Title

Prime-boost immunization with DNA followed by a recombinant vaccinia virus expressing P50 induced protective immunity against *Babesia gibsoni* infection in dogs

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Abstract

A heterologous prime-boost immunization regime with priming DNA followed by recombinant vaccinia virus expressing relevant antigens has been shown to induce effective immune responses against several infectious pathogens. In this study, we constructed a recombinant plasmid and vaccinia virus, both of which expressed P50 of *Babesia gibsoni*, to investigate the immunogenicity and protective efficacy of a heterologous prime-boost immunization against canine babesiosis. The dogs immunized with the prime-boost regime developed a significantly high level of specific antibody against P50 when compared with the control groups, and the antibody level was strongly increased after a booster immunization with a recombinant vaccinia virus. The prime-boost immunization regime induced a specific IgG2 antibody response and IFN-γ production in dogs. Two weeks after the booster immunization with a recombinant vaccinia virus expressing P50, the dogs were challenged with *B. gibsoni* patasites. The dogs immunized with the prime-boost regime showed partial protection, manifested as a significantly low level of parasitemia and a 2-day delay of the peak parasitemia. These results indicated that such a heterologous prime-boost immunization approach might be useful against *B. gibsoni* infection in dogs.

1. Introduction

Babesia gibsoni is a tick-borne hemoprotozoan parasite that causes piroplasmosis in dogs. This parasite causes severe disease and sometimes death [1-3]. *B. gibsoni* infection is endemic in many regions of Asia, Africa, Europe, and the Americas [4, 5]. The disease is frequently present in dogs and has recently become a serious clinical problem [6-9]. For the control and alleviation of *B. gibsoni* infection in dogs, vaccination is generally considered to be the most effective means. Until recently, most vaccine development efforts against protozoan diseases aimed at stimulating the humoral immune response, for example, the production of protective antibodies. However, for intracellular protozoan infections such as malaria or babesiosis, it is known that the induction of immune responses at both the humoral and cellular levels is required for protection against these pathogens [10, 11]. Therefore, a vaccine may be needed to induce both types of immune responses and provide optimal protection.

Previous studies have shown the use of an intramuscular injection of plasmid DNA inducing both antibodies and cell-mediated immune responses in a number of animal models. The use of DNA vaccines has been implemented to elicit durable responses against intracellular pathogens, such as *Plasmodium* or *Toxoplasma* [12, 13]. However, a DNA vaccine used alone could induce only modest cell-mediated immune responses, including antigen-specific killer T-cell responses in animals. To improve the DNA vaccines, various strategies have been considered, such as cytokine augmentation and ballistic epidermal delivery; however, the cell-mediated immune response induced with a DNA vaccine alone remained modest [14, 15]. In other cases, recombinant vaccinia viruses expressing antigens derived from pathogens have often been used to induce both antibodies and cell-mediated immune responses [16-18]. However, in a mouse malaria model, immunization with a recombinant vaccinia virus alone failed to induce a protective immune response [19, 20].

Recently, it has been demonstrated that a prime-boost immunization

regime with a DNA plasmid and a recombinant vaccinia virus, both of which expressed the same antigen of pathogens, could induce a strong immune response, including cell-mediated immunity [21-23]. Using a mouse malaria model, it was shown that immunization with priming DNA followed by a recombinant vaccinia virus, both of which expressed pre-erythrocytic antigens, induced complete protection against sporozoite challenge [24]. Therefore, it was considered that such a heterologus prime-boost immunization regime might provide the basis for preventative and therapeutic vaccination against protozoan diseases of dogs. However, in dogs, the number of reports of the use of a prime-boost regime is quite limited [25].

In our previous studies, we identified a type-I transmembrane protein P50 expressed on the surface of *B. gibsoni* merozoites [26, 27] and demonstrated that the P50 was recognized as an immunodominant antigen by the host immune system in dogs infected with *B. gibsoni* [26]. Furthermore, we showed that the antiserum against recombinant P50 produced in a rabbit significantly inhibited the parasite growth on *B. gibsoni*-infected canine red blood cell-substituted severe combined immunodeficiency (Ca-RBC-SCD) mice. These results indicated that P50 is a useful vaccine candidate for the control of canine *B. gibsoni* infection. In this study, we constructed the recombinant plasmid and vaccinia virus expressing P50 to investigate the immunogenicity and protective efficacy of a heterologus prime-boost immunization regime against *B. gibsoni* infection in dogs.

2. Materials and methods

2.1. Parasites

The NRCPD strain of *B. gibsoni* parasites was used [28, 29]. The *B. gibsoni*-infected red blood cells (RBCs) used for challenges were collected from a dog experimentally infected with *B. gibsoni*.

