



Babesia microti Protein *BmSP44* Is a Novel Protective Antigen in a Mouse Model of Babesiosis

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Babesiosis caused by *Babesia* species imposes an increasing threat to public-health and so far, there is no effective vaccine to prevent *Babesia* infections. *Babesia* surface antigen may participate in the invasion of erythrocytes. In our previous study, a surface antigen of *B. microti* merozoites, named as *BmSP44* was identified as a dominant reactive antigen by protein microarray screening. To evaluate its potential applications in diagnosis and prevention of Babesiosis, the open reading frame encoding *BmSP44* was cloned and the recombinant protein was expressed. In consistent with the protein microarray result, recombinant *BmSP44* (*rBmSP44*) can be recognized by sera from *B. microti* infected mice. Immunofluorescence assays (IFA) confirmed that *BmSP44* is a secreted protein and localized principally in the cytoplasm of the parasites. The parasitemia and *Babesia* gene copies were lower in mice administered *rBmSP44* antisera compared with normal controls. Active immunization with *rBmSP44* also afforded protection against *B. microti* infection. The concentrations of hemoglobin in *rBmSP44* immunization group were higher than those in the control group. Importantly, vaccination of mice with *rBmSP44* resulted in a Th1/Th2 mixed immune response with significantly elevated IL-10 and IFN- γ levels during the early stage of infection. Taken together, our results indicated that *rBmSP44* can induce a protective immune response against *Babesia* infection. Thus, *BmSP44* can be used as both a diagnosis marker and a vaccine candidate.

Keywords: *Babesia microti*, secreted protein, vaccine, antigens, diagnosis marker

INTRODUCTION

Babesia is a tick-borne intraerythrocytic protozoan parasite belonging to the phylum Apicomplexa. *B. microti* causes babesiosis in animals and humans worldwide. Although most human babesiosis cases were reported in the United States (1), babesiosis is regarded as an emerging vector-borne parasitic disease in other countries during recent years (2, 3). Babesiosis is normally a benign infection and most of the cases can be asymptomatic or present with mild symptoms. But in populations of neonates or immunocompromised patients, the infection of *Babesia* can be fatal (4, 5). Currently, no vaccine is available to control *Babesia* infection, and drugs for babesiosis are limited, suggesting the importance and necessity to explore potential vaccine based on relative antigen molecules (6, 7).

In recent years, several proteins involved in cell invasion and immunity have been developed as vaccine candidates and their protections against *Babesia* infection in animal models have been evaluated (8–13). But all these candidates as vaccines exhibited limited protection from the infection of *Babesia*. Thus, more work is required for identification of novel targets which can induce stronger protection against babesiosis.

The process of parasite invasion and enveloping within host cells is highly dependent on the interaction between the parasite and host-surface molecules (14, 15). The surface proteins produced by *Babesia* parasite enable it to adhere to and invade the erythrocytes, where it survives and grows (16, 17). Surface secreted proteins usually play a key role in facilitating parasite invasion, host cell remodeling and can be targeted or activated by the humoral immune response in the host (18–21). The surface proteins present in early infective stages may be useful for developing a diagnostic test for babesiosis as well as vaccine.

In our previous study, protein microarray screening was performed using a *Babesia* genomic expression library against murine sera from different stages of infection. Ten *B. microti* antigens were identified as targets of host humoral immune responses (22). However, the antigenicity, immunogenicity, function, and subcellular localization of these surface antigens are not clearly understood.

In the current study, the functions of a dominant *Babesia* antigen *BmSP44* was evaluated in a mouse model of babesiosis. *BmSP44* was confirmed as a secretory protein in the parasite of *B. microti*. The protection effect of this antigen against *Babesia* infection in a mouse model was examined by passive and active immunization strategies. Meanwhile, the changes of cytokine expressions after active immunization were examined to systematically evaluate the function of the protein. Our results suggested that *BmSP44* may serve as a potential vaccine candidate as well as a diagnostic antigen.

MATERIALS AND METHODS

Ethics Statement

All animal procedures were conducted in compliance with the principle for the Care and Use of Medical Laboratory Animals (Ministry of Health, People's Republic of China) and approved by the Institutional Animal Care and Use Committee (IACUC) of Soochow University for the use of laboratory animals (Permit Number: ECSU-201800091). All efforts were made to minimize suffering.

Animals and *Babesia* Infections

Six-to-eight-week-old female BALB/c mice were provided by the Experimental Animal Center of Soochow University (Suzhou, China), and kept under specific pathogen-free conditions. The Peabody strain (ATCC, PRA-99) of *B. microti* was obtained from ATCC. One mouse was initially infected via intraperitoneal injection and blood from the mouse (5 days post infection, the parasitemia is approximately 60%) was taken from the eyelids, anticoagulated with ethylenediaminetetraacetic acid (EDTA), mixed with sterile 0.9% physiological saline in a ratio of 1:2, and infected by 100 μ l per BALB/c mouse via intraperitoneal

injection. The infections were performed with this strain of *Babesia* by intraperitoneal (i.p.) injection with 1×10^7 *B. microti*-infected red blood cells (iRBCs). Totally, 6 mice equally divided into two groups, 3 mice in the immunized group, and 3 ones set as the control were use in the passive experiments. And there were totally 10 mice in the active immunized experiment, each group with 5 mice and the same protocols were applied in independently.

