 14. Immunohistochemistry in the spleen of *L. donovani* infected RAG2$^{-/-}$ and BALB/c mice. The spleen sections were stained using the anti-CD3ε affinity purified goat polyclonal antibody and counterstained with hematoxylin. Scale bar: 200 µm.

Figure 15. Immunohistochemistry in the liver of *L. donovani* infected RAG2$^{-/-}$ and BALB/c mice. The liver sections were stained using the purified rat anti-mouse CD45R(B220) antibody (BD

Pharmingen) and counterstained with hematoxylin. Arrows indicate the B220 positive cells. Scale bar: 200 µm.

Figure 16. Immunohistochemistry in the spleen of *L. donovani* infected RAG2$^{-/-}$ and BALB/c mice. The spleen sections were stained using the purified rat anti-mouse CD45R(B220) antibody (BD Pharmingen) and counterstained with hematoxylin. Scale bar: 200 µm.

Figure 17. Immunohistochemistry in the liver of *L. donovani* infected RAG2$^{-/-}$ and BALB/c mice. The liver sections were stained using the anti-MRP8 goat polyclonal (sc-8115) antibody (SANTA CRUZ BIOTECHNOLOGY, INC) and counterstained with hematoxylin. Scale bar: 200 µm (inset: 20 µm).

Figure 18. Immunohistochemistry in the spleen of *L. donovani* infected RAG2$^{-/-}$ and BALB/c mice. The spleen sections were stained using the anti-MRP8 goat polyclonal (sc-8115) antibody and counterstained with hematoxylin. Scale bar: 200 µm (inset: 20 µm).

Figure 19. Immunohistochemistry in the liver of *L. donovani* infected RAG2$^{-/-}$ and BALB/c mice. The liver sections were stained using the anti-MRP14 goat polyclonal (sc-8113) antibody (SANTA CRUZ BIOTECHNOLOGY, INC) and counterstained with hematoxylin. Scale bar: 200 µm (inset: 20 µm).

Figure 20. Immunohistochemistry in the spleen of *L. donovani* infected RAG2$^{-/-}$ and BALB/c mice. The spleen sections were stained using the anti-MRP14 goat polyclonal (sc-8113) antibody and counterstained with hematoxylin. Scale bar: 200 µm (inset: 20 µm).

Figure 21. Cytokine expression in the liver and spleen of *L. donovani* infected RAG2$^{-/-}$ and BALB/c mice. The result of cytokine expression at 0, 4, 8 and 12 weeks post infection in the spleen and liver of BALB/c and RAG2$^{-/-}$ mice. RNA was extracted from the spleen and liver, and cytokine mRNA levels were examined by RT-PCR. Targeted cytokines in this study were Interferon gamma, tumor necrosis factor alpha, interleukin-4 and interleukin-10. Beta-actin was included for RT-PCR analysis as a housekeeping gene.

Tables

Figures

Table 1. Antibodies used in this study

Antibodies	Reactivity	Target cell	Source
Purified Rat anti-mouse CD45R(B220)	Mouse	B cell	BD Pharmingen
Anti- CD3ε affinity purified goat polyclonal (sc-1127)	Mouse	T cell	SANTA CRUZ BIOTECHNOLOGY, INC
Anti- MRP14 goat polyclonal (sc-8113)	Mouse,	Macrophage	SANTA CRUZ BIOTECHNOLOGY, INC
Anti- MRP8 goat polyclonal (sc-8115)	Mouse, rat	Macrophage	SANTA CRUZ BIOTECHNOLOGY, INC

Table 2. Kinetics of immune cells during experimental VL

Spleen

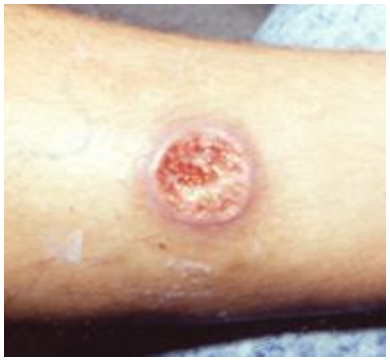
	BALB/c			RAG2-/-		
	naïve	p.i.4 wks	p.i. 8 wks	naïve	p.i.4 wks	p.i. 8 wks
CD3ε	(++)	(+++)	(+++)	(±)	(±)	(±)
B220	(++)	(+++)	(+++)	(+)	(+)	(+)
MRP8	(+)	(++)	(+++)	(+)	(++)	(++)
MRP14	(+)	(++)	(+++)	(+)	(++)	(++)

Liver

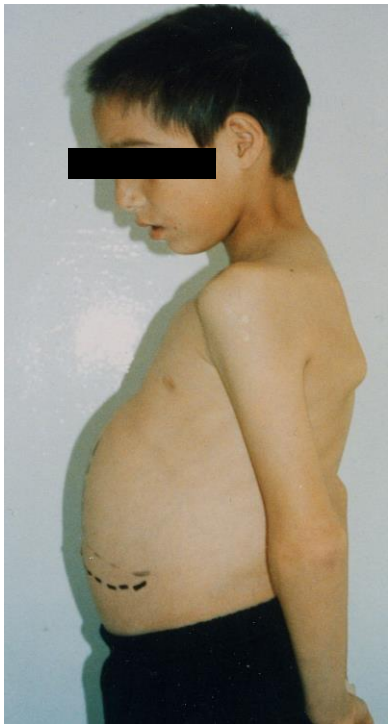
	BALB/c			RAG2-/-		
	naïve	p.i.4 wks	p.i. 8 wks	naïve	p.i.4 wks	p.i. 8 wks
CD3ε	(±)	(+)	(++)	(±)	(±)	(±)
B220	(±)	(+)	(++)	(±)	(±)	(±)
MRP8	(±)	(+)	(++)	(±)	(+)	(+)
MRP14	(±)	(+)	(++)	(±)	(+)	(+)

Table.3. Primers used for reverse transcription PCR

Gene	Sequences (5’-3’)	Product size (bp)
IFN- γ	F: ACTGCCACGGCACAGTAA	401
	R: GCGACTCCTTTTCCGCTT	
TNF-α	F: GAAAGCATGATCCGCGACGTGG	678
	R: GTAGACCTGCCCGGACTCCGCAA	
IL-4	F:GAATGTACCAGGAGCCATATCC	370
	R:AATCCATTTGCATGATGCTCTT	
IL-10	F: GACAATAACTGCAAGTGCATCATCG	443
	R: ATCATCATGTATGCTTCTATGC	
β-actin	F: GTTACCAACTGGGGACGACA	460
	R: TGGCCATCTCCTGCTCGAA	



A. Cutaneous leishmaniasis



B. Visceral leishmaniasis

Fig. 1

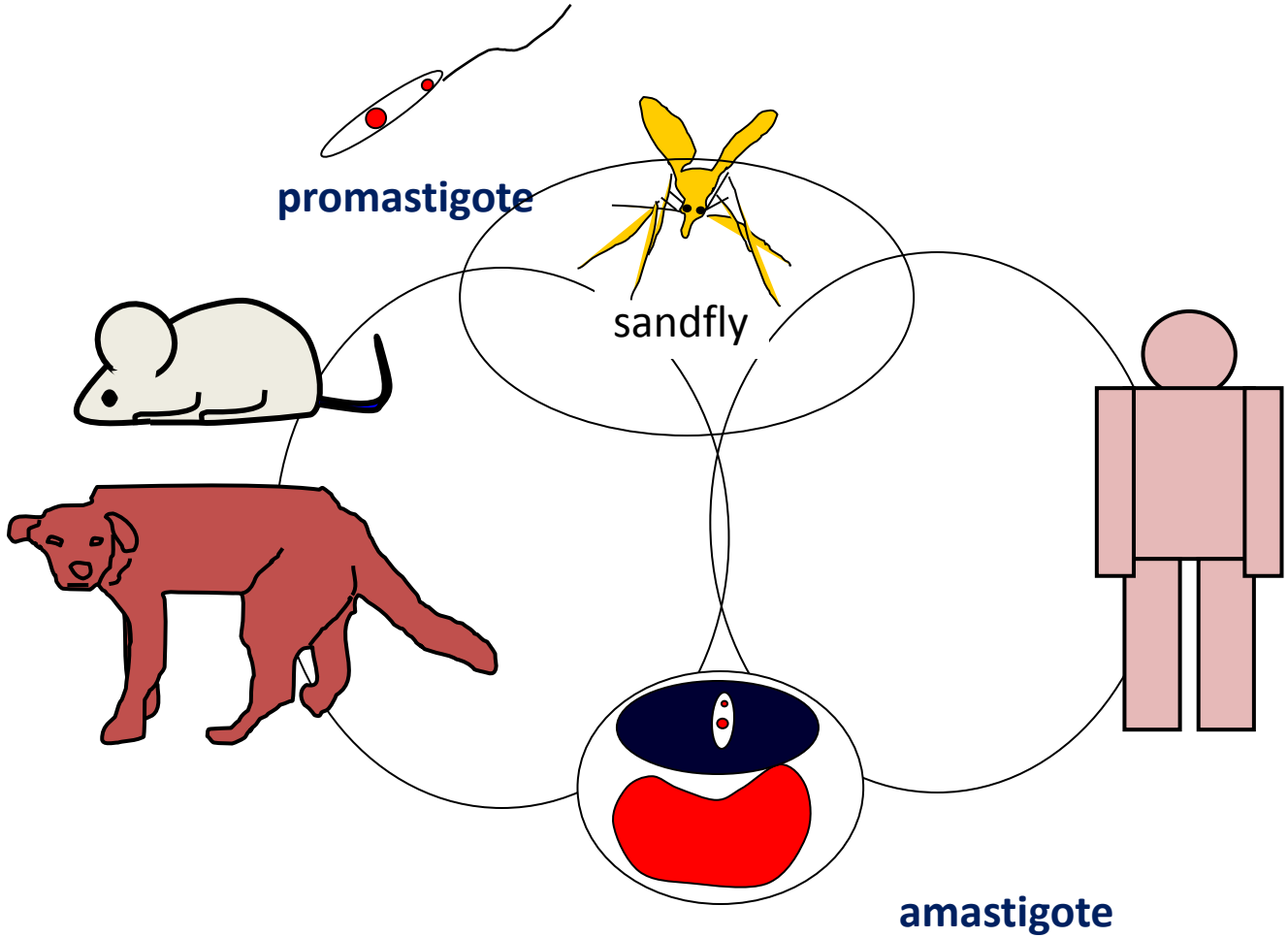
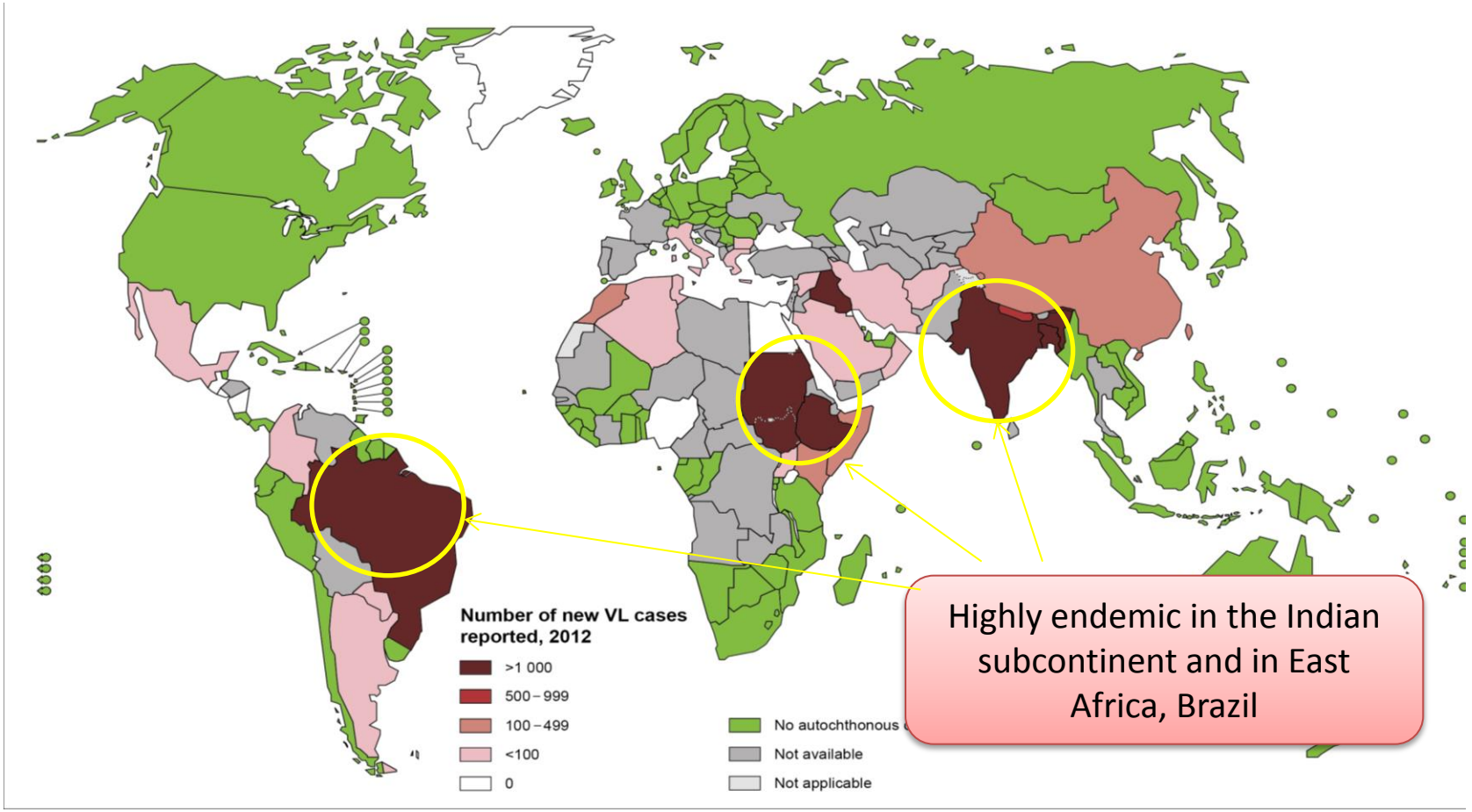


