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SHORT COMMUNICATION

Recombinant methionine aminopeptidase protein of *Babesia microti*: immunobiochemical characterization as a vaccine candidate against human babesiosis

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Abstract Human babesiosis is the most important zoonotic protozoan infection in the world. This is the first report of the cloning, expression, purification, and immunobiochemical characterization of a methionine aminopeptidase 1 (MetAP1) protein from *Babesia microti* (*B. microti*). The gene encodes a MetAP1 protein of *B. microti* (BmMetAP1) of approximately 66.8 kDa that includes glutathione S-transferase (GST) tag and shows MetAP activity. BmMetAP1 was detected in a lysate of *B. microti* and further localized in cytoplasm of the *B. microti* merozoite. rBmMetAP1 was found to be immunogenic, eliciting a high antibody titer in mice. Moreover, rBmMetAP1 stimulated the production of IFN-γ and IL-12 but not IL-4. Finally, rBmMetAP1 was able to provide considerable protection to mice against a *B. microti* challenge infection based on a reduction in peak parasitemia levels and earlier clearance of the parasite as compared with control mice. Taken together, these results suggest that rBmMetAP1 confers significant protection against experimental *B. microti* infection and might be considered a potential vaccine target against human babesiosis.

Keywords Babesiosis · Enzyme assay · Immunization · Mice

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Introduction

Human and rodent babesioses are caused by *Babesia microti* and transmitted by the bite of the ixodid tick. Since the first reported case of human babesiosis in New York was from Long Island in 1975 (Dammin et al. 1981), this disease has emerged as important zoonosis worldwide. Although infection usually is asymptomatic, the immunocompromised and elderly develop mild to moderate fever, headache, body aches, fatigue, and anorexia. If proper clinical treatment for babesiosis is not administered, it can become life threatening and cause death (Kjemtrup and Conrad 2000). Currently, there is no unified understanding of a gold standard drug or optimal treatment regimen for human babesiosis. Therefore, it is necessary to develop a novel vaccine candidate for the control of babesiosis.

In the past few decades, aminopeptidases have emerged as exciting novel drug targets and vaccine candidates for various diseases, offering new options to control diseases (Chang et al. 1992; Molina et al. 2002; Chen et al. 2006; Narayanan et al. 2008; Kang et al. 2012, 2015). Methionine aminopeptidases (MetAPs), a family of aminopeptidases, play an important role in the N-terminal excision of methionine from polypeptides during protein synthesis, and they have been identified in numerous microorganisms, plants, vertebrates, and invertebrates (Lowther and Matthews 2000; Addlagatta et al. 2005). In addition to their involvement in N-terminal methionine excision, MetAPs are involved in the general metabolism of amino acids and proteins, the activation and inactivation of biologically active peptides, and antigen processing to be presented to the major histocompatibility system (Mercado-Flores et al. 2004).

Recently, the putative gene encoding MetAP1 was found in the genome of *B. microti* (Cornillot et al. 2012). However, the characterization and enzymatic activity of MetAP1 in murine *Babesia* species have not been well studied yet. Therefore, in

this study, we immunologically characterized a member of the methionine aminopeptidase family in *B. microti* as a potent vaccine candidate against human babesiosis.

Materials and methods

Parasites and mice

The Munich strain of *B. microti* was maintained in BALB/c mice by serial passaging. Infections were initiated by intraperitoneal (i.p.) injection of 1×10^7 infected erythrocytes (Igarashi et al. 1999). Eight-week-old female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). All mice used in the present study were cared for in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine (Obihiro, Japan).