Preparing Secreted Protein Microarray and Acquiring Immunoreactivity Profiles

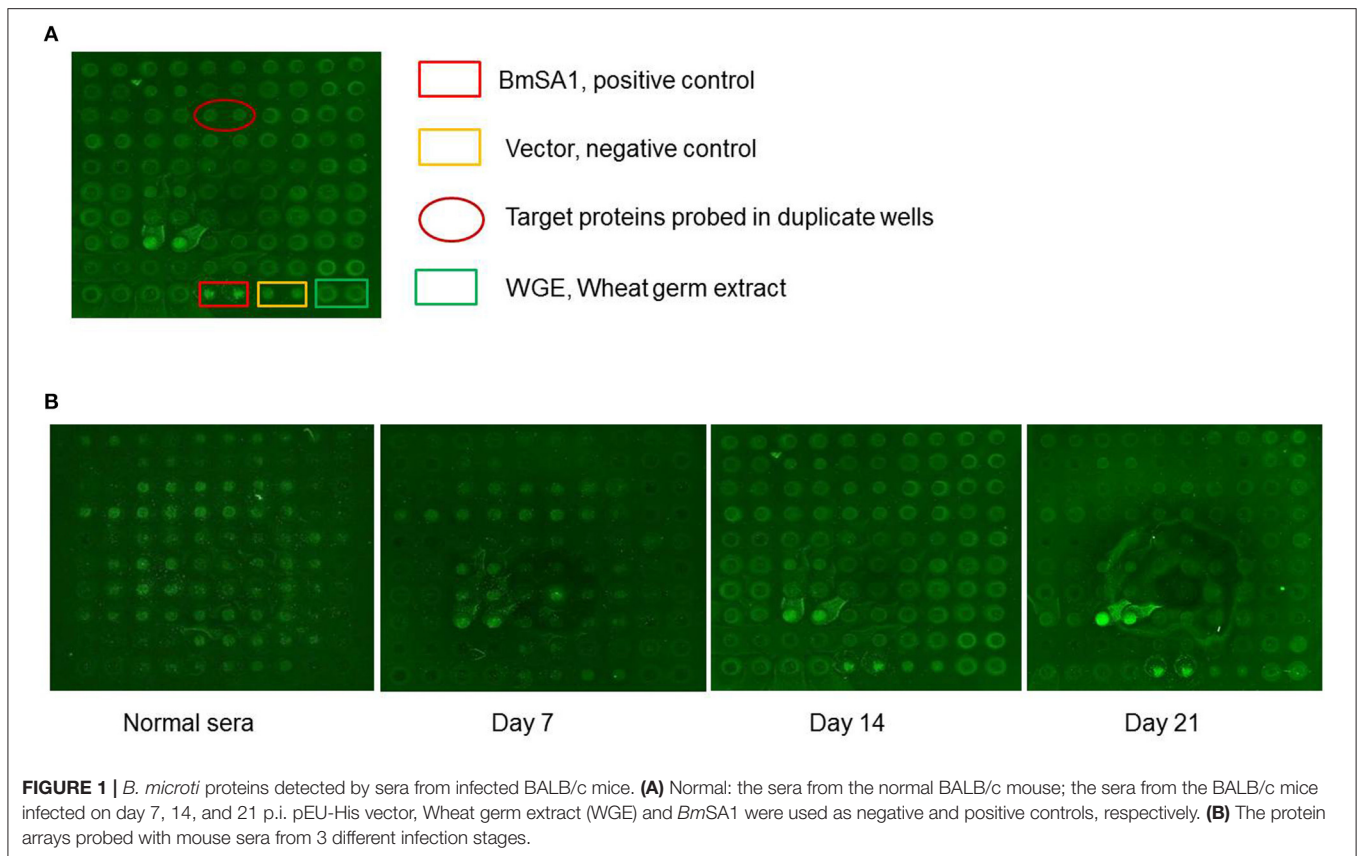
Based on the SignalP software (<http://www.cbs.dtu.dk/services/SignalP/>) and EuPathDB database (<http://piroplasmadb.org/piro/>), ORFs encoding *Babesia* proteins containing signal peptides were selected and cloned in-frame into the pEU-E01-His-TEVMCS-N2 (pEU, Cell Free Sciences, Matsuyama, Japan) vector. The cell-free protein synthesis system, wheat germ cell-free (WGCF) system was applied in the high throughput expression assay. The ORF sequences were amplified from cDNA of *B. microti* PRA99 strain and the recombinant protein r*BmSA1* (23) expressed in *E. coli* DH5 α strain was used as the positive control. The wheat germ lysate with expression of an empty vector served as the negative control. Detailed protocols with the high throughput assay were described in our previously study (22).

Bioinformatic Analysis of the Gene Coding for *BmSP44*

Antigenic epitopes were predicted using ABCPred (<http://www.imtech.res.in/raghava/abcpred/>). The hydrophobicity and the signal peptide were predicted using Expasy (<http://www.expasy.org/>) and the SignalP4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>), respectively.

Expression and Purification of Recombinant Protein *BmSP44*

Expression of *BmSP44* with glutathione S-transferase (GST) fusion proteins was conducted using the pGEX vector system. ORF of *BmSP44* was amplified with the proof-reading Polymerase (Pfu) (Transgene, Beijing) from the cDNA of *B. microti* using gene-specific primers (forward-TTCCAGGGGCCCTGGGATCCATGCATATCAACTACAAATTAATTA and reverse-TCACGATGCGCCGCTCGAGTTAAGCAGCATTAGGTGTGTGAT). The fragment was then cloned into pGEX-6P-2 (Invitrogen, Carlsbad, NM) vector by digestion with *Bam*HI and *Xho*I (Vazyme, USA). Validated pGEX constructs were re-transfected into *E. coli* strain BL21 (DE3) for recombinant protein expression. Briefly, 1,000 ml of LB medium containing 1 ml of ampicillin were incubated with bacterium in constant temperature shaker. After 4h, protein expression was induced with 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Then, the soluble recombinant GST-tagged fusion proteins were purified using GST affinity agarose (GE Healthcare, Sweden) and the GST tag was



removed by on-column enzyme digestion of Prescission Protease (Sigma).

Preparing Rabbit Antisera Against rBmSP44

For generation of Rabbit antisera against rBmSP44, NZW rabbit was immunized with 100 μ g of rBmSP44 together with complete Freund's adjuvant (Sigma-aldrich, USA). The rabbit was received 2 boosts (400 μ g rBmSP44) at 2-week intervals in incomplete Freund's adjuvant. Two weeks after the final boost, sera were collected and the antibody titers were evaluated with the standard ELISA procedures described above.