2.2. Construction of vaccine plasmid

The entire P50 gene in pBluescript SK(+) vectors [26] was recovered after digestion with *Eco* RI and *Xho* I, blunt-ended with a Klenow fragment of DNA polymerase, and then inserted into the blunt-ended *Eco* RI restriction enzyme site under the control of the CAG promoter of the mammalian expression vector pCAGGS [30, 31], designated as pCAGGS-P50. pCAGGS-P50 was amplified in DH5α *Escherichia coli*, and the purification was performed using the QIAGEN Plasmid Mega Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The expression of P50 was analyzed using rabbit kidney (RK13) cells *in vitro* prior to an immunization trial of the dogs. The pCAGGS-P50 plasmid or control empty plasmid pCAGGS was transfected to RK13 cells using a lipofectine reagent (Gibco BRL, Rockville, MD) by the standard method [32]. The expression of P50 was analyzed by the indirect immunofluorescence antibody test (IFAT) or Western blotting using anti-P50 monoclonal antibody (MAb) 31D [27] as a primary antibody 2 days after transfection. IFAT and Western blotting were performed as described in our previous paper [26].

2.3. Construction of the recombinant vaccinia virus expressing P50

The entire P50 gene in pBluescript SK(+) vectors [26] was recovered after digestion with *Eco* RI and *Xho* I, blunt-ended with a Klenow fragment of DNA polymerase, and then ligated into the *Sal* I site of the vaccinia virus transfer vector pAK8 [33]. The structure of the recombinant plasmid pAK8-P50 was checked by restriction enzyme analysis. Rabbit kidney (RK13) cells infected with the vaccinia virus LC16mO (mO) strain [33] were transfected with the pAK8-P50 using a lipofectine reagent (Gibco BRL). Thymidine kinase-negative (TK-) viruses were isolated by a plaque assay on 143TK- cells in the presence of 5-bromo-2'-deoxyuridine

at a concentration of 100 μg/ml [33]. The recombinant vaccinia virus expressing P50 (vvP50) was propagated in RK13 cells in Eagle's minimum essential medium (Sigma, Tokyo, Japan) supplemented with 7.5% fetal bovine serum (FBS). To analyze the expression of P50 *in vitro*, RK13 cells were inoculated with 5 plaque-forming units (PFU) of vvP50 or mO per cell. Two days after inoculation, the cells were harvested and then subjected to IFAT or Western blotting as described above.

2.4. Dogs and immunization

Purebred female specific-pathogen-free Beagle dogs (14-15 months old) purchased from the Chugai Medical Animal Institute (Nagano, Japan) were used. Nine dogs were randomly divided into three groups (n-=3). One group was immunized with pCAGGS-P50 and vvP50. The negative control group was immunized with the empty plasmid pCAGGS and the parent vaccinia virus mO strain. The remaining group was used as the no-immunization control. Dogs were injected intramuscularly (i.m.) in the quadriceps muscle with the plasmid DNA. A 1-ml insulin syringe with a 21G-inch needle was used for all injections, and each single dose consisted of 200 μg dissolved in 1 ml of PBS containing 25% (w/v) sucrose. The dogs were immunized with plasmid DNA three times at two-week intervals. Two weeks after the last plasmid DNA immunization, the dogs were boosted intravenously (i.v.) with an injection of either 5 x 10^8 PFU of the recombinant vaccinia virus vvP50 or mO.

2.5. Reactivity analysis against B. gibsoni merozoites

Two weeks after the booster immunization with the recombinant vaccinia virus, serum samples were collected from dogs. The reactivity of the sera against *B. gibsoni* parasites was analyzed by Western blotting using merozoites purified from infected dog RBCs. Purification of the merozoites, sample preparation, sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE), and Western blotting were performed as previously described [27].

2.6. Challenge infection

Two weeks after the booster immunization, dogs were i.v. infected with 2 x 10^8 of *B. gibsoni*-infected RBCs collected from a dog experimentally infected with the *B. gibsoni* parasite (NRCPD strain). Parasitemia in peripheral blood and the packed cell volume (PCV) were monitored at one-day intervals.

2.7. Determination of the IgG subclass by the enzyme-linked immunosorbent assay (ELISA)

The increase in the antibody responses against the P50 protein in dogs was measured using the ELISA with glutathion *S*-transferase (GST)-P50 as an antigen. The ELISA was performed as described in our previous paper [34]. All serum samples were used in 1:200 dilutions for ELISA. The antigen-specific isotype profile was determined using horseradish peroxidase-conjugated anti-dog IgG, IgG1, and IgG2 antibodies (Bethyl Laboratories, Montgomery, TX) as the secondary antibodies. The IgG subclass against P50 of the sequential sera collected from a dog infected with *B. gibsoni* was also analyzed.