Fig. 2

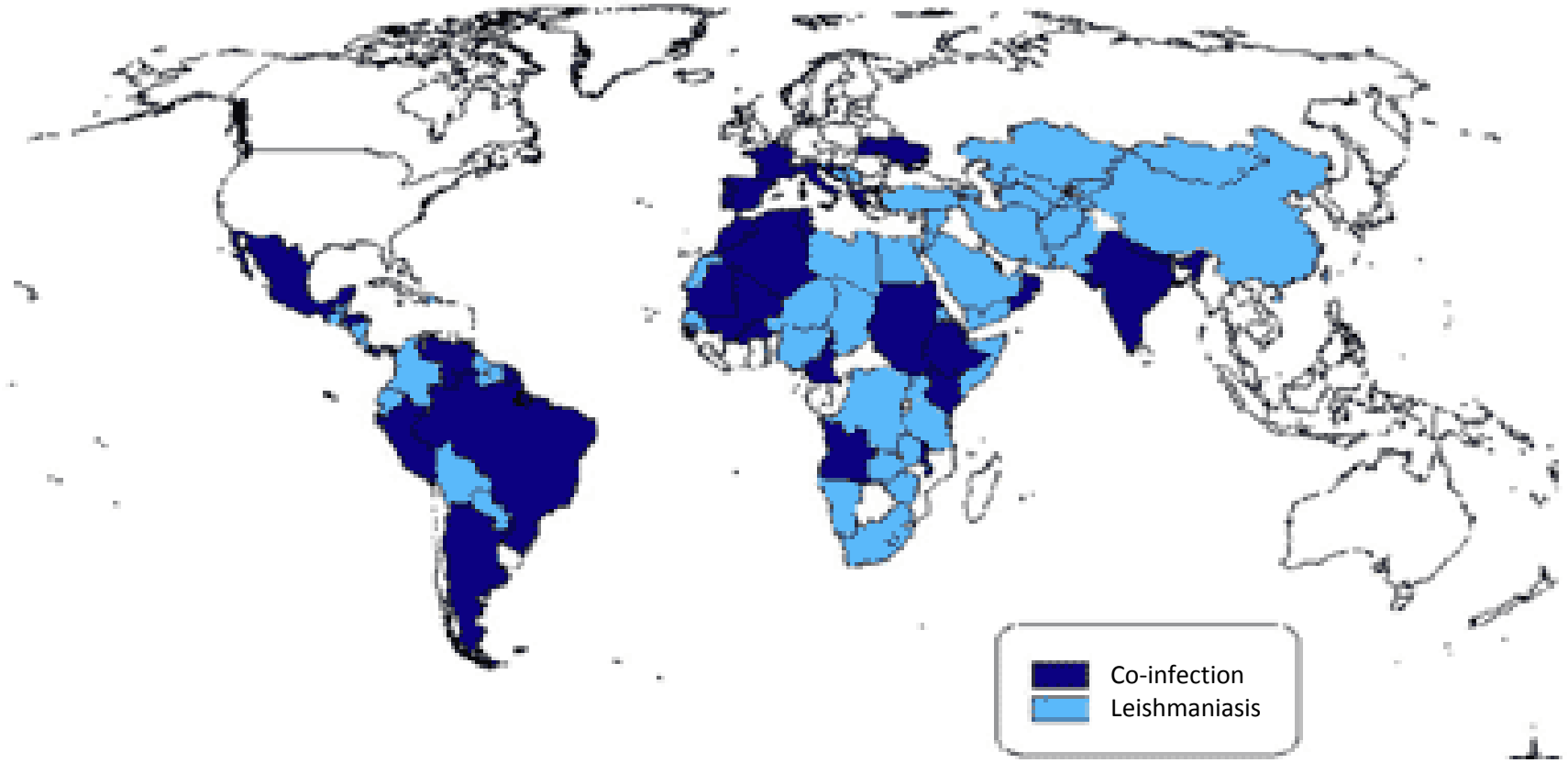


(Adopted from WHO report, 2012)

http://gamapserver.who.int/mapLibrary/Files/Maps/Leishmaniasis_VL_2013.png?ua=1

Number of patients (yearly incidence): Visceral leishmaniasis 200,000 - 400,000

Fig. 3



http://www.who.int/leishmaniasis/burden/hiv_coinfection/burden_hiv_coinfection/en/
Desjeux P.; Alvar J. 2003, Annals of Tropical Medicine & Parasitology, Vol. 97, Supplement No. 1, S3–S15