Western Blot Analysis

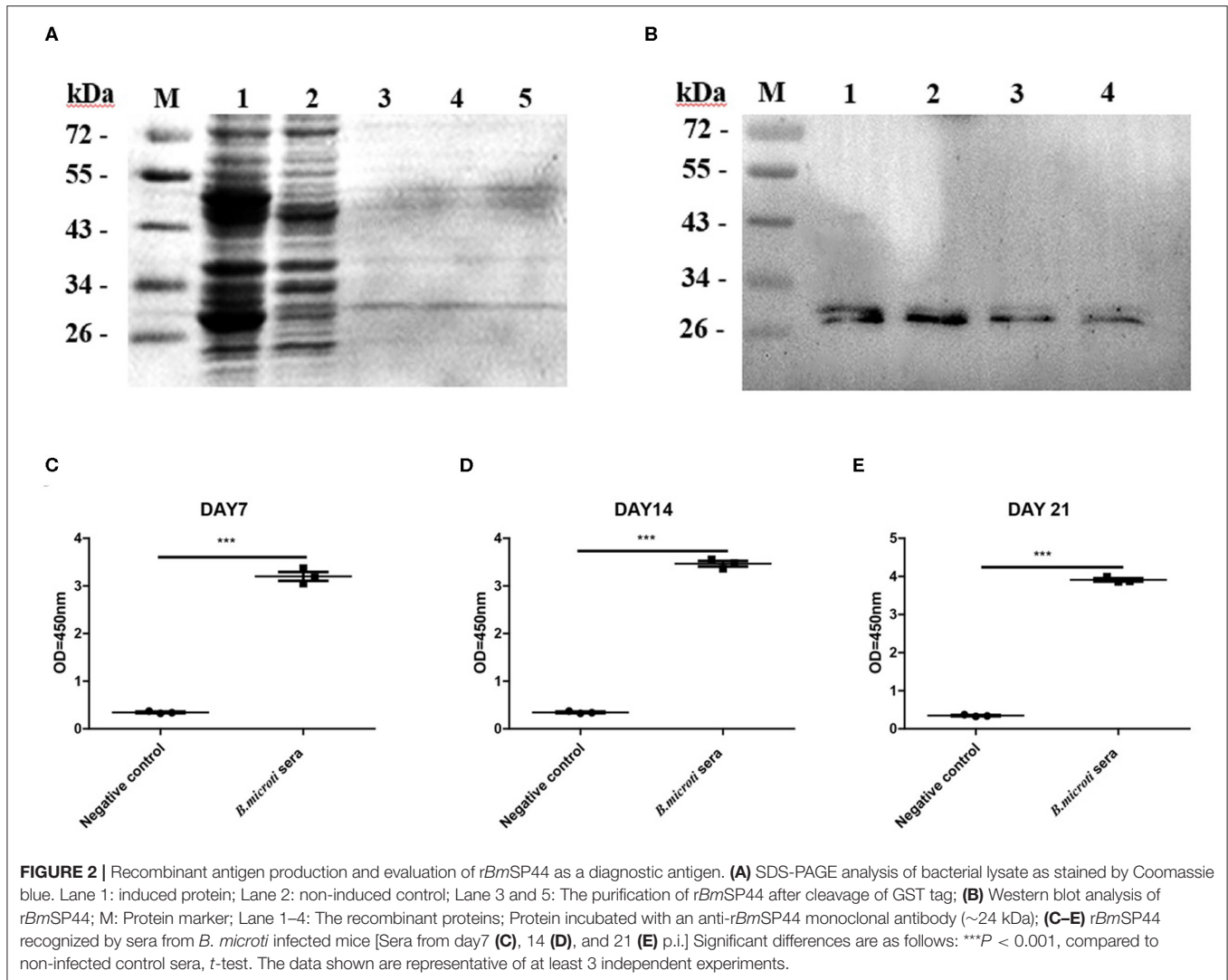
Total protein samples were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a 0.45 μ m polyvinylidene difluoride (PVDF) membranes. The blot was blocked with 5% skim milk diluted in TBS containing 0.05% Tween (TBST) for 1 h at room temperature. The *B. microti* hyperimmune sera were diluted (1:5,000) in TBST containing 2% skim milk and incubated overnight at 4°C. The blot was washed with TBST three times and then incubated in an HRP-conjugated goat anti-mouse IgG (H+L) secondary antibody (Bioworld, USA) (1:10,000) for 1 h at room temperature. After three washes with TBST, the signal was detected with an enhanced chemiluminescence ECL Plus kit (Thermo, USA).

Evaluation of rBmSP44 as a Diagnostic Antigen

Enzyme-linked immunosorbent assay (ELISA) plates were coated with the rBmSP44 protein (to the final concentration of 5 μ g/ml) and incubated overnight at 4°C. After three washes with PBST, the plates were blocked with 2% BSA. One hundred microliters of sera from different infection stages (7, 14, and 21 days post infection), and negative mouse sera (from healthy mice) were diluted (1:2,000) in 2% BSA and incubated for 30 min. After incubating with peroxidase-conjugated rabbit anti-mouse IgG antibody, the reaction was examined with 3, 3', 5', 5'-Tetramethylbenzidine–hydrogen peroxide substrate (TMB) (Biolegend, USA) according to standard protocols.

Immunofluorescent Assay (IFA) and Confocal Microscopy

Anticoagulated blood collected from mice infected with *B. microti* with approximately 30% parasitemia was smeared on slides using cytospin centrifugation (Thermo Fisher Scientific) and fixed with 4% PFA-PBS for 10 min. After washed three times with PBS, the slides were permeabilized with 0.4% Triton X100 for 10 min, and then treated with protease K (20 μ g/ml) for 5 min. After washes, the slides were blocked with 5% FBS for 30 min to reduce non-specific binding and then incubated overnight at 4°C with a 1:300 dilution of



mouse anti-BmSP44 serum. After washing three times again, the slides were incubated with Alexa Fluor488 goat anti-mouse IgG for 1 h (Invitrogen) diluted at 1:500 in PBS for 1 h at room temperature. After washes, the slides were incubated with 0.5 $\mu\text{g}/\text{ml}$ 6-diamidino-2-phenylindole (DAPI) for 15 min and imaged using a Nikon C2 Confocal microscope system (Nikon, Tokyo, Japan).

Passive Immunization

The rabbit was immunized with *rBmSP44* 100 μg mixed with complete Freund's adjuvant (Sigma-aldrich, USA) in ratio of 2:1 and boosted twice at 2-week intervals by injection of 200 μg protein mixed with incomplete Freund's adjuvant also in ratio of 2:1. Two weeks after the final boost, sera were collected and the antibody titers were evaluated with the standard ELISA procedures described above. For passive immunization, mice were administered of *rBmSP44* rabbit antisera (200 μl each) or control antisera (5 per group). Twenty-four hours later, the animals were challenged with intraperitoneal