2.8. Determination of the peripheral IFN-γ response

The serum samples collected at 0 and 8 days post-infection were used for the assay. The samples were kept at -80 ˚C until use. IFN-γ was measured using a capture ELISA (R&D Systems, Minneapolis, MN) for dogs following the manufacturer's instructions.

2.9. Statistical analyses

The parasitemia and antibody response in the dog group immunized with P50 and the immunized and non-immunized controls were statistically analyzed by the Student's *t*-test with Stat view.

All animal experiments described in this article were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

3. Results

3.1. Expression of P50 in vitro by a vaccine plasmid

To investigate whether the pCAGGS-P50 plasmid expresses P50, we transfected the plasmid to RK13 cells and analyzed it by IFAT and Western blotting. In the IFAT analysis, the anti-gene 10-P50 mouse serum specifically reacted to the RK13 cells transfected with the pCAGGS-P50 plasmid but not to the cells transfected with the control pCAGGS plasmid (Fig. 1). In Western blotting with the anti-gene 10-P50 mouse serum, a specific band with a molecular weight of 50 kDa was detected in the RK13 cells transfected with the pCAGGS-P50 plasmid (data not shown). The molecular weight of P50 expressed by pCAGGS-P50 plasmid was similar to that of the native P50 from *B. gibsoni*.

3.2. Expression of P50 in vitro by the recombinant vaccinia virus

RK13 cells were infected at 5 PFU/cell with a vvP50 or with a control vaccina virus mO. After incubating for 2 days, cells infected with vvP50 were analyzed by IFAT or Western blotting using MAb 31D. In the IFAT analysis, specific fluorescence was detected in the cells infected with vvP50 but not in the cells infected with the control mO (Fig. 2A). In

Western blotting, a specific band with a molecular weight of 50 kDa was detected (Fig. 2B). The molecular weight of the recombinant P50 expressed by vvP50 was similar to that of the native P50 from *B. gibsoni*.

3.3. IgG subclass against P50 of a dog experimentally infected with B. gibsoni

The IgG subclass against P50 of the sequential serum samples collected from experimentally *B. gibsoni*-infected dog was measured. As shown in Fig. 3, the IgG2 antibody response against P50 was detected as a major subclass in comparison with the IgG1 antibody response. A higher IgG2 subclass response was detected until the acute and chronic phases of infection.

3.4. Peripheral IgG and IFN-γ response in immunized dogs

To confirm the immunogenicity of the vaccine plasmid and virus, sera were collected 2 weeks after each immunization from dogs and examined by ELISA, IFAT, or Western blotting. Figure 4 shows the antibody responses determined by ELISA with GST-P50 protein as the antigen. As shown in Fig. 4a, the IgG response against the P50 protein moderately increased after each immunization with the vaccine plasmid pCAGGS-P50. Two weeks after the booster immunization with the recombinant vaccinia virus vvP50, the mean optical density (OD) strongly increased. Neither the vaccine-immunized control group nor the non-immunized control group developed a specific antibody response against P50. The isotype of the antibody was also analyzed. As shown in Fig. 4b and c, the IgG2 responses were determined as a major subset when compared with those of IgG1. To determine the antigenicity of the vaccine, the sera obtained 2 weeks after the booster immunization with vvP50 were examined for IFAT or Western blotting using *B. gibsoni* merozoites as the antigen. In IFAT analysis, all sera derived from dogs

immunized with the vaccine plasmid and virus strongly reacted to the *B. gibsoni* merozoites, but sera derived from the control dogs did not (data not shown). As shown in Fig. 5, the sera from dogs immunized with pCAGGS-P50 and vvP50 specifically recognized the 50-kDa band of the native P50 protein of *B. gibsoni* merozoites. The peripheral IFN-γ response of dogs after the challenge infection was analyzed by ELISA. As shown in Table 1, at 0 days post-infection, all dog groups showed an undetectable level of IFN-γ. At 8 days post-infection, the dog group immunized with pCAGGS-P50 and vvP50 showed a significantly higher level of the IFN-γ response but not detected in control dogs immunized with pCAGGS and mO or non-immunized.

3.5. Protective efficacy against challenge infection

Two weeks after the booster immunization with the recombinant vaccinia virus vvP50, the dogs were challenged by an intravenous injection of the NRCPD strain of *B. gibsoni*-infected RBCs collected from an experimentally infected dog on day 0. Parasitemia in peripheral blood was monitored every 1 to 2 days. Parasite growth was significantly inhibited ($P<0.05$, on day 20) in dogs immunized with P50 in comparison with that in control immunized and mO or not immunized (Fig. 7). There was no significant difference between the two control groups ($P>0.2$). At the peak of parasitemia, the ratio of the inhibitory effect was 48.5% compared to that of the immunization control group and 49.2% compared to that of the non-immunized group. The PCV of venous blood was measured to determine the anemia level. There was no significant difference between the group immunized with P50 and the immunized and non-immunized control groups.