Fig. 4

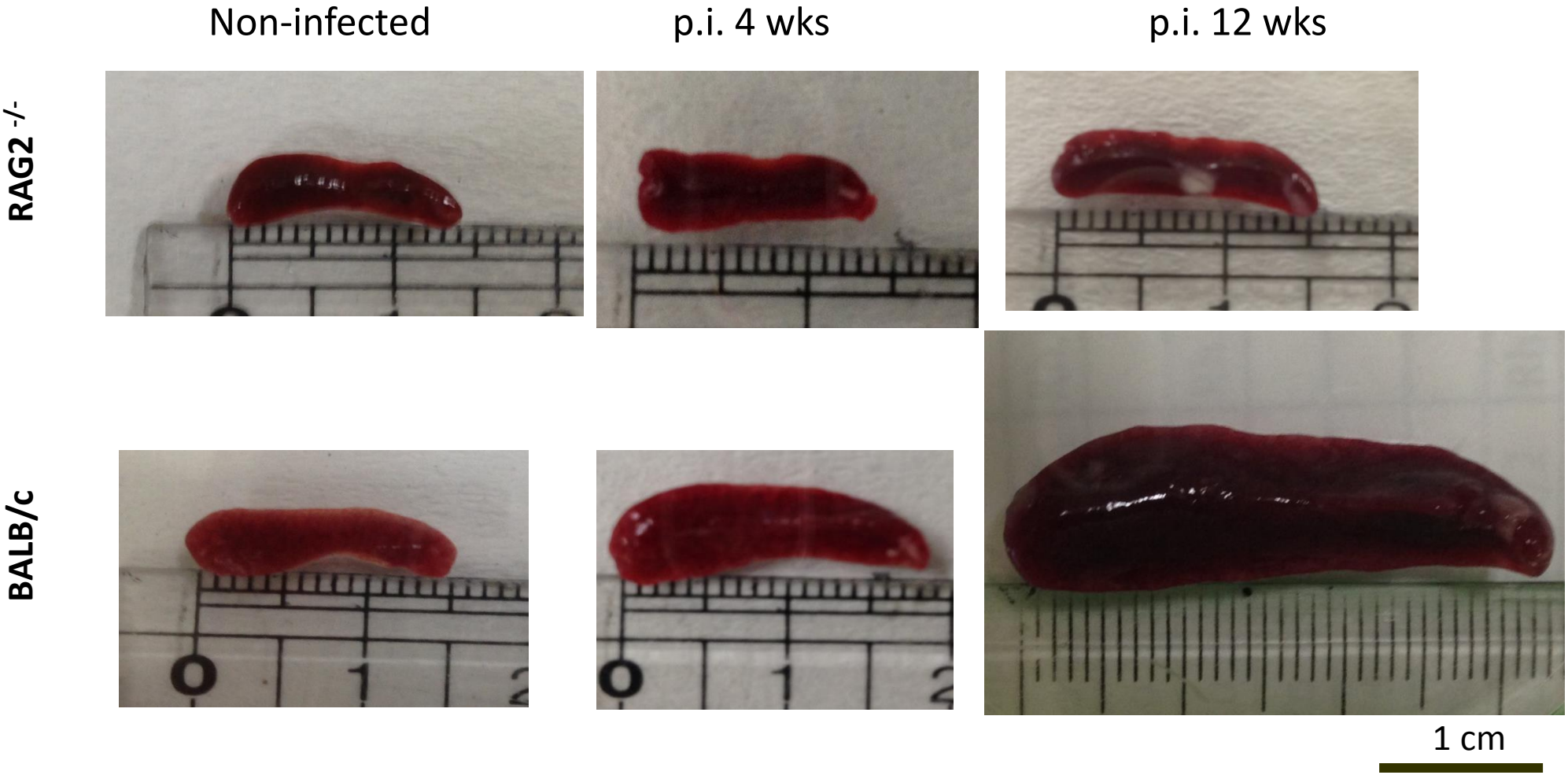


Fig. 5

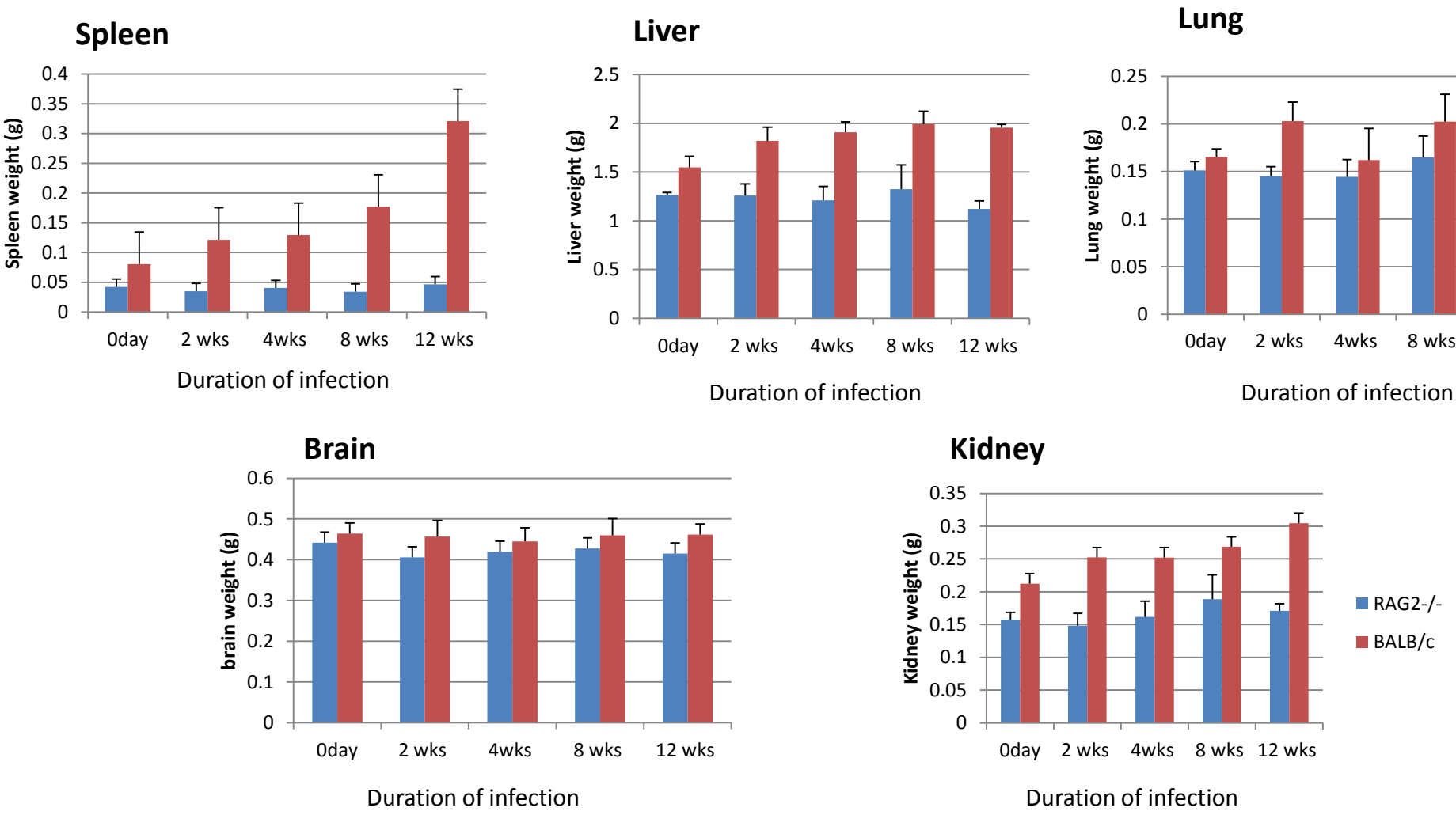

Spleen
Spleen weight (g)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0day
2 wks
4wks
8 wks
12 wks
Duration of infection
Liver
Liver weight (g)
2.5
2
1.5
1
0.5
0
0day
2 wks
4wks
8 wks
12 wks
Duration of infection
Lung
Lung weight (g)
0.25
0.2
0.15
0.1
0.05
0
0day
2 wks
4wks
8 wks
12 wks
Duration of infection
Brain
brain weight (g)
0.6
0.5
0.4
0.3
0.2
0.1
0
0day
2 wks
4wks
8 wks
12 wks
Duration of infection
Kidney
Kidney weight (g)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0day
2 wks
4wks
8 wks
12 wks
Duration of infection
RAG2-/-
BALB/c

Fig. 6

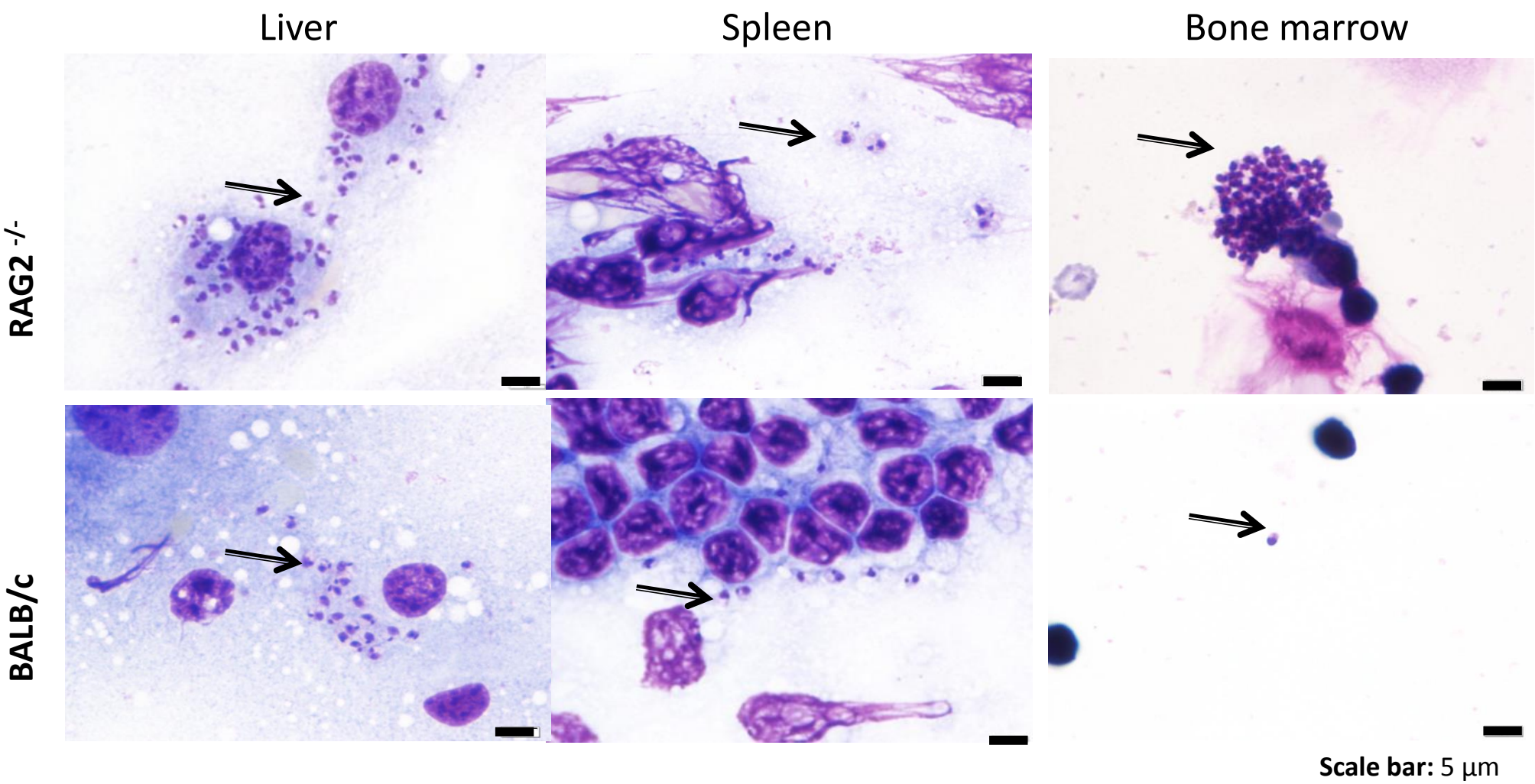
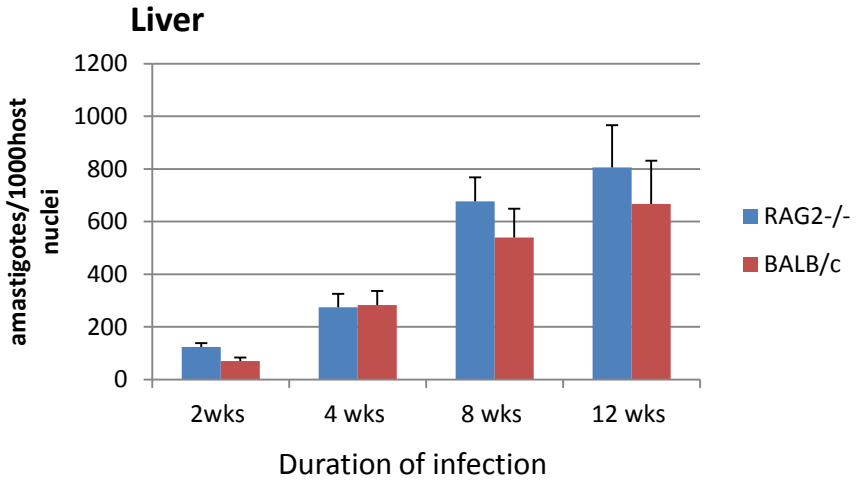
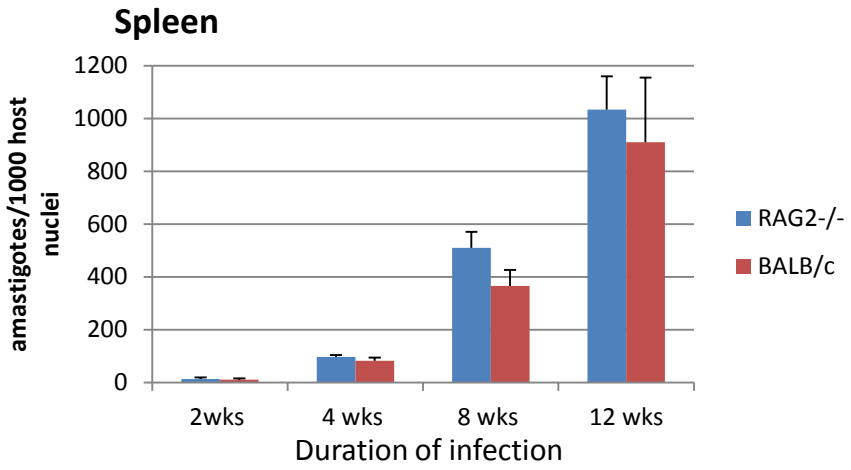