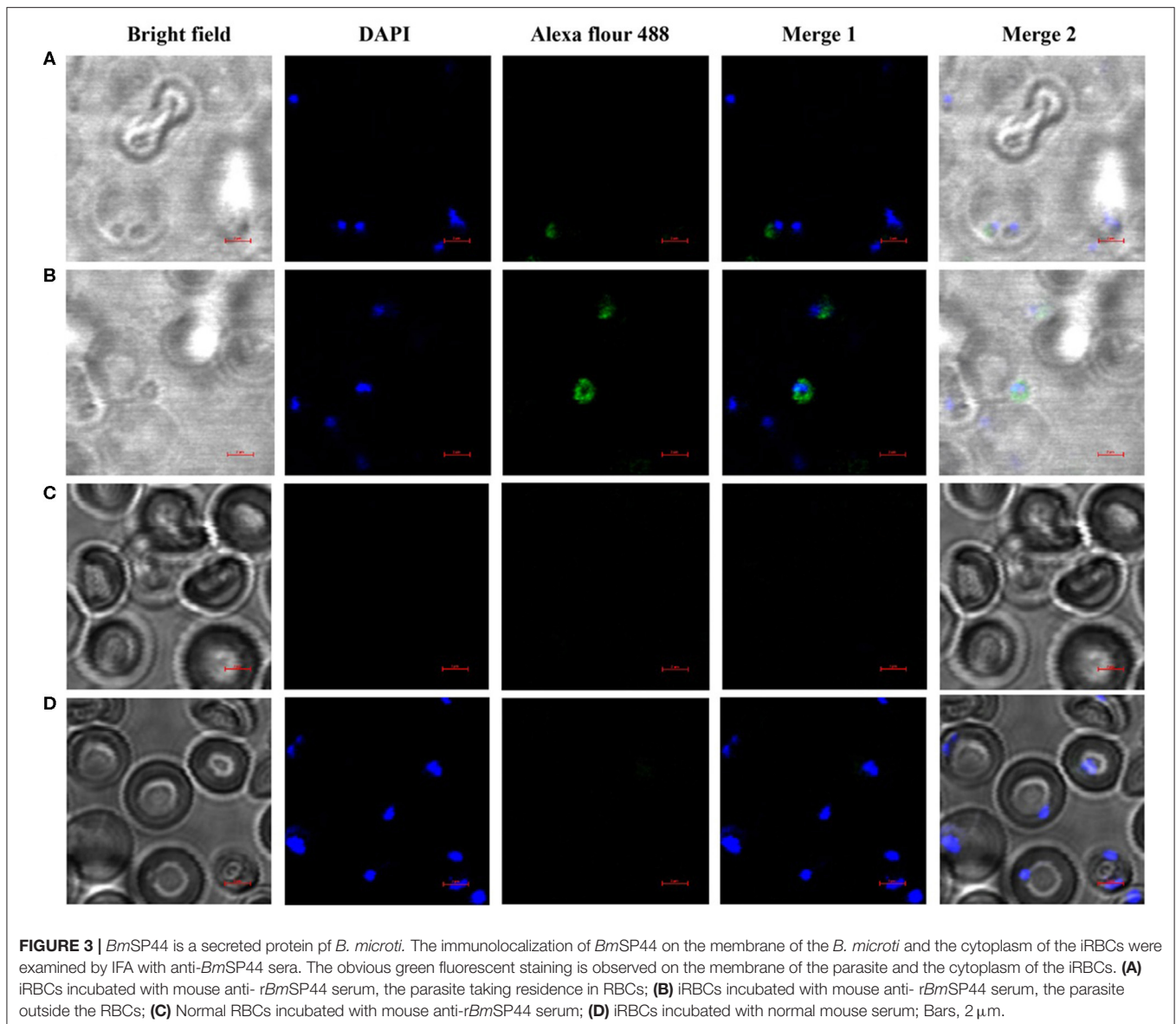
inoculation of 1×10^7 *B. microti*-parasitized red blood cells (iRBCs).

Active Immunization

For active immunization, mice (5 per group) were immunized with *rBmSP44* (20 $\mu\text{g}/\text{each}$) mixed with complete Freund's adjuvant while the control group receiving Freund's adjuvant only. Animals were received 2 boosts (40 μg *rBmSP44*) at 2-week intervals in incomplete Freund's adjuvant. Two weeks after the final boost, sera were collected and evaluated with the standard ELISA procedures described above.

Parasitemia and Babesia Load

The concentration of parasites in the blood was determined by blood smears examination and quantitative RT-PCR (qRT-PCR). Thin films from the peripheral blood were prepared every third days after *B. microti* inoculation, and stained with Giemsa's solution. The numbers of infected and non-infected erythrocytes were counted per 50 microscopic fields to calculate



parasitemia. The degree of infection in each group is presented as the geometric mean of the parasitemia percentage. The blood RNA was extracted using the blood RNA kit (OMEGA, USA). Briefly, the blood RNA was reverse-transcribed using the PrimeScript Master Mix kit (TaKaRa, Japan). PCR was performed by using iTaq SYBR Green Supermix (Monad, China) on a CFX96 real time PCR system (Eppendorf, USA) and involved an initial denaturation at 95°C for 30 s, 40 cycles of 5 s at 95°C, and 30 s at 60°C. At the end of each reaction, a melting curve (70–95°C) was checked to confirm the identity of the PCR product. *B. microti* 18S rRNA primers: forward- AGCGTTTTCGAAGGTA TGTTGC and reverse-AGCAGATACATCCTTACTAGGGAAA. Mouse GAPDH (control): forward- GGCCTTCCGTGTTTCCT ACC and reverse - AGCCCAAGATGCCCTTCAGT were applied during the amplification. And the mouse GAPDH gene was amplified as an internal control.

Severity of the Disease

Besides the parasitemia and *Babesia* load, the weight, temperature, and hemoglobin level of mice were also measured to evaluate the severity of the disease. The weight and the temperature were monitored daily post *B. microti* infection. To measure the hemoglobin (Hb) concentration, 10 μ l of blood was diluted in 2,490 μ l of Drabkin's reagent (Sigma-Aldrich, St. Louis, MO, USA) and quantified at 540 nm using a biophotometer (Eppendorf, USA). The absorbance and hemoglobin concentration (Hb) were counted using a commercial Hb standard curve.

Detection of Cytokine Levels in Serum

After receiving active immunization with the recombinant protein, mice were challenged with 1×10^7 *B. microti* infected erythrocytes. Sera samples were prepared from each immunized

or control mouse on day 0, 3, 6, and 9 after challenge. The concentration of cytokines such as IFN- γ , TNF- α , and IL-10 were determined by ELISA assay according to the manufacturer's instructions (Biolegend, USA).

Statistical Analysis

GraphPad Software of Prism 7 software was used in charts and statistical analyses. Statistical differences were analyzed by Student's *t*-test and ANOVA. A value of $p < 0.05$ was considered statistically significant.

RESULTS

High-Throughput Screening of Secreted Proteins by Protein Arrays

A total of 55 proteins containing signal peptides from *B. microti* were selected and screened by sera collected from *B. microti* infected mice at different stages of infection. One secreted protein, named *BmSP44*, showed a higher immune reactivity with the sera collected on day 7, 14, and 21 post infection compared to other antigens tested (Figures 1A,B).

Molecular Characterization and Sequence Analysis of *BmSP44*

The nucleotide sequence coding *BmSP44* was 654 bp long and predicted to encode a protein of 218 amino acid residues (Supplementary File 1 with a predicted molecular weight and isoelectric point of 24 kDa and 5.39. Signal peptide sequence was found in *BmSP44* protein based on SignalP4.1 and antigenic epitopes were predicted using ABCPred bioinformatic server (Supplementary Figures 1A,B). However, no homologous genes were identified in other species of *Babesia* based on the current genome sequences available from NCBI database.

Evaluation of *BmSP44* as a Diagnostic Antigen

To investigate the characteristics of *BmSP44*, a recombinant protein without the GST tag (~24 kDa) was produced in the *E. coli* expression system (Figure 2A) and used to immunize mice. Western blot suggested that *rBmSP44* reacted with the sera from *rBmSP44*-immunized mice but not from the controls (Figure 2B).

To validate the potential of *rBmSP44* as a diagnostic antigen, an indirect ELISA was set to detect *BmSP44* specific antibodies from sera of *B. microti* infected mice. The results suggested that *rBmSP44* can be detected with mouse serum collected on day 7, 14, and 21 p.i. (Figures 2C–E), which were consistent with the data from high throughput protein microarray. Thus, *BmSP44* can be used as a diagnostic antigen since it can be detected with host serum at different stages of infection.