4. Discussion

For protection against intracellular pathogens, including protozoan

parasites, increasing the cell-mediated immune response has been considered important. Traditionally, vaccine formulations were designated to mainly induce antibodies; however, recent efforts have aimed at inducing strong cellular immune responses. In recent studies, it has been reported that a heterologous prime-boost immunization regime priming DNA followed by a recombinant vaccinia virus, both of which expressed the same antigen, appears to be particularly promising at inducing cellular and humoral immune responses [21-23]. These observations have been extended to protozoan parasite infection in animal models [25, 35-37]. Specifically, this immunization regime induced complete protection against *P. berghei*, the same intraerythrocytic parasite as *B. gibsoni*, infection in mice [24]. Previously, we identified the immunodominant protein P50 of *B. gibsoni* and demonstrated that the antiserum against P50 significantly inhibited parasite growth in Ca-RBC SCID mice infected with *B. gibsoni* [38]. These results indicated that P50 might be a useful vaccine candidate for the control of canine *B. gibsoni* infection. In this study, we determined the immunogenicity and protective efficacy of P50 using a heterologous immunization regime with priming DNA followed by a recombinant vaccinia virus in dogs. The dogs immunized with P50 developed a strong immune response and showed partial protection against experimental intravenous inoculation of *B. gibsoni*-infected RBCs.

For the vaccination trial against *B. gibsoni* infection, we generated the mammalian expression vector pCAGGS-P50 and the recombinant vaccinia virus vvP50. In Western blotting, it was confirmed that both vaccine components correctly expressed the 50-kDa band in RK13 cells. To examine the immunogenicity of these vectors, a group of dogs (n=3) was immunized three times with priming DNA and once with a boost of the recombinant vaccinia virus. A previous study showed that this immunization regime was more effective for the induction of a strong immune response against malaria infection than other vaccination regimes in humans [39]. After a booster immunization with vvP50, the dogs

developed a significantly higher level of IgG antibody against P50 than that before the booster immunization. These antibody responses were higher than those induced in control dog groups after a challenge infection with *B. gibsoni* parasites. These results indicated that the immunization regime used in this study was an effective method for dogs as well. The sera collected from dogs after the booster immunization showed strong reactivity against *B. gibsoni* merozoites in IFAT and specifically reacted to the native P50 in Western blotting. These results demonstrated that P50 expressed by those vectors in dogs is similar to the native P50 in terms of its molecular structure and antigenicity. We also determined the type of immune response elicited in dogs by measuring the ratio of IgG1 and IgG2 anti-P50 antibodies. The IgG subclasses were considered to be a representative marker for the Th1/Th2-type immune response [40-42]. The dog group immunized with P50 showed an increase in the IgG2 response over the IgG1 subclass. The response of the IgG subclass was similar to that of a dog infected with *B. gibsoni*. The higher IgG2 response was maintained at all infection periods after challenge. Moreover, the P50-immunized dog group showed a significantly higher level of IFN-γ after challenge infection with the parasite. These results might indicate an induction of a Th1-type T-cell-mediated immune response in dogs immunized with P50.

To confirm the protective efficacy of the immunization with priming pCAGGS-P50 followed by vvP50, the dogs were inoculated with *B. gibsoni*-infected RBCs derived from i.v. infected dogs. The dogs immunized with P50 showed partial protection, manifested as a significantly low level of parasitemia; complete protection, characterized as the absence of apparent parasitemia, was not observed. Furthermore, the dogs immunized with P50 showed a 2-day delay of the peak parasitemia and 49.5% inhibition on parasite growth when compared to the control groups. These results indicated that P50 might be a useful candidate for the development of a vaccine for the control of canine *B. gibsoni* infection.

Acknowledgements

We are grateful to J. Miyazaki of Osaka University for the pCAGGS plasmid. This work was supported by a grant from The 21st Century COE Program (A-1) and a Grant-in-Aid for Scientific Research (No. 13556049) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. S. Fukumoto was supported by a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists.

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Figure legends

Fig. 1. Expression of P50 in RK13 cells transfected with pCAGGS-P50. The expression of P50 was analyzed by IFAT using MAb 31D recognizing P50. P50, cells transfected with pCAGGS-P50; con, cells transfected with control plasmid pCAGGS.

Fig. 2. Expression of P50 in RK13 cells infected with vvP50. (A) Western blot analysis of P50 expressed in RK13 cells. The expression of P50 was detected using MAb 31D. Lane 1, lysates of recombinant vaccinia virus vvP50-infected cells; lane 2, control lysates of vaccinia virus mO-infected cells; lane 3, lysates of *B. gibsoni*-infected RBCs; lane 4, control lysates of healthy-dog RBCs were used as the antigen. (B) IFAT analysis of P50 expressed in RK13 cells. The cells were stained with MAb31D. P50, cells infected with vvP50; con, cells infected with the control parent virus mO.