Cloning and sequencing of the *MetAP1* gene

Total RNA was extracted from the *B. microti*-infected erythrocytes using a commercial RNeasy Mini Kit (Qiagen, Germany). The concentration of the purified total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., USA). The cDNA was prepared from approximately 5 μg of the total RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen, USA). The polymerase chain reaction (PCR) was carried out with a forward *Bam*HI-metAP1 protein of *B. microti* (BmMetAP1)-F (5′-CCGGATCC ATGACTGAAGTATGTATTTC-3′) and a reverse *Xho*I-BmMetAP1-R (5′-TTCTCGAGTTCAA AGTCTAGCGGAGGCG-3′) (the underlined sequences contain a *Bam*HI and *Xho*I restriction site, respectively) primer set to amplify a 1086-bp fragment of BmMetAP1 (GenBank accession no. XP_012649271) (Cornillot et al. 2012). The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen, Germany). The purified product was cloned in a pCR® 2.1 Vector (Invitrogen, USA) and was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Japan). Amino acid alignment was performed using ClustalW software (European Bioinformatics Institute, Cambridge, UK). Percent identities between nucleotide and amino acid sequences were calculated using EMBOSS Needle, an online program. A putative N-terminal signal peptide in identical sequences was predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/), and functional motifs and domains were predicted using the ExPASy proteomics server (http://www.myhits.isb-sib.ch/cgi-bin/motif_scan).

Expression of recombinant MetAP1 protein and antibody production

The entire DNA fragment encoding the BmMetAP1 was subcloned into a pGEX-6P-2 vector (Amersham Pharmacia Biotech, USA) using the *Bam*HI and *Xho*I enzyme sites and expressed as glutathione S-transferase (GST)-fusion proteins in an *Escherichia coli* (*E. coli*) BL21 strain. The recombinant protein was purified from the soluble fraction of *E. coli* lysate using Glutathione Sepharose 4B beads (Amersham Biosciences, USA), and then endotoxins were removed using DetoxiGel™ Endotoxin Removing Gel (Piece Biotechnology, USA) in accordance with the manufacturer's instructions. The quantity of recombinant protein was measured using a Pierce BCA Protein Assay Kit (Pierce Biotechnology, USA).

Eight-week-old BALB/c mice were immunized i.p. with 100 μg of purified rBmMetAP1 or GST emulsified in 100 μl of Freund's complete adjuvant (Sigma-Aldrich, USA). Two boosters were given i.p. using 50 μg of the same antigens emulsified in Freund's incomplete adjuvant (Sigma-Aldrich, USA) at 14-day intervals. Sera were collected 2 weeks after the last booster and kept at −30 °C until use.

Enzyme assay

The aminopeptidase activity of BmMetAP1 was assayed fluorometrically based on the hydrolysis of L-methionine 4-methyl-coumaryl-7-amide (Met-MCA; Peptides International, Louisville, KY, USA) in a 96-well microplate format, as described previously (Munkhjargal et al. 2016a). Enzyme activity was assayed in buffers with various pHs, ranging from pH 5.0 to 9.0. Further, the requirement of metal ions for optimal BmMetAP1 activity and the effects of different protease inhibitors on BmMetAP1 activity were analyzed. Kinetic values were calculated using a Michaelis–Menten curve fit and a Lineweaver–Burk plot using GraphPad Prism 5 (GraphPad Software Inc., USA).

Western blot and immunofluorescence assays

rBmMetAP1 or whole parasite lysates were resolved by reducing 12 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Immunobilon™-P, Millipore, USA) using an electroblotter (HorizeBlot Type AE-6677) (Terkawi et al. 2014). The membranes were probed with *B. microti*-infected mouse serum or anti-rBmMetAP1 mouse serum (1:100). The membrane was reprobed with a secondary antibody (1:2000) of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Bethyl Laboratories, USA) and stained with 3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 (Dojindo, Japan). Acetone-fixed thin blood smears of the *B. microti* were probed with anti-rBmMetAP1 mouse serum (1:100), and bound antibodies were visualized with Alexa

Fluor® 488-conjugated goat anti-mouse IgG (1:2000) (Molecular Probes, Dallas, TX, USA). The parasite nuclei were visualized with propidium iodide (5 μg/ml) (Molecular Probes, USA). The slides were viewed with a confocal laser scanning microscope (TCS-SP5; Leica, Mannheim, Germany).

Enzyme-linked immunosorbent assay

The sera of mice were collected by tail vein bleeding prior to each immunization and before challenge for IgG response determination. Briefly, a flat-bottom 96-well plate was pre-coated with each 50 μl of rBmMetAP1 or rGST in a 50-mM carbonate–bicarbonate buffer (pH 9.6) overnight at 4 °C. The subsequent protocols were performed as described previously (Munkhjargal et al. 2016b).