Fig. 7


Liver
amastigotes/1000host nuclei
1200
1000
800
600
400
200
0
2wks
4 wks
8 wks
12 wks
Duration of infection
RAG2-/-
BALB/c


Spleen
amastigotes/1000 host nuclei
1200
1000
800
600
400
200
0
2wks
4 wks
8 wks
12 wks
Duration of infection
RAG2-/-
BALB/c

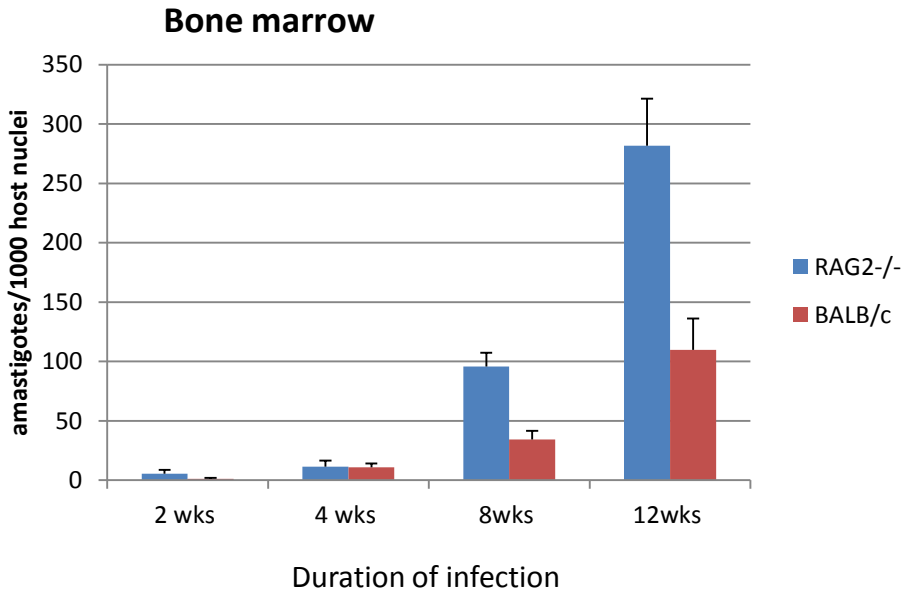
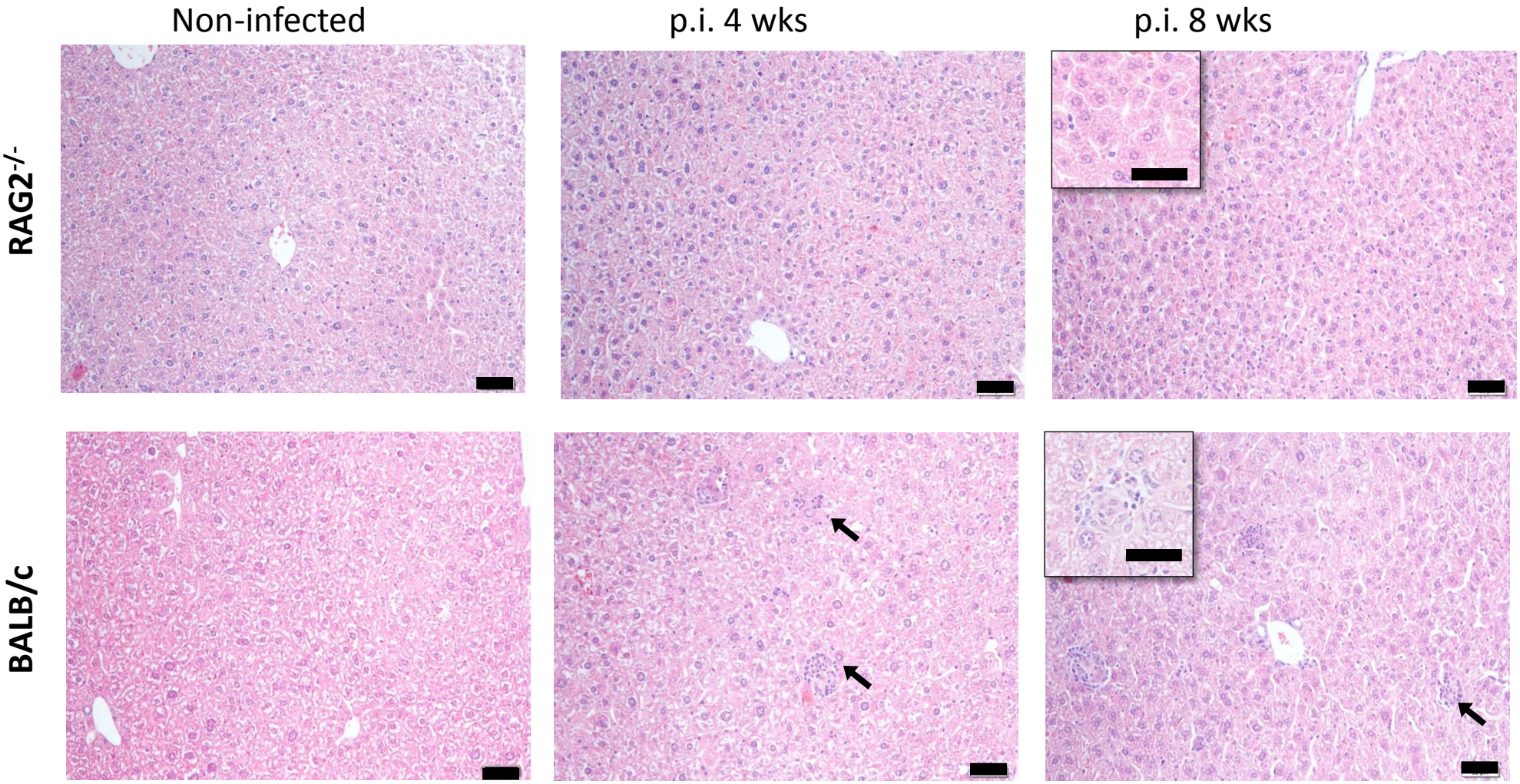
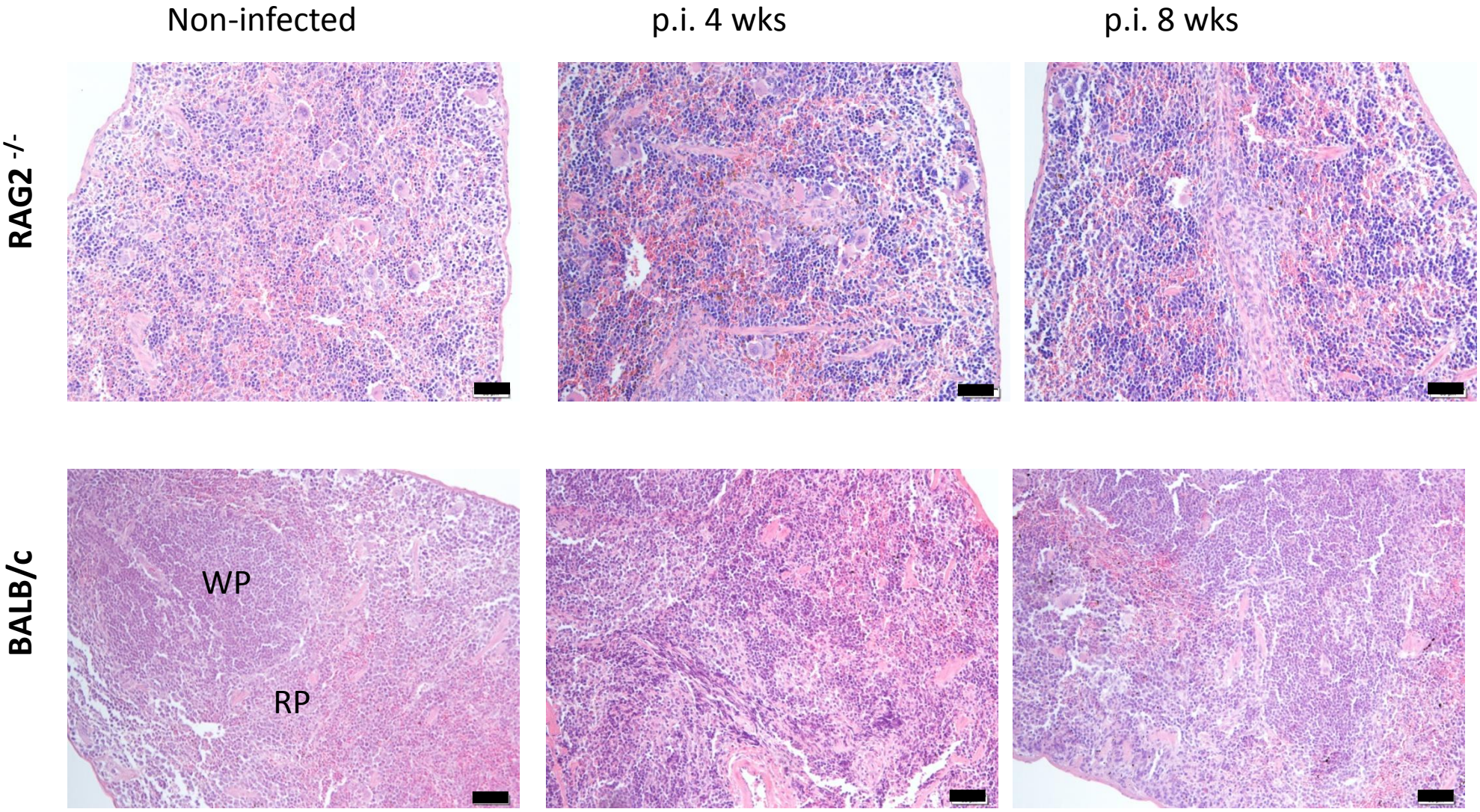

Bone marrow
amastigotes/1000 host nuclei
350
300
250
200
150
100
50
0
2 wks
4 wks
8wks
12wks
Duration of infection
RAG2-/-
BALB/c