Fig. 3. Determination of the IgG subclass against P50 of the sequential sera from a dog infected with *B. gibsoni*. (A) IgG subclass response against P50 of a dog infected with *B. gibsoni*. (B) Parasitemia and hematocrit value of the same dog.

Fig. 4. Determination of the antibody response against P50 of dogs immunized with P50. (a) Total IgG, (b) IgG1, and (c) IgG2. P50, sera collected from dogs immunized with pCAGGS-P50 and vvP50; immune-con, sera collected from dogs immunized with control antigen pCAGGS and mO; non-immune, sera collected from non-immunized control dogs. The asterisks show the significant difference ($P<0.05$) between the dog groups immunized with P50 and the control groups. The results are shown as the mean values, and the error bars represent the standard deviations.

Fig. 5. Western blot analysis of the reactivity of the sera collected from dogs immunized with P50. Lanes 1 and 2, MAb 31D; lanes 3 to 5, each

serum collected from dogs immunized with pCAGGS-P50 and vvP50; lanes 6 to 8, each serum collected from dogs immunized with control pCAGGS and mO; lanes 9 to 11, each serum from non-immunized control dogs. Lanes 1 and 3 to 11, lysates of *B. gibsoni*-infected RBCs; lane 2, control lysates of healthy-dog RBCs were used as the antigen.

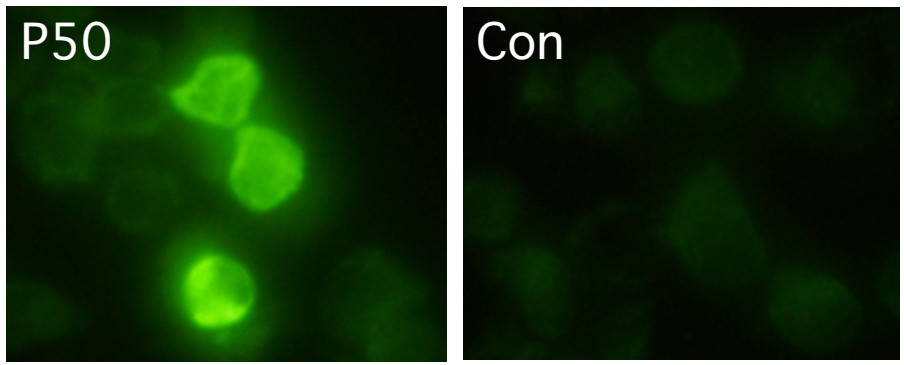
Fig. 6. Parasitemia of dogs after challenge infection with *B. gibsoni*-infected RBCs. P50, dogs immunized with pCAGGS-P50 and vvP50; immune-con, dogs immunized with control plasmid pCAGGS and mO; non-immune, non-immunized control dogs. The results are shown as the mean values, and the error bars represent the standard deviations. The asterisks show the significant difference ($P<0.05$) between the dog groups immunized with P50 and the control groups.

Table 1. Peripheral IFN-gamma response of the immunized dogs infected with *B. gibsoni*.