Proliferation assay and cytokine detection

To evaluate the immunogenic efficacy of rBmMetAP1, BALB/c mice were immunized once with 100 μg of rBmMetAP1 and GST. After 14 days, the mice were killed, and their blood and spleens were obtained. Total IgG and subclass titers were measured by enzyme-linked immunosorbent assay (ELISA). Splenocytes were suspended in RPMI-1640 medium (Sigma-Aldrich, USA) supplemented with a 10 % fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. The mononuclear cells were isolated using Histopaque-1077 (Sigma-Aldrich, USA) in accordance with the manufacturer's instructions. The mononuclear cells (5×10^5) were cultured in 96-well plates at 37 °C for 48 h in 200 μl of medium in the absence of any stimuli and in the presence of different concentrations of antigens (5, 10, 15, or 50 μg/ml of rBmMetAP1 or GST), 5 μg/ml concanavalin A (Con A), and 10 μl of Cell Counting Kit-8 reagent (Dojindo, Japan). After 4 h of incubation at 37 °C in 5 % CO^2, the optimal density was determined using an ELISA reader at 450 nm. For cytokine detection, 1×10^6 mononuclear cells were cultured in 48-well plates for 48 h at 37 °C in 400 μl of medium either in the presence or in the absence of any stimuli. The release of cytokines in the supernatants of splenocytes derived from mice and the sera of immunized mice was measured using commercial ELISA kits (BioSource International, USA) in accordance with the manufacturer's instructions.

Vaccination and challenge infection

A total of 15 female BALB/c mice were divided into three groups ($n = 5$). In the treated group, 100 μg of rBmMetAP1 emulsified in 100 μl of Freund's complete adjuvant (Sigma-Aldrich, USA) was administered i.p. followed by three additional boosters (50 μg) i.p. without adjuvant at 14-day intervals. Control mice received either GST protein or no immunization. Two weeks after the final boosting, mice were challenged i.p. with 1×10^7 *B. microti*-infected erythrocytes. Parasitemia was monitored daily for 30 days by the examination of Giemsa-stained smears.

Statistical analysis

The significant differences (GraphPad Prism 5, GraphPad Software Inc., USA) among the means of all variables were examined with a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for the pairwise comparison of data from the multiple groups (Li et al. 2012). Results were considered to be statistically significant when $P < 0.05$.

Results and discussion

Bioinformatics analysis of the *B. microti MetAP1* gene

The *MetAP1* gene isolated from *B. microti* consisted of an open reading frame of 1086 bp, encoding polypeptides of 361 amino acids with a predicted molecular mass of 40.867 kDa with a predicted isoelectric point of 6.97. The SignalP 4.1 server showed that BmMetAP1 had no signal peptide. The secondary structure of BmMetAP1 is similar to those of other known enzymes (Vedro and Chang 2002; Chen et al. 2006; Kang et al. 2012, 2015; Munkhjargal et al. 2016a), consisting of an MYND-like zinc finger at a less-conserved N-teriminal domain, the signature of the MetAP1 and APP_MetAP subfamily at the highly conserved catalytic C-terminal domain, and a metallopeptidase family M24 domain but no transmembrane structure or signal peptide (Fig. 1a). Five amino acid residues responsible for metal binding were found in catalytic domains of BmMetAP1. The residues, including His^{179}, Asp^{196}, Asp^{207}, His^{270}, Gly^{303}, and Gly^{334}, were conserved in mammals (Fig. 1b). Homological analysis also showed over 47 % identities with mammalian MetAP1, indicating that BmMetAP1 possesses a similar function to that of mammalian MetAP1. In addition, BepiPred 1.0 server analysis predicted the presence of many potential B cell epitopes in the BmMetAP1 protein, suggesting it may be a good antigen for vaccine development.