Localization of *BmSP44* in *B. microti*

The localization of *BmSP44* in *B. microti* and infected RBCs was detected by IFA using mouse anti-*BmSP44* sera. *BmSP44* appeared to localize on the surface of *B. microti*, supporting that it is a potential secreted antigen of *B. microti* (Figures 3A,B). As

negative controls, red blood cells from non-infected mice did not show any specific fluorescent signals (Figures 3C,D).

Passive Immunization With Antisera Against *rBmSP44* Interferes With *B. microti* Infection

In order to examine whether *rBmSP44* antiserum thwarts infection with *B. microti*, *rBmSP44* antisera were administered into the mice 24 h before *B. microti* challenge. The titers of the rabbit antisera against *rBmSP44* were confirmed by ELISA before inoculation (Supplementary Figure 2). qRT-PCR analysis suggested that *Babesia* gene copies in mice receiving rabbit antisera against *rBmSP44* were reduced on day 3, 6, and 9 p.i. compared with that in control animals (Figure 4A). The results from the blood smears also showed a decrease parasitemia in passive immunization group compared with the control group (Figures 4B–H).

Active Immunization With *rBmSP44* Reduces *Babesia* Infection in Mice

We then examined whether active immunization of mice with *rBmSP44* influenced *Babesia* infection. Following active immunization, high levels of *rBmSP44* antibodies were detected in mouse sera (Figure 5A). After challenged with *B. microti*, mice immunized with *rBmSP44* displayed reduced *Babesia* gene copies in whole blood samples on day 3, 6, and 9 p.i. compared with controls (Figure 5B). A reduced *Babesia* parasitemia also observed in blood smears from *rBmSP44* immunized mice compared to controls (Figures 5C–I). Therefore, active immunization with *rBmSP44* also protects against *B. microti* infection of mice.

Parasitemia is a major indicator for evaluating the severity of the disease. Meanwhile, weight, body temperature, and concentration of hemoglobin were also introduced to evaluate the severity of Babesiosis. Our results showed that the concentrations of hemoglobin in *rBmSP44* immunized mice were significantly higher than that of controls, while there are no obvious differences regarding the weight and the temperature between the two groups (Figures 6A–F).

Cytokine Profiles of *rBmSP44* Immunized Mice

In order to analyze the levels of Th1 and Th2 cytokines in infected mouse sera, the expressions of IFN- γ , TNF- α , and IL-10 were determined by ELISA kits. Compared with the control groups, serum IFN- γ , and IL-10 expression were significantly higher in mice immunized with *rBmSP44* on day 6 p.i. Serum TNF- α was also slightly higher in the immunized group than the controls on day 6 and 9 p.i., but the differences were not significant. These data suggested that a Th1/Th2-mixed immune response was induced in *rBmSP44* immunized mice during the early stage of infection. This Th1/Th2-mixed immune response may contribute to the protective efficacy of *rBmSP44* against *Babesia* parasites infection (Figures 7A–C).

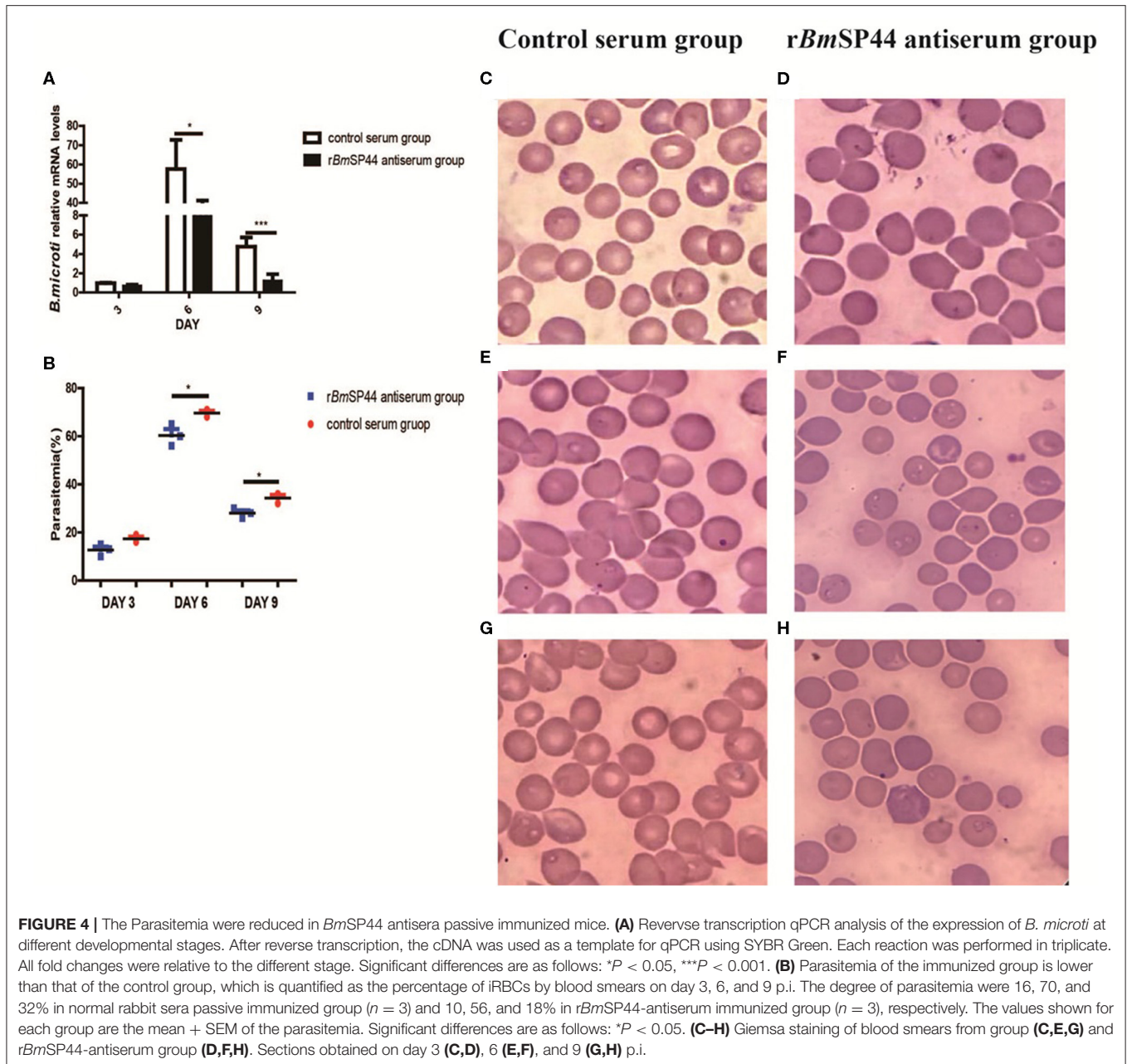


FIGURE 4 | The Parasitemia were reduced in *BmSP44* antisera passive immunized mice. **(A)** Reverse transcription qPCR analysis of the expression of *B. microti* at different developmental stages. After reverse transcription, the cDNA was used as a template for qPCR using SYBR Green. Each reaction was performed in triplicate. All fold changes were relative to the different stage. Significant differences are as follows: * $P < 0.05$, *** $P < 0.001$. **(B)** Parasitemia of the immunized group is lower than that of the control group, which is quantified as the percentage of iRBCs by blood smears on day 3, 6, and 9 p.i. The degree of parasitemia were 16, 70, and 32% in normal rabbit sera passive immunized group ($n = 3$) and 10, 56, and 18% in *rBmSP44*-antiserum immunized group ($n = 3$), respectively. The values shown for each group are the mean + SEM of the parasitemia. Significant differences are as follows: * $P < 0.05$. **(C–H)** Giemsa staining of blood smears from group **(C,E,G)** and *rBmSP44*-antiserum group **(D,F,H)**. Sections obtained on day 3 **(C,D)**, 6 **(E,F)**, and 9 **(G,H)** p.i.