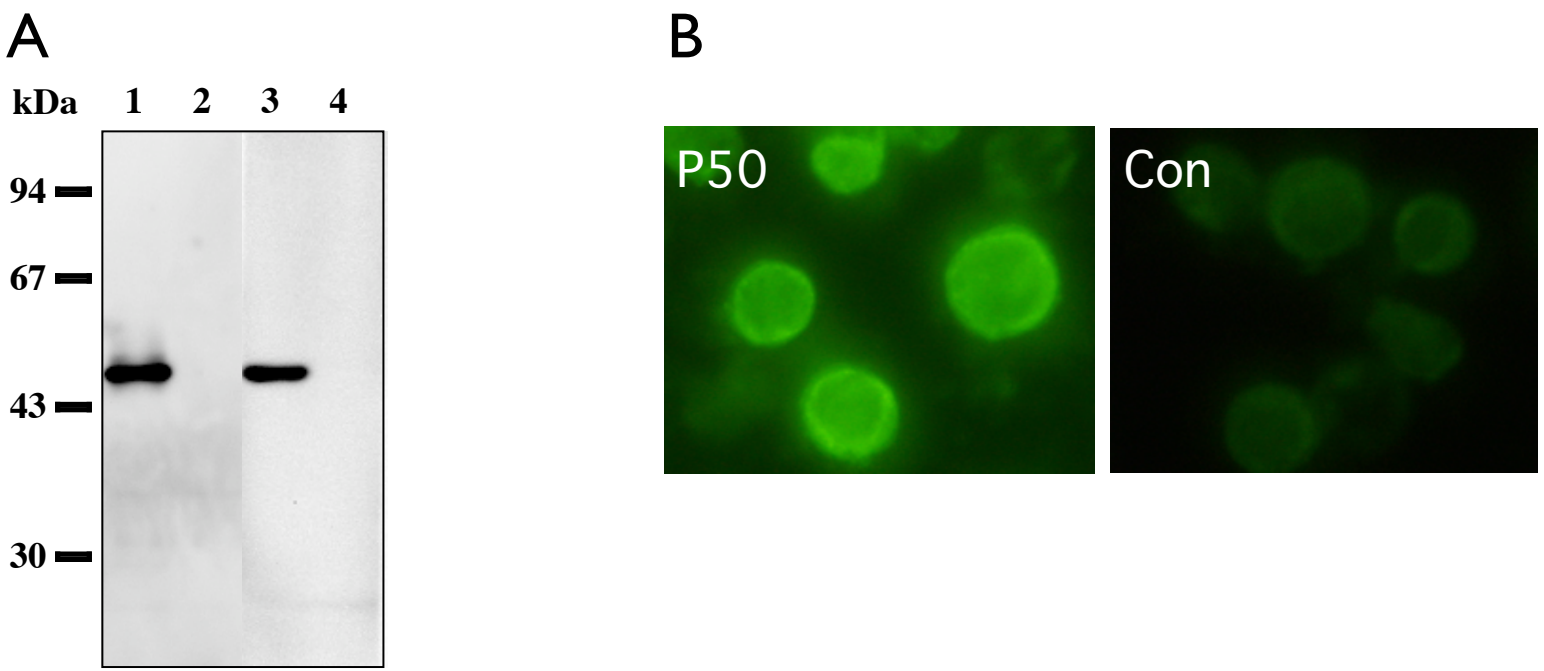
Dog group (n=3)	IFN-γ response (pg/ml)	
	day 0	day 8
P50	U.D.[a]	57.3±11.08[b]
Immune-con	U.D.	U.D.
No-immune	U.D.	U.D.

[a]U.D.: Under detectable level

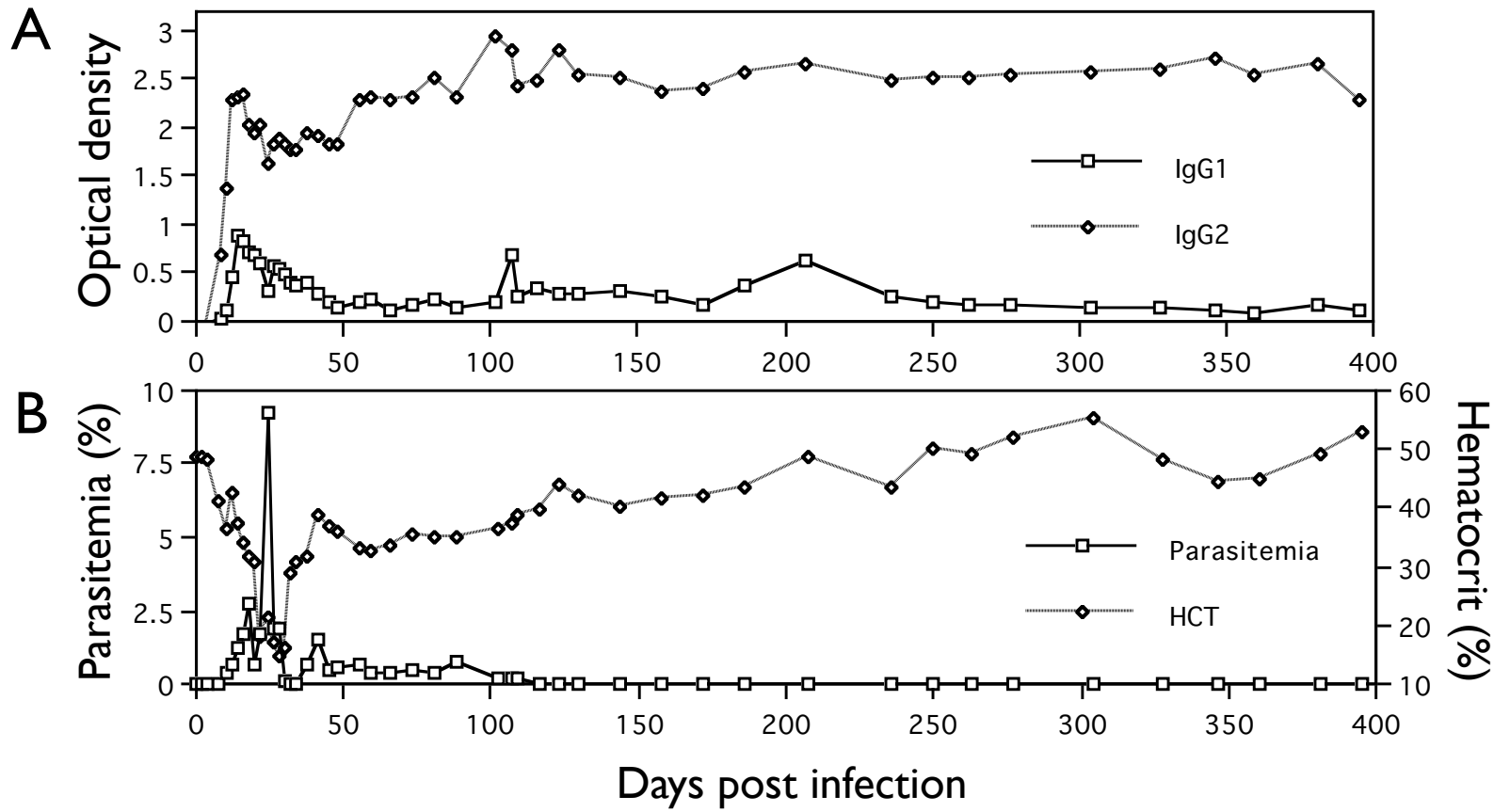
[b]Result was shown in mean ± standard deviation