Characterization of MetAP1 from *B. microti*

Recombinant MetAP enzymes expressed in *E. coli* are known to be suitable for the characterization of MetAPs of parasitic pathogens (Narayanan et al. 2008; Marschner and Klein 2015;

Fig. 1 Bioinformatics analysis of a translated BmMetAP1 polypeptide. **a** The predicted functional domains of BmMetAP1 are shown by BLASTp. **b** MAP amino acid sequences for *B. microti* (XP_0126492711) and *Homo sapiens* (NP_05595582) were aligned using the ClustalX program. Gaps were introduced to maximize the alignment. The putative zinc finger motifs are in the *blue box* on the sequence. The signature of the MetAP1 subfamily is in *bold*, and the metallopeptidase family M24 domain is in the *red box* on the sequence. The conserved metal-binding residues are shown in *triangles*

Kang et al. 2012, 2015; Munkhjargal et al. 2016a). In the present study, we have successfully expressed rBmMetAP1 protein with a molecular mass of approximately 66.8 kDa in *E. coli* as a GST-fusion protein (Fig. 2a, lane 1). rBmMetAP1 reacted with *B. microti*-infected mouse serum but not with uninfected mouse serum (Fig. 2a, lanes 2 and 3, respectively), suggesting the maintenance of antigenicity of *E. coli*-expressed rBmMetAP1. Furthermore, mouse anti-rBmMetAP1 reacted with *B. microti* lysates, yielding a specific single band that corresponded to an approximately 40.8 kDa native MetAP (Fig. 2a, lane 4). In contrast, pre-immunized mouse serum did not react with *B. microti* lysates (data not shown). On further analysis using IFAT and confocal microscopy, the mouse anti-BmMetAP1 serum probed with endogenous MetAP1 in *B. microti* yielded a specific green fluorescence, mainly in the parasite cytosol (Fig. 2b, upper panels). No reaction was observed when pre-immunized mouse serum was probed with endogenous MetAP1 in *B. microti* (Fig. 2b, lower panels).

Enzymatic profiles of rBmMetAP1

BmMetAP1 shared similar biochemical properties with MetAPs of other organisms (Kang et al. 2012, 2015), including *Babesia* (Munkhjargal et al. 2016a). BmMetAP1 was found to be catalytically active, as seen by hydrolysis of Met-MCA, the substrate used for the fluorometric determination of methionine aminopeptidases. The steady-state kinetic parameters for the BmMetAP1 enzyme revealed K_m values of 14.32 ± 7.809 and V_{max} values of 21.62 ± 15.06 with respect to the substrate, Met-MCA (Fig. 3a). Furthermore, the BmMetAP1 enzyme showed a broad optimal pH range (pH 7.5–8.5), with maximum activity at pH 7.5 (Fig. 3b). The activity of the enzyme was highly enhanced in the presence of divalent manganese cations; however, other metal ions showed less effect on BmMetAP1 activity (Table 1), suggesting that a divalent manganese cation is a main cofactor for BvMAP in vitro. Moreover, the activity of the BmMetAP1 enzyme was effectively inhibited by a metal chelator