DISCUSSION

B. microti, a major etiological agent of tick-borne babesiosis invading only erythrocytes in human has been sequenced to have the smallest nuclear genome among all apicomplexan parasites (24). Several novel antigens of the parasite were characterized using protein microarrays (25–27). Some of them can trigger host immune response and be associated with genes encoding the secretome and the surface proteome of the parasite (28–30). These studies provide candidates for the development of improved diagnostic assays and vaccines.

A panel of secreted proteins containing signal peptides were identified in previous studies (12, 22), some of which have

been characterized as diagnostic antigens of babesiosis. Relative fewer were considered as candidates in vaccine development (31–33). What's more, increased drug resistance of *Babesia* and modest effect of new vaccine candidates were reported (34, 35). In this study, the gene encoding *BmSP44* was cloned and characterized in *B. microti*. Bioinformatics analysis suggested that *BmSP44* has no homologous genes in other species of *Babesia*; in addition, *BmSP44* does not contain any conserved domains, making it difficult to predict its function. *BmSP44* is a secreted protein that can be recognized by the host's immune system and therefore considered as an attractive vaccine candidate and a diagnostic marker. Our microarray and ELISA data all indicated that *rBmSP44*

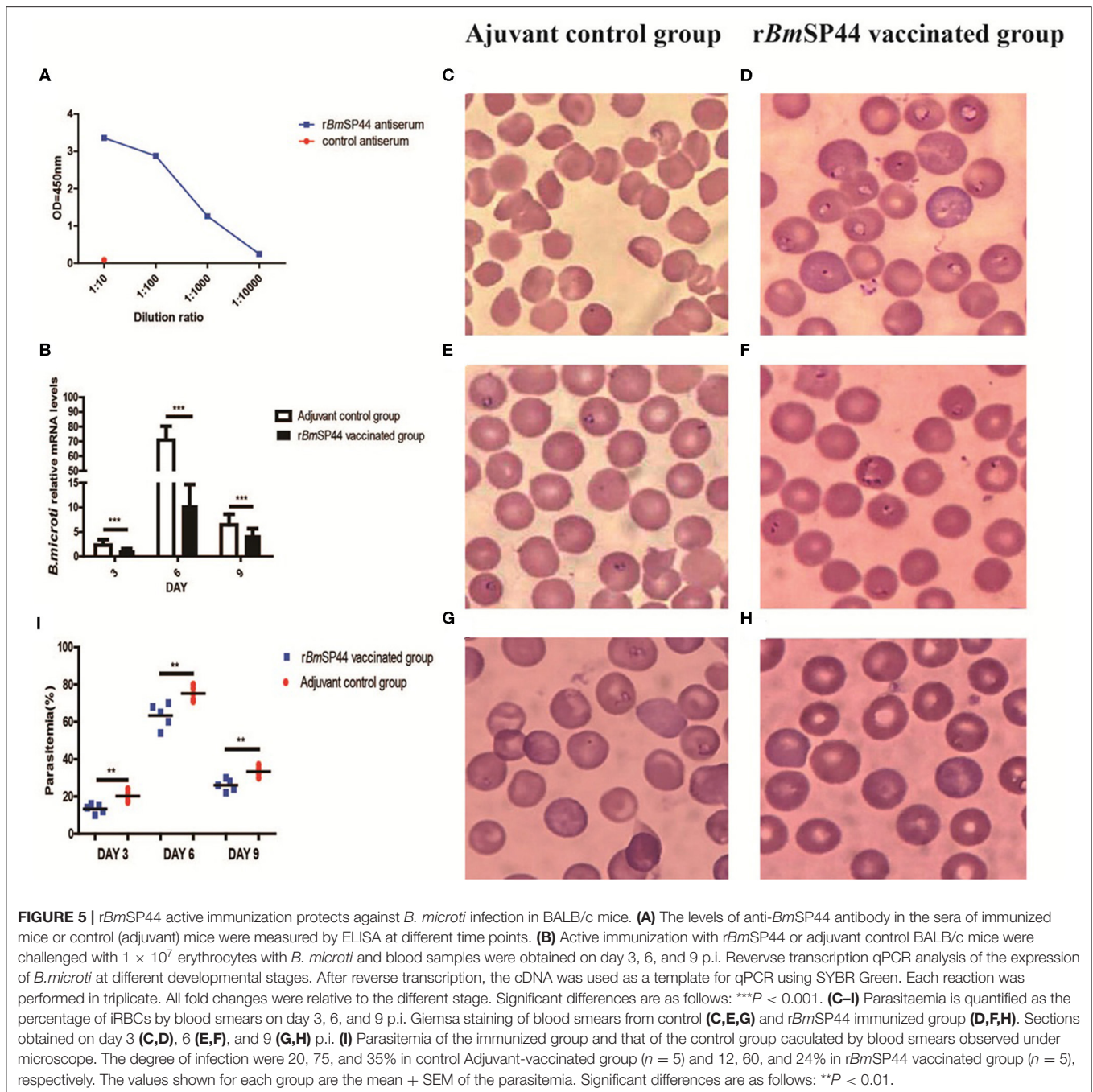


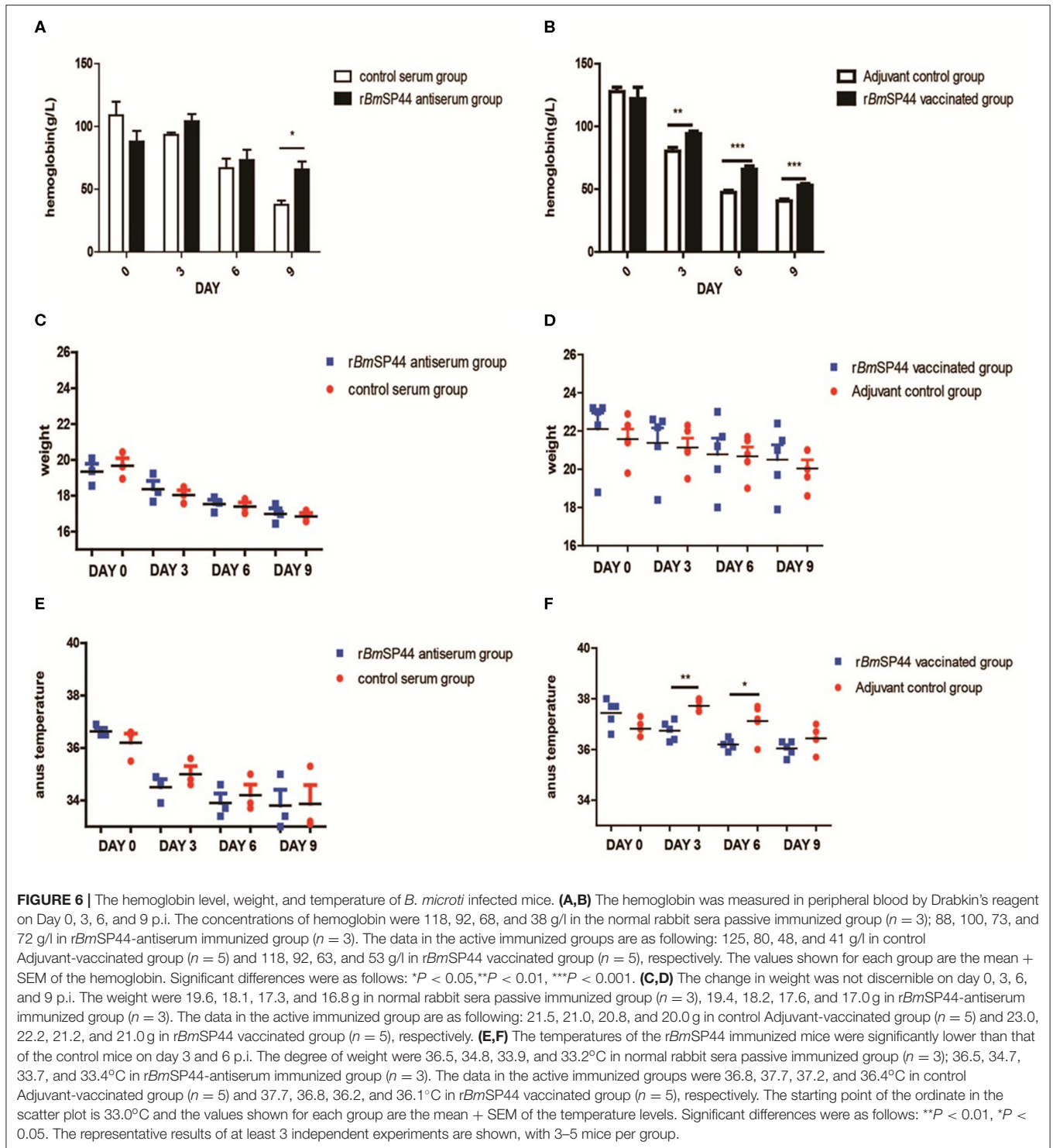
FIGURE 5 | rBmSP44 active immunization protects against *B. microti* infection in BALB/c mice. **(A)** The levels of anti-rBmSP44 antibody in the sera of immunized mice or control (adjuvant) mice were measured by ELISA at different time points. **(B)** Active immunization with rBmSP44 or adjuvant control BALB/c mice were challenged with 1×10^7 erythrocytes with *B. microti* and blood samples were obtained on day 3, 6, and 9 p.i. Reverse transcription qPCR analysis of the expression of *B. microti* at different developmental stages. After reverse transcription, the cDNA was used as a template for qPCR using SYBR Green. Each reaction was performed in triplicate. All fold changes were relative to the different stage. Significant differences are as follows: *** $P < 0.001$. **(C–I)** Parasitaemia is quantified as the percentage of iRBCs by blood smears on day 3, 6, and 9 p.i. Giemsa