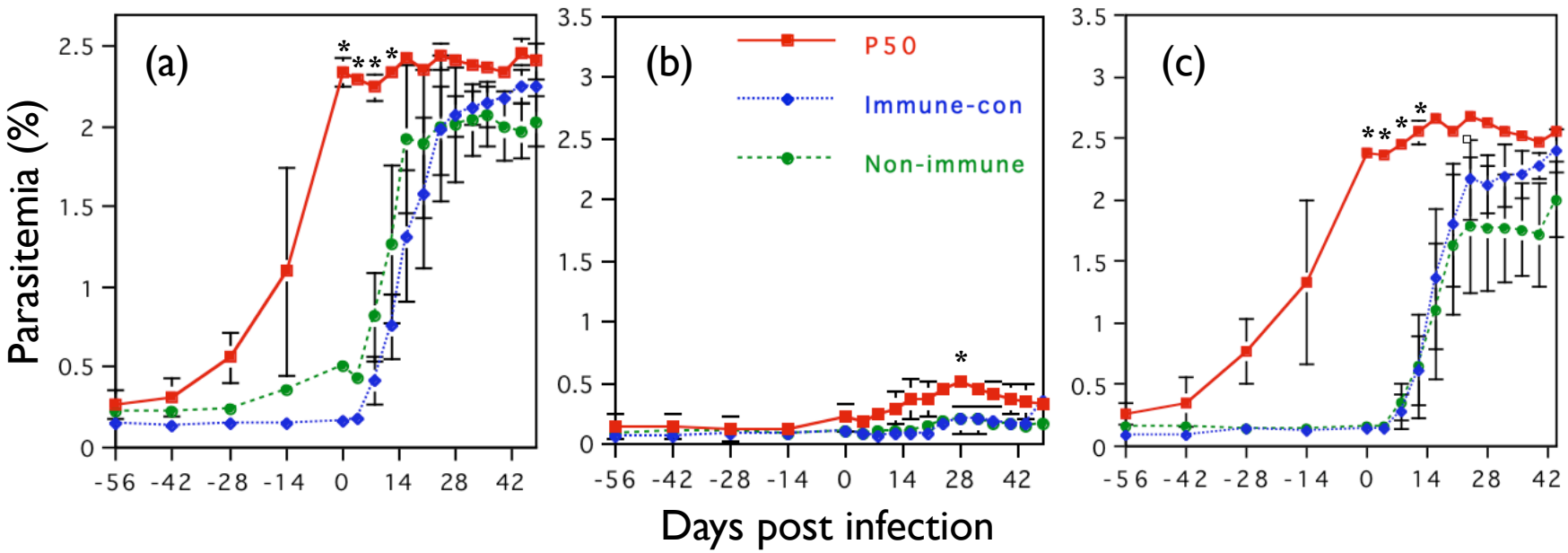
Fukumoto et al. Fig. 1



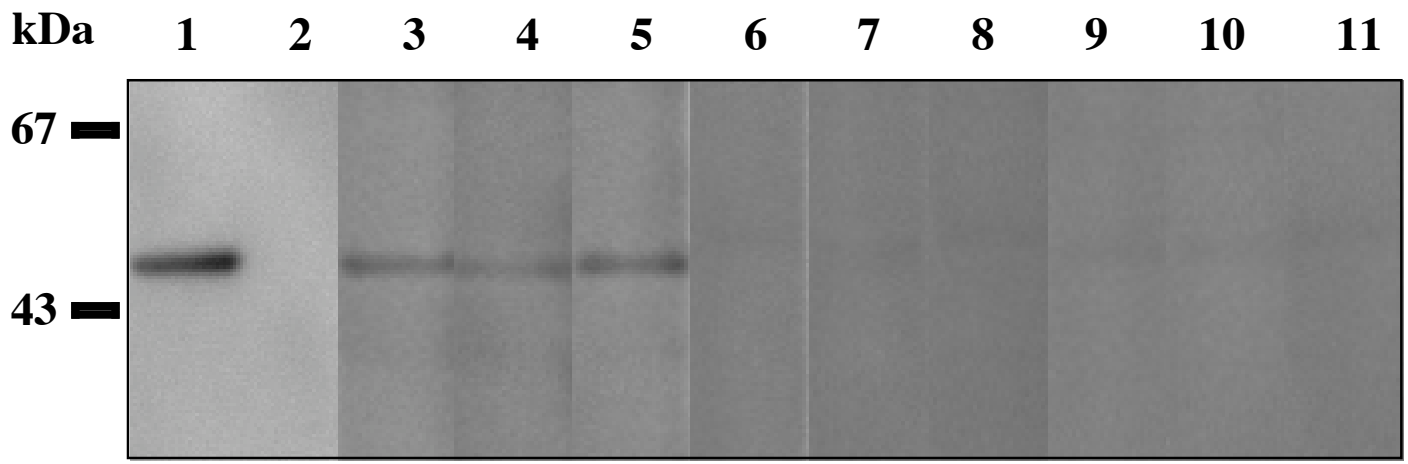
Fukumoto et al. Fig. 2