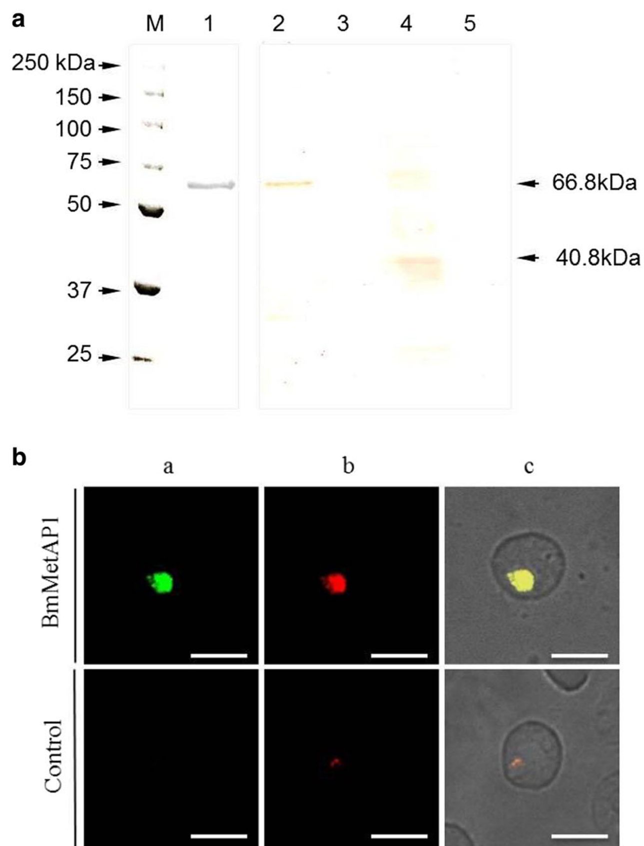

Fig. 2 Molecular characterization of a native BmMetAP1 enzyme. **a** SDS-PAGE and Western blot analysis. *Lane M*: molecular size marker. *Lane 1*: purified recombinant BmMetAP1 fused with GST after analysis by SDS-PAGE. *Lanes 2 and 3*: rBmMetAP1 reacted with *B. microti*-infected mouse serum but not with uninfected mouse serum, respectively. *Lanes 4 and 5*: the lysates of *B. microti* and non-parasitized murine erythrocytes, respectively, probed with anti-rBmMetAP1 serum raised in a mouse. **b** Immunofluorescence analysis of the endogenous BmMetAP1. The endogenous BmMetAP1 probed with anti-rBmMetAP1 mouse serum (*upper panels*) and pre-immunized mouse serum (*lower panels*). *a* shows specific green staining of the anti-rBmMetAP1, which is absent in the pre-immunized mouse serum. *b* shows parasite nuclei visualized with propidium iodide. *c* shows a merger of *a* and *b*. *Bars*, 5 μm

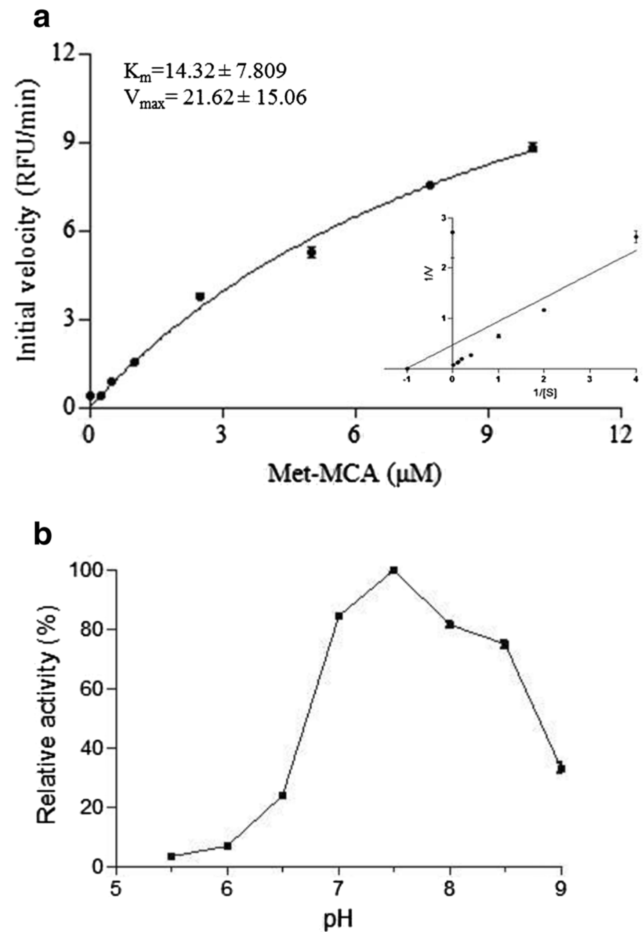

Fig. 3 Enzymatic profiles of BmMetAP1. **a** Kinetic values of BmMetAP1 for the substrate Met-MCA. BmMetAP1 (1 μg) was added to Tris–HCl (pH 7.5) containing 5 μM Met-MCA and 0.1 mM $MnCl_2$, and the enzyme activity was measured. **b** Optimal pH: the optimal pH for the maximum activity of BmMetAP1 was assessed in sodium acetate (pH 5.0–5.5), sodium phosphate (pH 6.0–6.5), Tris–HCl (pH 7.0–8.5), and glycine–NaOH (pH 9.0). For each pH, the appropriate blank without BmMetAP1 was individually measured as a control. Maximal activity is shown as 100 %

(ethylenediaminetetraacetic acid (EDTA)) but not by other classes of protease inhibitors (PMSF and E-64) (Table 2). Significantly, aminopeptidase inhibitors (amastatin and bestatin) partially inhibited the activity of BmMetAP1 enzymes in a dose-dependent manner (Table 2). No inhibition was observed for the assay mixture with the DMSO control (data not shown).