Fig. 8



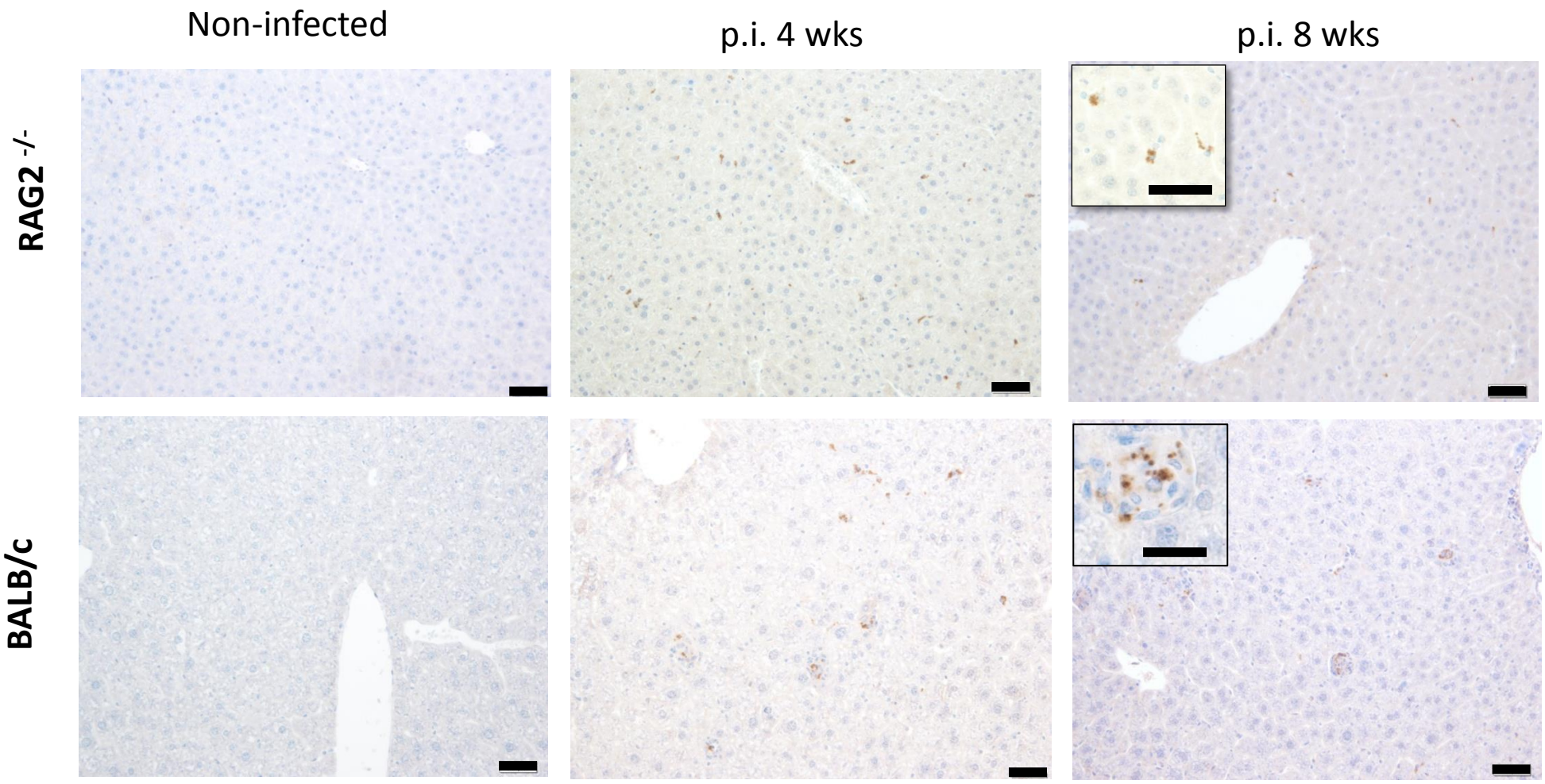
Hematoxylin and eosin, Scale bar: 50 µm (inset: 20 µm)

Fig. 9



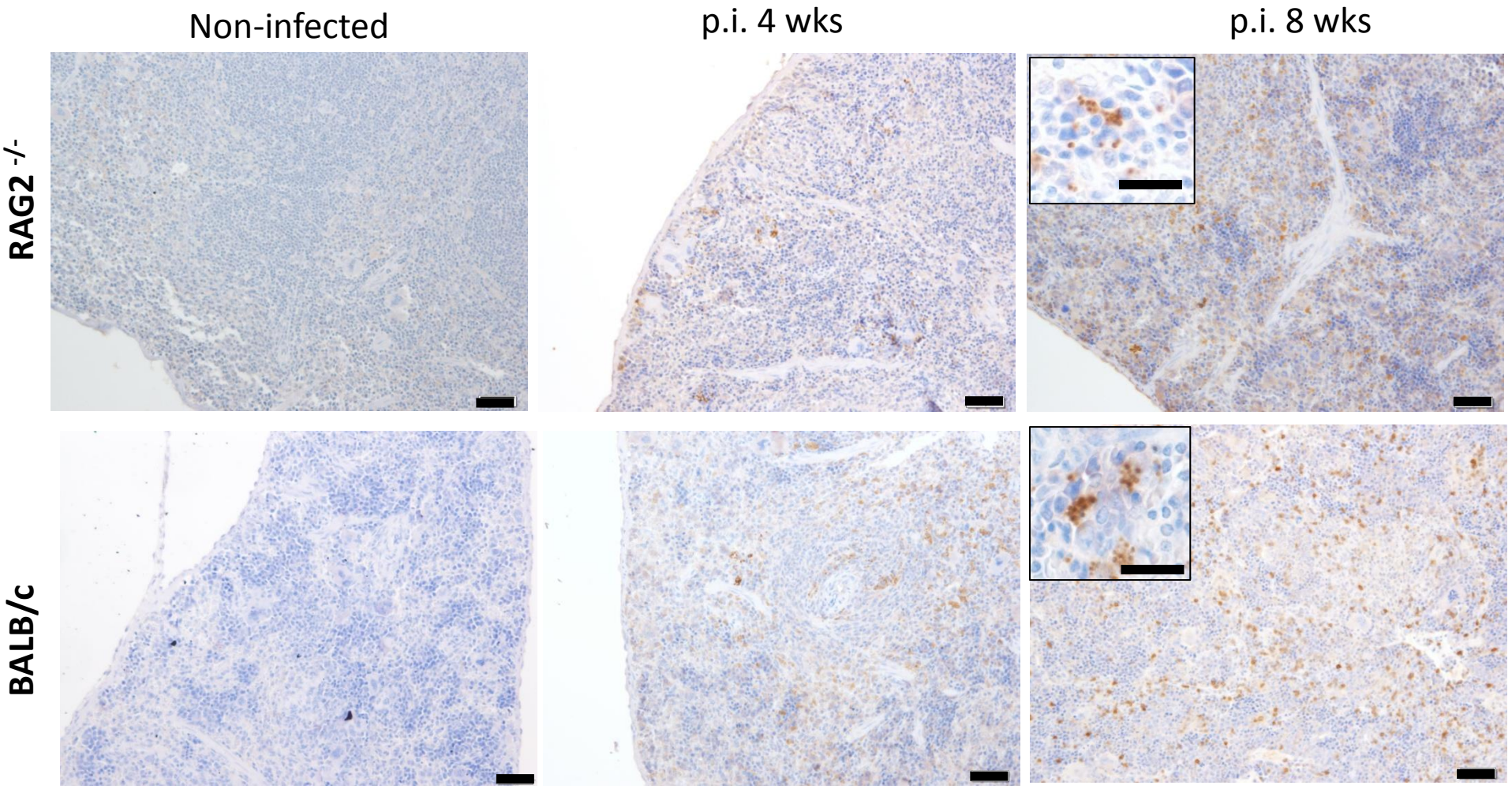
Hematoxylin and eosin, Scale bar: 50 µm

Fig. 10



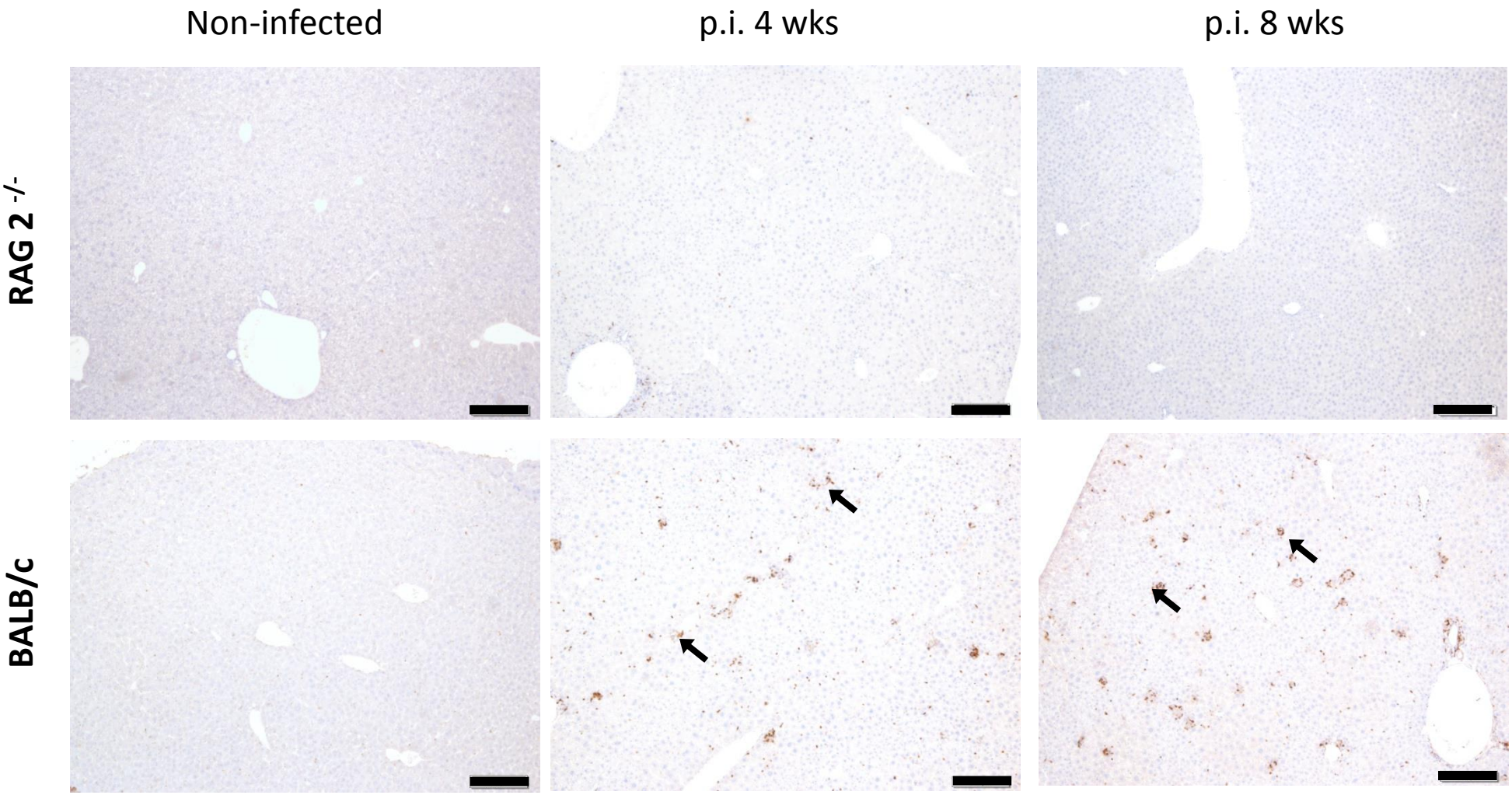
Antibody: C11C antibody (2.5 µg/ml); **Scale bar:** 50 µm (inset: 20 µm)