staining of blood smears from control **(C,E,G)** and rBmSP44 immunized group **(D,F,H)**. Sections obtained on day 3 **(C,D)**, 6 **(E,F)**, and 9 **(G,H)** p.i. **(J)** Parasitemia of the immunized group and that of the control group calculated by blood smears observed under microscope. The degree of infection were 20, 75, and 35% in control Adjuvant-vaccinated group ($n = 5$) and 12, 60, and 24% in rBmSP44 vaccinated group ($n = 5$), respectively. The values shown for each group are the mean + SEM of the parasitemia. Significant differences are as follows: ** $P < 0.01$.

could be an antigen for serological diagnostics marker for human babesiosis.

As BmSP44 elicits strong immunoreactions, we extended the study to test the role of rBmSP44 in altering *Babesia* infection. In our active immunization experiments, the mice immunized with rBmSP44 demonstrated significant protection against *Babesia* infection. The passive immunization with rBmSP44 rabbit antiserum also delivered protection in immunized mice; however the protection rates were lower than that of active immunization experiments. Although rabbit antibodies have

the advantages of recognizing small epitopes (36, 37), the antibody titer of passive immunization was lower than that of active immunization in our study. Also, active immunization may result in cellular immunity in addition to humoral immunity. This may explain why the passive immunization inducing relatively weak protection compared to the active immunization.

As apicomplexan protozoa, the protective immunity induced by *Babesia* proteins like BmMetAP1, BmHSP70 involves mainly cell-mediated responses with high level of IgG1 and Th1



cytokines, such as IFN- γ and IL-12 (38, 39). The interaction between the erythrocyte receptors and MSP1 was critical for the invasion process of *Plasmodium*, another important intraerythrocytic protozoan. Thus, MSP1 is a major malaria vaccine candidate which protects malaria parasites in mouse models (40). Humoral immune responses may also contribute

to the protections in these parasite infections. Classically, the Th1 cytokines responses are characterized by the production of IFN- γ , IL-2, and TNF- α , while Th2 responses are represented by cytokine IL-4, IL-6, and IL-10. It was confirmed that cytokines such as IL-12, TNF- α , IFN- γ , and IL-2 play an important role in controlling the proliferation of *Babesia* in