Immunogenicity of rBmMetAP1

rBmMetAP1 induced significantly higher antibody responses than did controls with GST immunization or no immunization. rBmMetAP1 elicited responses mainly consisting of total IgG and IgG2a, while GST immunization failed to elicit an IgG isotype response (Fig. 4a). Furthermore, mononuclear cells were obtained and cultured either in the presence or absence of stimuli to analyze cellular responses in vitro. While stimulation with 50 μg/ml rBmMetAP1 caused proliferation in tested groups, splenocytes obtained from rBmMetAP1-immunized mice showed significantly higher increases than those of control mice (Fig. 4b). Moreover, measurement of IFN-γ, IL-12, and IL-4 levels released after restimulation indicated significantly high levels of IFN-γ and IL-12, in contrast to control mice in which IFN-γ and IL-12 were not detected (Fig. 4c). Taken together, these results indicate that rBmMetAP1 might have an immunogenic effect specific to the Th1 response, as evidenced by the presence of the serum IgG2a and high levels of IFN-γ and IL-12 in the culture supernatant of splenocytes after priming with antigens. In addition, significant differences in the response of GST to

Table 1 Influence of divalent metal ions on *B. microti* MetAP1 activity

Metal ions	Concentrations (μM)	Relative activity (100 %)
None		100
Mn(II)	0.1	344.4 ± 295
	1	864.9 ± 808.6
Zn(II)	0.1	122.2 ± 87.9
	1	143.3 ± 114.7
Mg(II)	0.1	146 ± 87.9
	1	104.3 ± 88.4
Ca(II)	0.1	120.3 ± 117.3
	1	119.7 ± 117.8
K(II)	0.1	93.7 ± 84.6
	1	95.7 ± 85.9

The requirement of metal ions for optimal activity for BmMetAP1 containing one of the following metal chlorides: $CaCl_2$, KCl_2, $MgCl_2$, $MnCl_2$, and $ZnCl_2$. The relative enzyme activity was calculated compared to the control, which did not contain any metal ions or DMSO (dissolvent) in distilled water (0.01 %). All experiments were carried out in triplicate, and the mean and standard deviation (SD) was calculated

rBmMetAP1 and control indicated that MetAP has an adjuvant's capacity to induce a cellular immune response in the absence of an adjuvant. Similar findings that observed that rMetAP proteins induced cellular responses in cured hamsters infected with *Leishmania* and generated Th1-type cytokines from peripheral blood mononuclear cells of cured patients suggest that MetAP have strong vaccine potential for *Leishmania* (Gupta et al. 2012).

Table 2 Effect of protease inhibitors on *B. microti* MetAP1 activity

Inhibitors	Concentrations (μM)	Relative activity (100 %)
None		100
Amastatin	1	37.6 ± 27.4
	10	28.2 ± 22.7
Bestatin	1	35.9 ± 26.4
	10	28.6 ± 13.7
EDTA	1	37.8 ± 20.9
	10	31.3 ± 18.3
PMSF	0.1	82.8 ± 70.9
	1	79.3 ± 65.3
E-64	1	89.1 ± 74.6
	10	87.2 ± 75.5

BmMetAP1 enzyme pre-incubated with different concentrations of each protease inhibitors for 20 min at room temperature and the residual enzyme activity was measured. The following inhibitors were used in this study: phenylmethylsulphonyl fluoride (PMSF); *trans*-epoxy-succinyl-L-leucylamido (4-guanidino) butane (E-64); ethylenediaminetetraacetic acid (EDTA); bestatin ((−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-L-leucine); and amastatin (2S,3R)-3-amino-2-hydroxyl-5-methylhexanoyl-val-val-asphydrochloridehydrate) (Sigma-Aldrich). The relative enzyme activity was calculated compared to the control, which did not contain any inhibitors or DMSO (dissolvent) in distilled water (0.01 %). All experiments were carried out in triplicate, and the mean and standard deviation (SD) was calculated