Fig. 11



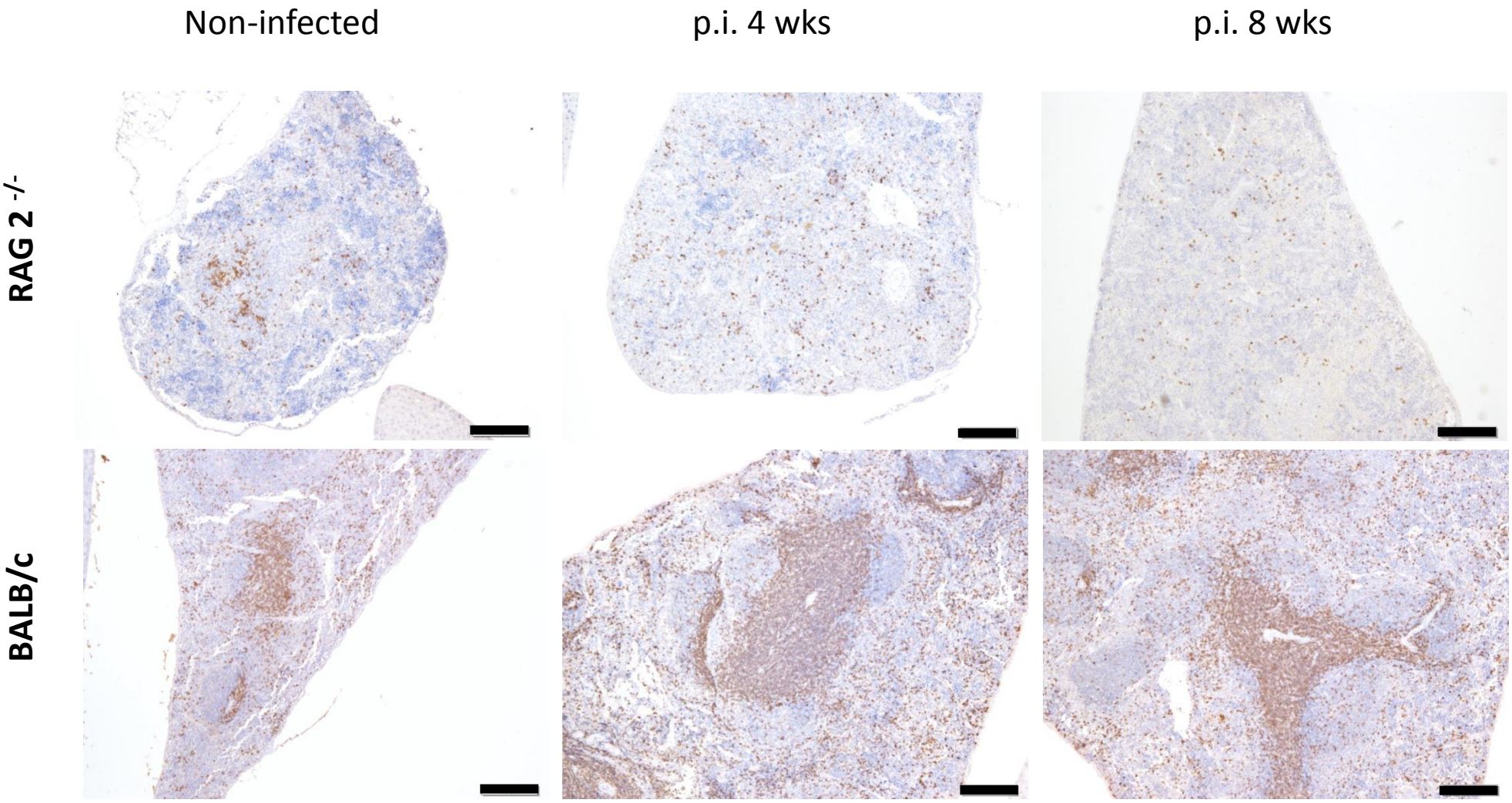
Antibody: C11C antibody (2.5 µg/ml); **Scale bar:** 50 µm (inset: 20 µm)

Fig. 12



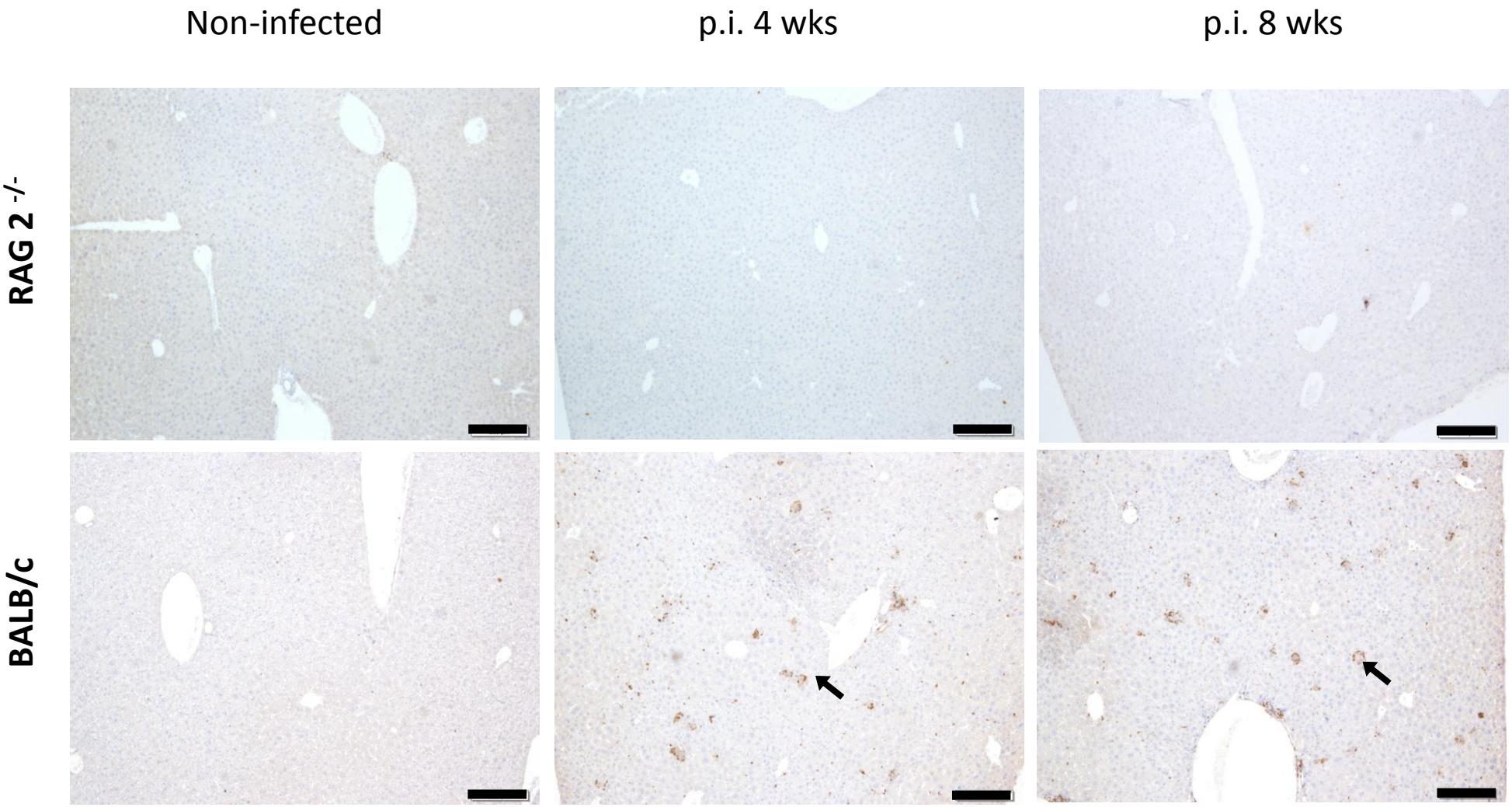
Antibody: α-CD3ε antibody; **Scale bar:** 200 μm

Fig. 13



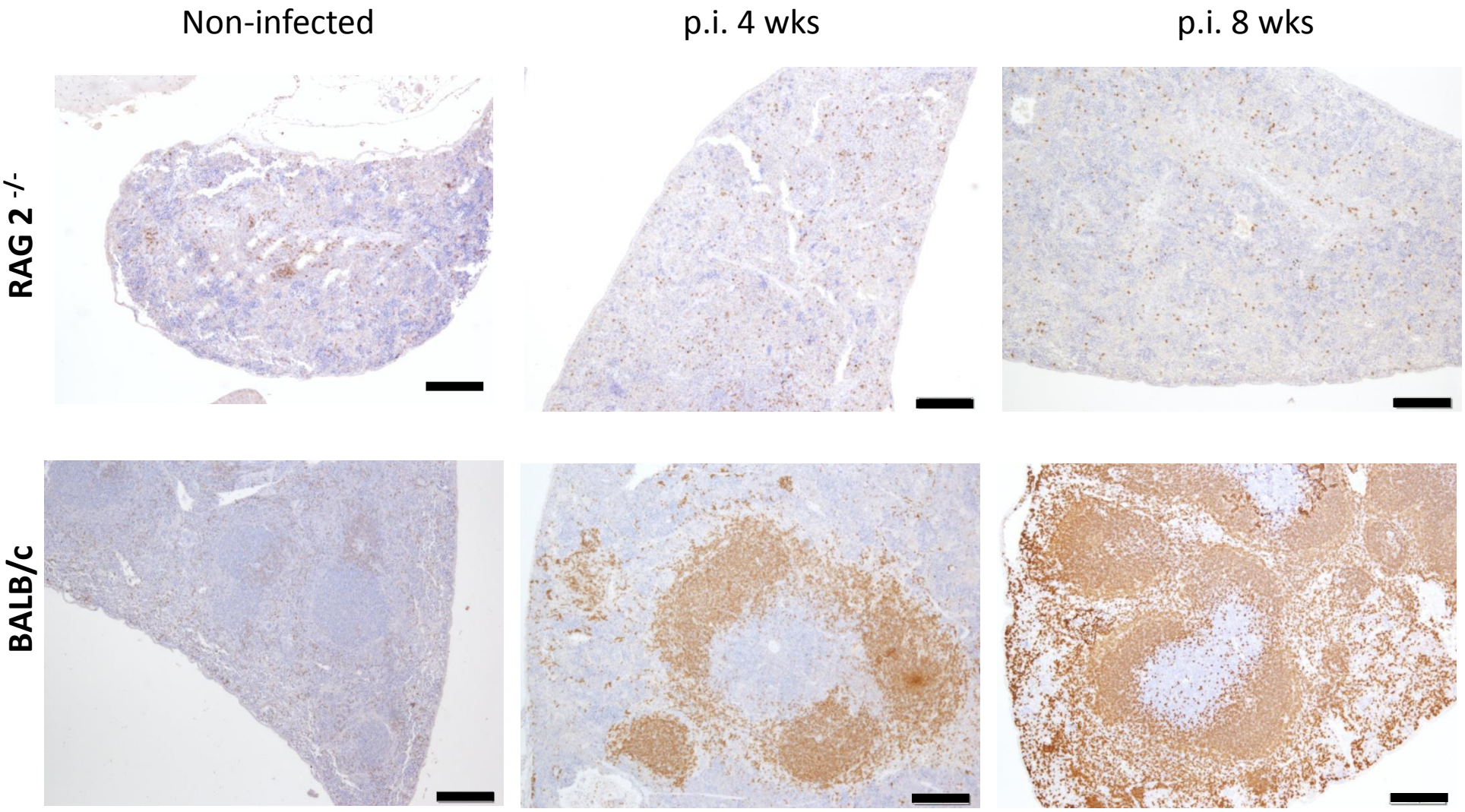
Antibody: α-CD3ε antibody; **Scale bar:** 200 μm