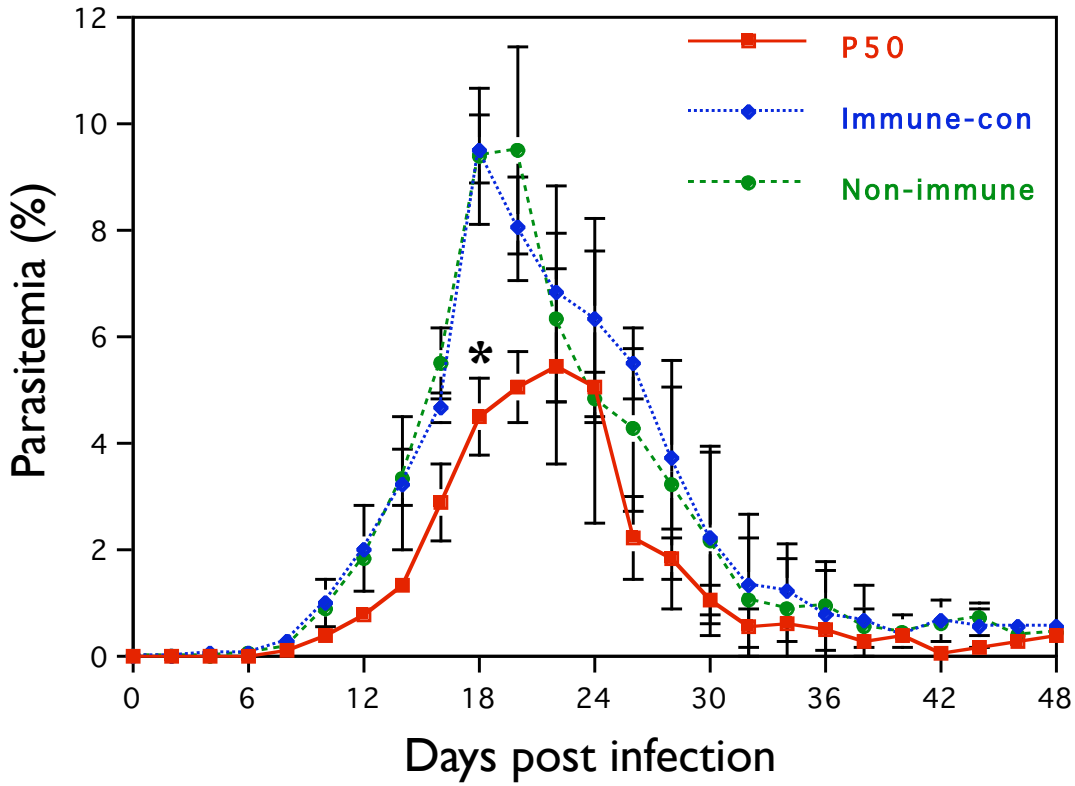
Fukumoto et al. Fig. 3



Fukumoto et al. Fig. 4



Fukumoto et al. Fig. 5



Fukumoto et al. Fig. 6