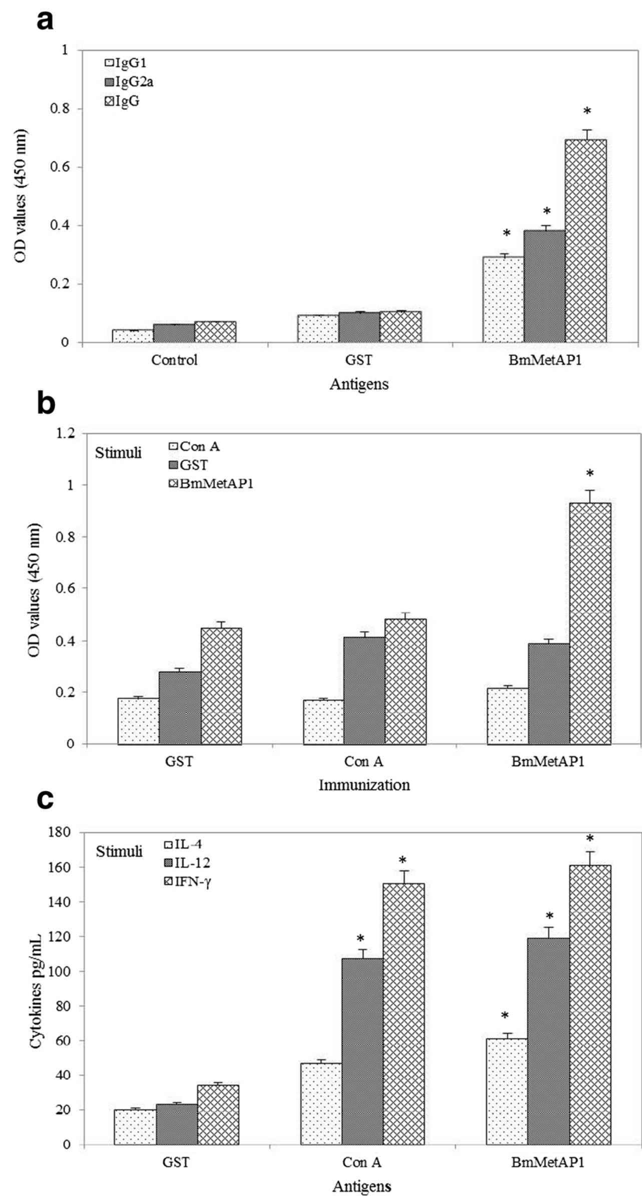

Fig. 4 The immunogenic efficacy of MetAP1 of *B. microti*. **a** ELISA determination of specific serum antibody responses of BALB/c mice with a single immunization of rBmMetAP1 and GST. **b** The specific splenocyte proliferative responses to rBmMetAP1 of immunized mice after restimulation with the same antigen in vitro. **c** The specific cytokine release of splenocytes after restimulation with the same antigen in vitro. Results are expressed as the mean of triplicate wells ± the standard deviation

Effective protection against *B. microti* infection after vaccination with rBmMetAP1

MetAP is an important target for chemotherapeutic drugs and vaccine candidates (Chen et al. 2006; Peng et al. 2010; Gupta et al. 2012; Kang et al. 2015). The vaccination efficacy of

rBmMetAP1 was evaluated against a challenge infection with *B. microti* in BALB/c mice. On day 14 after the last immunization with rBmMetAP1, the levels of total IgG and IgG2a were higher than with rGST immunization or no immunization in BALB/c mice (Fig. 5a). Moreover, rBmMetAP1 strongly induced IFN-γ and IL-12 responses but not an IL-4 response in BALB/c mice 2 weeks after the last immunization (Fig. 5b).