Fig. 14



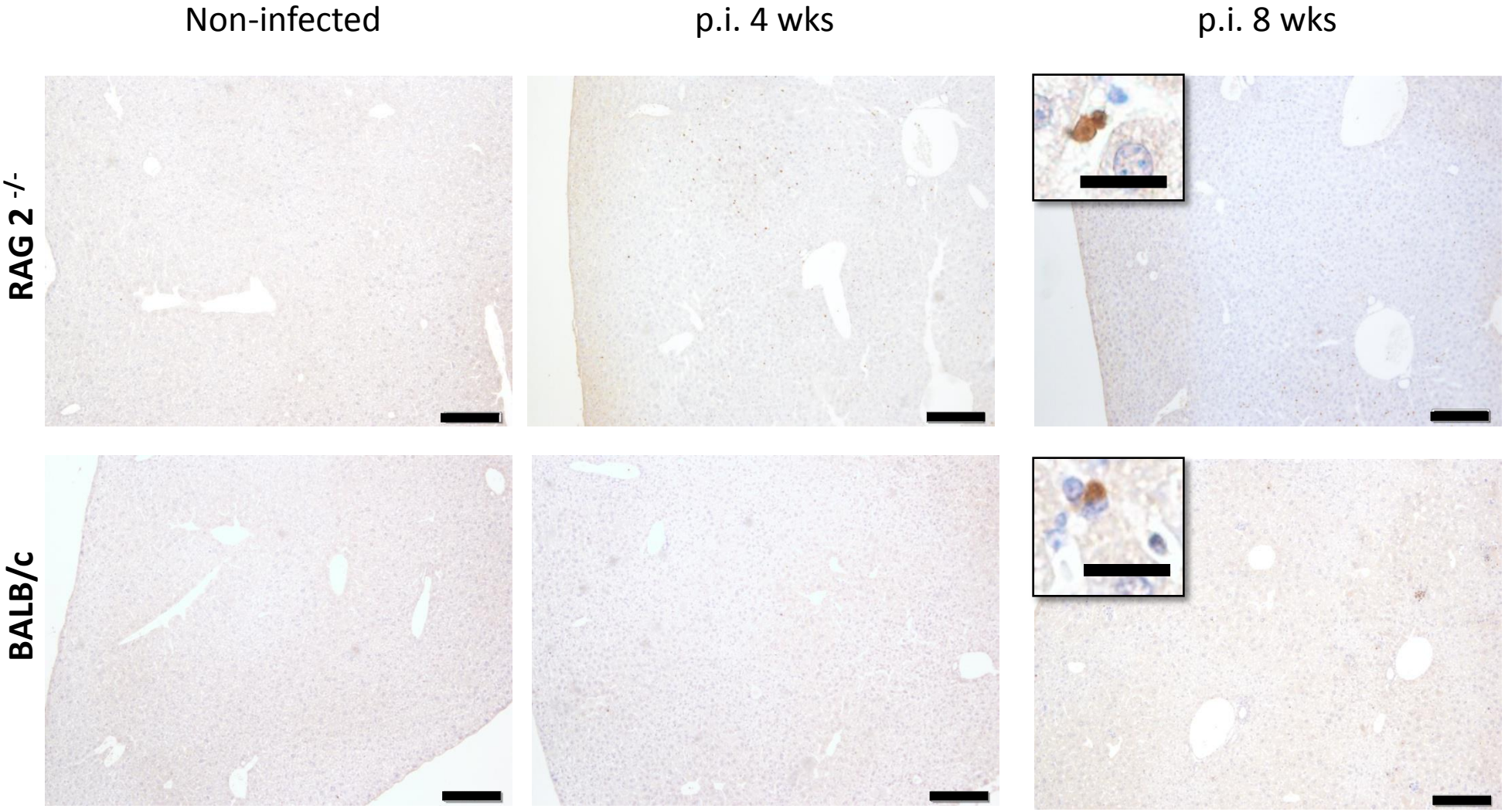
Antibody: α-B220 antibody; **Scale bar:** 200 μm

Fig. 15



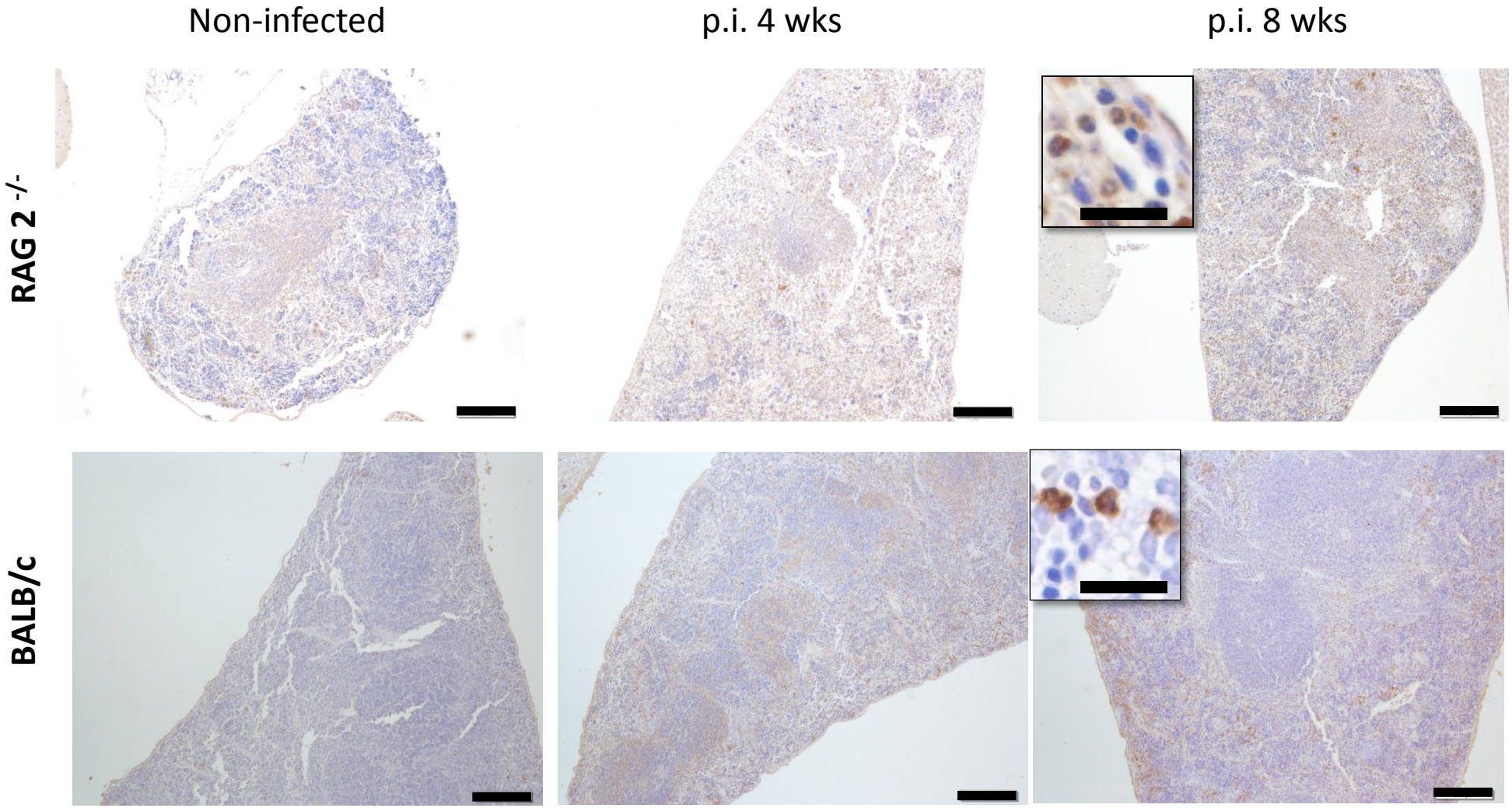
Antibody: α-B220 antibody; **Scale bar:** 200 µm