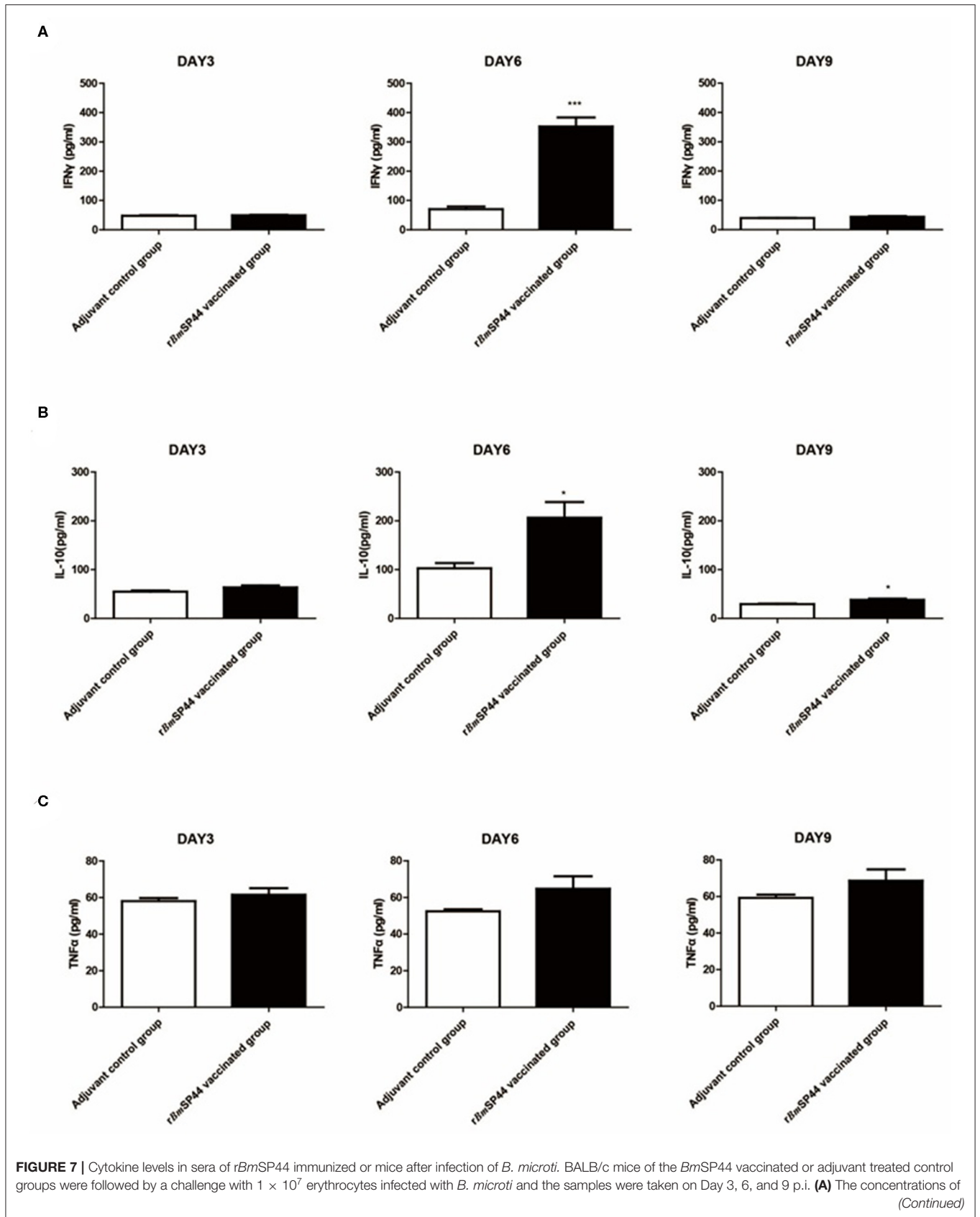


FIGURE 7 | IFN- γ in sera were 45.4, 60.5, and 40.0 pg/ml in Adjuvant-vaccinated control group ($n = 5$) and 52.3, 231.9, and 43.5 pg/ml in rBmSP44 vaccinated group ($n = 5$), respectively. Results are presented as the mean \pm SEM. The asterisks (*) indicate that the levels of IFN- γ by immunized mice was significantly higher (***) $P < 0.001$) than those of the adjuvant control group. **(B)** The concentrations of IL-10 in sera were 53.9, 101.5, and 28.8 pg/ml in Adjuvant-vaccinated control group ($n = 5$) and 64.4, 193.2, and 40.9 pg/ml in rBmSP44 vaccinated group ($n = 5$), respectively. Results are presented as the mean \pm SEM. The asterisks (*) indicate that the levels of IL-10 by immunized mice were significantly higher ($*P < 0.05$) than those of the adjuvant control group. **(C)** The concentrations of TNF- α in sera were 57.2, 51.9, and 57.9 pg/ml in Adjuvant-vaccinated control group ($n = 5$) and 60.3, 62.3, and 68.5 pg/ml in rBmSP44 vaccinated group ($n = 5$), respectively. Results are presented as the mean \pm SEM. The data represent two individual experiments. All the levels of cytokine in sera were measured by ELISA kits.

the early and acute stages of infection, while IL-10, IL-4, IL-5, and IL-6 may involve in chronic and low parasitemia stages of infections (41–45). Our current study suggested that immunization with rBmSP44 elicits IFN- γ , TNF- α , and IL-10 expression and results in a Th1/Th2 mixed humoral and cellular immune response, which may contribute to protect mice from *Babesia* infection.

The clinical manifestations of *Babesia* infections are diverse and the declining concentration of hemoglobin induced by babesiosis is considered to be a major feature of the infection (42). In our current study, the concentration of hemoglobin in the immunization group was slightly higher than that in controls, while changes in weight and temperature were not discernible. We only observed the condition within 2 weeks after infection, and the increment of temperature is not only associated directly to the concentration of parasites but also related to the immune status of the hosts.

In conclusion, our study indicated that BmSP44 is a secreted protein and localized principally in the cytoplasm of the parasites. BmSP44 can elicit immune responses in a mouse model of Babesiosis and can be recognized by immune serum from different stages of infection. Both active and passive immunization with rBmSP44 (or antisera) can afford protection to mice against *Babesia* infection. Thus, BmSP44 can be used as both a diagnosis marker and a vaccine candidate to combat Babesiosis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Protein ID: CCF73510.

ETHICS STATEMENT

All animal procedures were conducted in compliance with the principle for the Care and Use of Medical Laboratory Animals (Ministry of Health, People's Republic of China) and approved by the Institutional Animal Care and Use Committee (IACUC)

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AUTHOR CONTRIBUTIONS

XZ and JD conceived the study, collected and analyzed the data, and drafted the manuscript. HW, YW, and JH carried out the whole experiments and revised the manuscript. BX and JH conceived the project and provided technical support for data collection and analysis. All authors read and approved the final manuscript. Written consent to publish was obtained.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01437/full#supplementary-material>

Supplementary File 1 | The amino acid sequences of BmSP44.

Figure S1 | Predicated signal peptide and antigenic epitopes of BmSP44 **(A)** Signal peptide was detected in BmSP44 based on SignalP4.1 software; **(B)** Antigenic epitopes of BmSP44 were predicted by ABCPred bioinformatic serves.

Figure S2 | Quantification of the antibody titers in sera of rabbits immunized with rBmSP44. Rabbits immunized with 100 μ g **(A)** or 500 μ g **(B)** rBmSP44, respectively and the antibody tiers were examined by ELISA. Normal rabbit serum was served as a control.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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