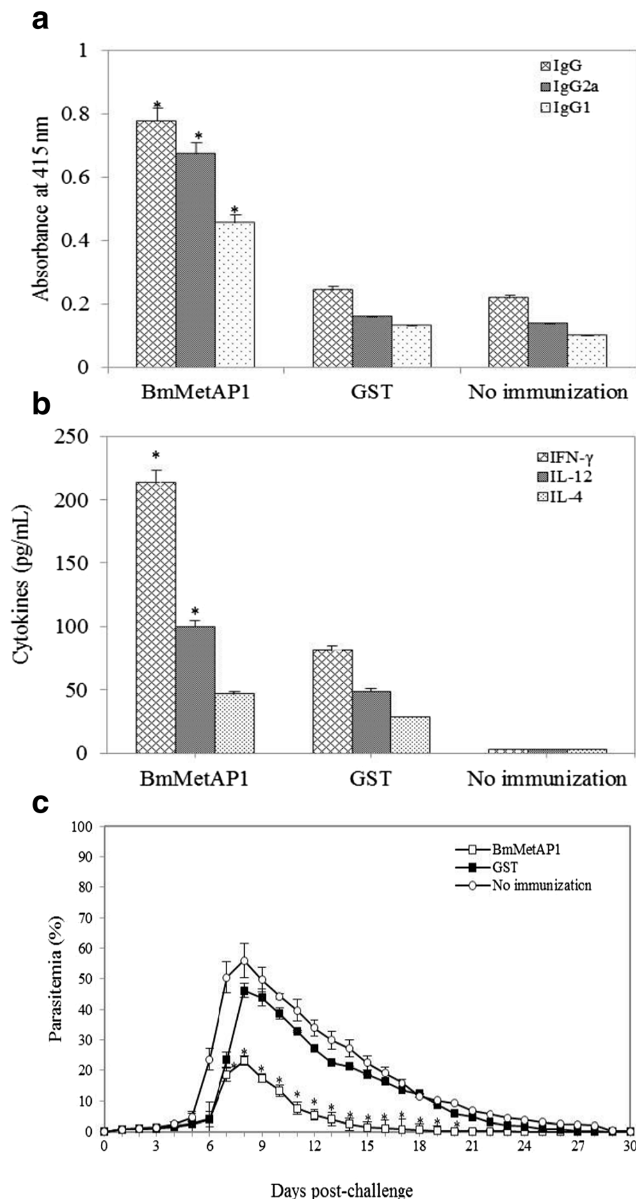

Fig. 5 Immunoprotective properties of rBmMetAP1 immunization on *B. microti* challenge infection in BALB/c mice. **a** ELISA determination of specific serum antibody responses to a protein immunization regime. **b** The specific cytokine productions (IFN-γ, IL-12, and IL-4) in the serum fraction of BALB/c mice immunized with rBmMetAP1 on day 14 after the last immunization. **c** Protective properties of rBmMetAP1 immunization on *B. microti* challenge infection in BALB/c mice. Each *bar* or *point* represents the mean ± SD, and each *asterisk* indicates a significant difference of five mice per group. Results are expressed as the mean of triplicate wells ± the standard deviation, and mean values were compared by one-way ANOVA

As a final test of the efficacy of rBmMetAP1 as a potential vaccine, all mice were challenged with 1×10^7 *B. microti*-infected erythrocytes 30 days after the last immunization, and parasitemia in the peripheral blood was monitored daily for 30 days. In rBmMetAP1-immunized mice, the levels of parasitemia were significantly lower as compared to those of the control mice that received either GST protein or no immunization ($P < 0.05$) from days 7 to 20 (Fig. 5c). Peak parasitemia reached an average of 46.2 and 56.1 % in the control groups (immunization with GST and no immunization, respectively) 8 days after the challenge infection and 23.1 % in the group immunized with rBmMetAP1 8 days postinfection. Parasites were completely eliminated 19 days postchallenge in the immunized groups. In contrast, the parasites were completely cleared 30 days postchallenge in the control groups (Fig. 5c). The protection elicited by rBmMetAP1 vaccination might be due to the strong Th1 response induced. Indeed, the early inducing of potent IL-12 and IFN-γ responses from $CD4^+$ T cell-driven immunity is hypothesized to play a critical role in controlling babesial infection by supporting high levels of immunoglobulin isotypes and the activation of macrophages, including their killing mechanisms (Igarashi et al. 1999; Terkawi et al. 2014). Therefore, rBmMetAP1 might be induced by a Th1 response containing high levels of IgG and cytokines (IFN-γ and IL-12), which is required for the control of *Babesia* infection.

In conclusion, we expressed and immunobiochemically characterized a member of the methionine aminopeptidase family of murine *Babesia* parasites and demonstrated its protective effects that seem to enhance Th1 responses based on high levels of cytokine (IFN-γ) and immunoglobulin (IgG2a) titer in the experimental model of human babesiosis. Our preliminary studies have indicated that rBmMetAP1 might be used as a potential vaccine candidate against human babesiosis, although more comprehensive studies are required to confirm this.

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Compliance with ethical standards All mice used in the present study were cared for in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine (Obihiro, Japan).

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