Fig. 16



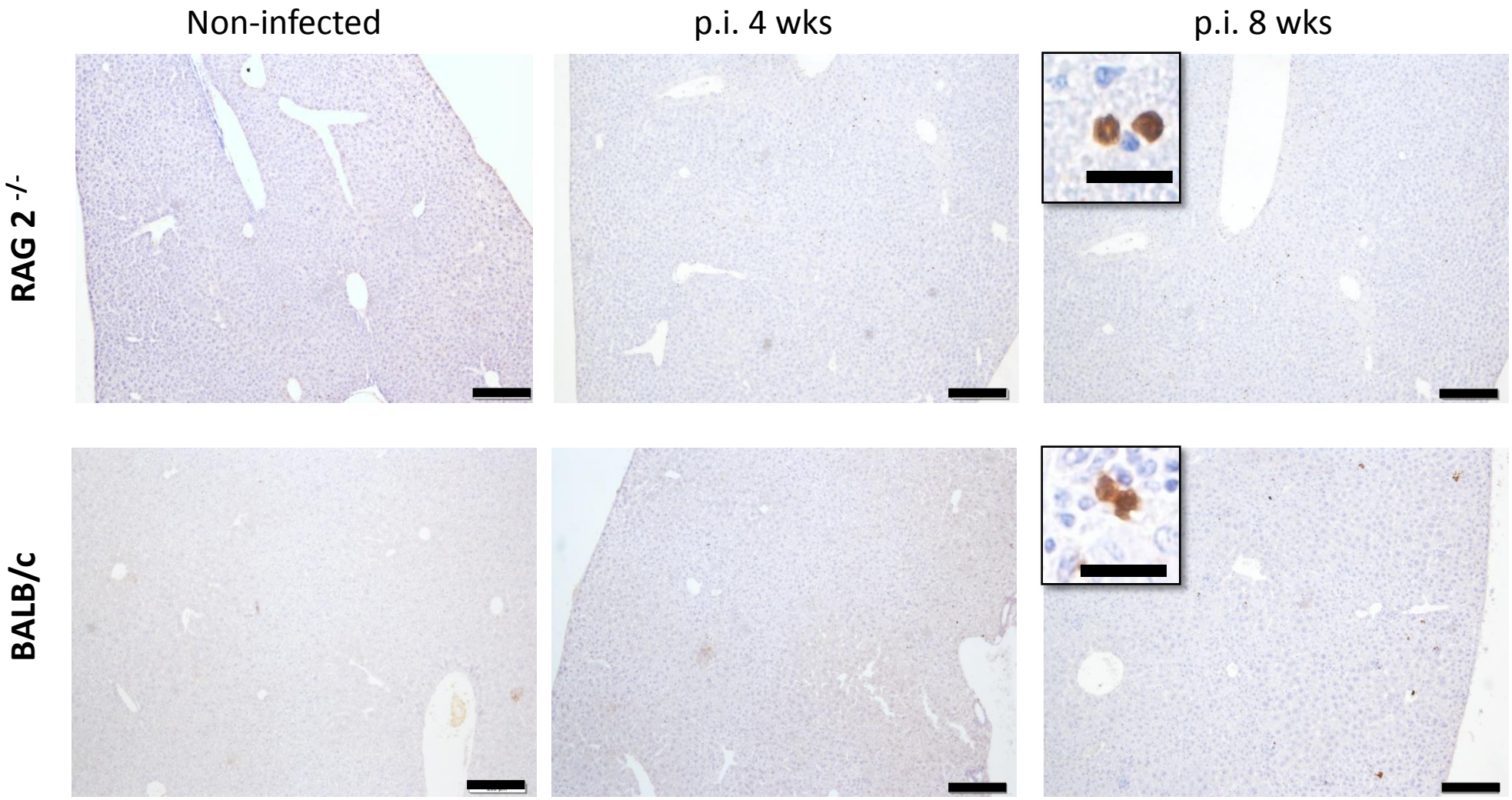
Antibody: α-MRP8 antibody; **Scale bar:** 200 µm (inset: 20 µm)

Fig. 17



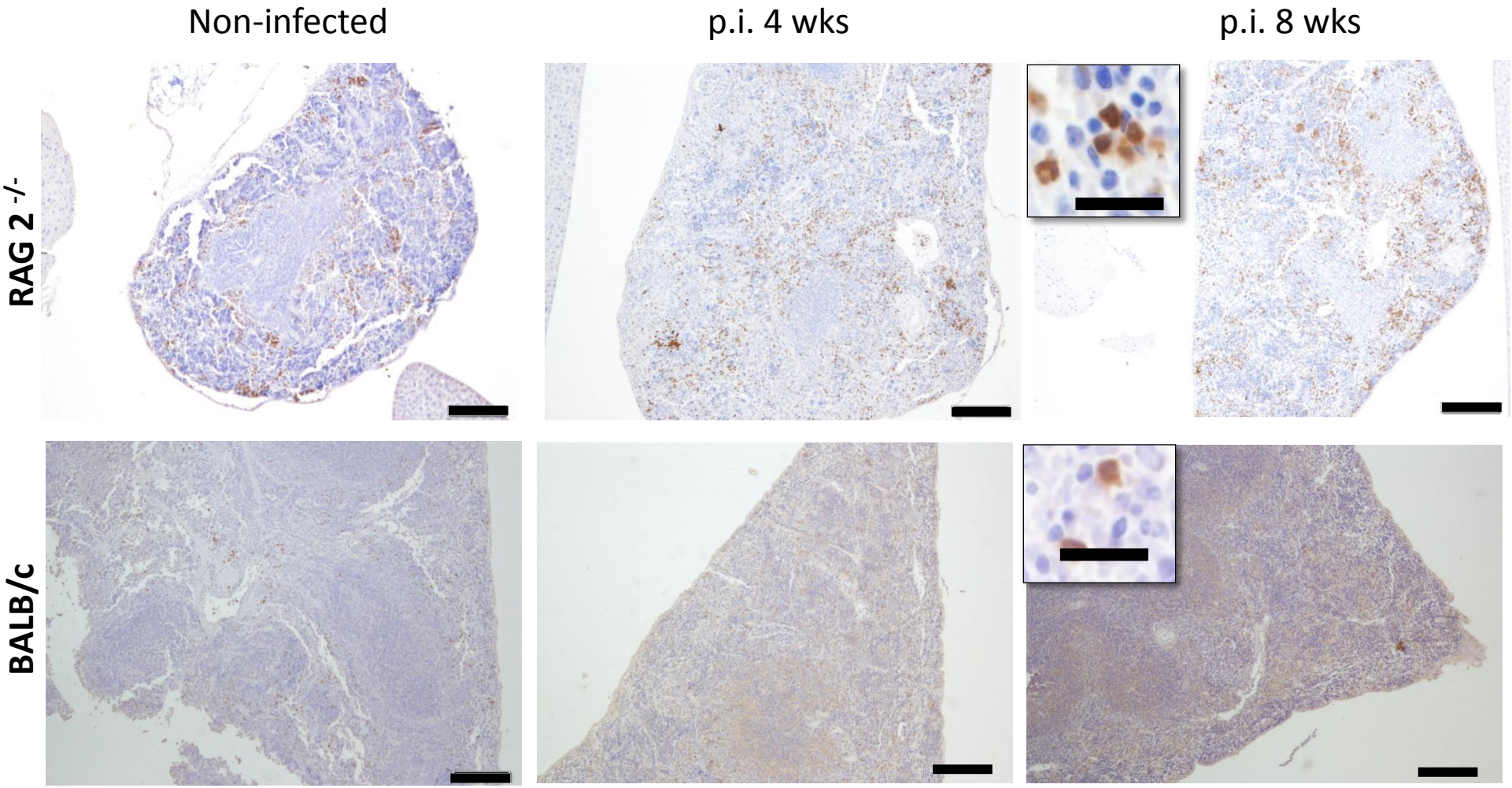
Antibody: α-MRP8 antibody; **Scale bar:** 200 µm (inset: 20 µm)

Fig. 18



Antibody: α-MRP14 antibody; **Scale bar:** 200 µm (inset: 20 µm)

Fig. 19



Antibody: α-MRP14 antibody; **Scale bar:** 200 µm (inset: 20 µm)

Fig. 20

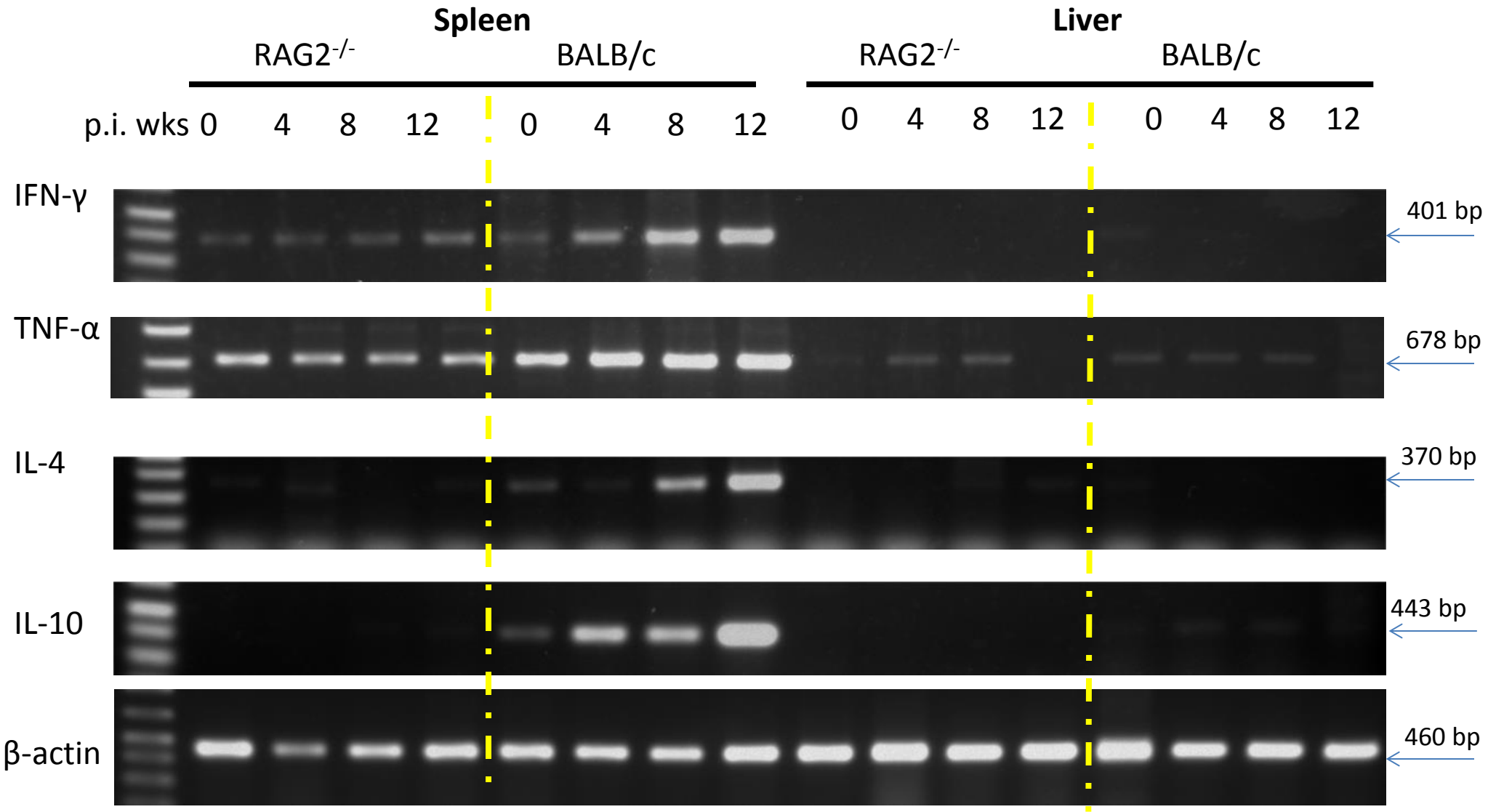

Spleen
Liver
RAG2-/-
BALB/c
RAG2-/-
BALB/c
p.i. wks 0 4 8 12 0 4 8 12 0 4 8 12 0 4 8 12
IFN-γ
401 bp
TNF-α
678 bp
IL-4
370 bp
IL-10
443 bp
β-actin
460 bp

Fig. 21