Bombyx mori Nucleopolyhedrovirus Displaying Neospora caninum Antigens as a Vaccine Candidate Against N. caninum Infection in Mice

Bombyx mori nucleopolyhedrovirus displaying *Neospora caninum* antigens as a vaccine candidate against *N. caninum* infection in mice

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Abstract Baculovirus display systems have been utilized for cell-specific gene transfer, regenerative medicine and as vaccine vectors. In particular, baculovirus particles displaying surface antigens have been used as vaccines against some parasites and viruses. In this study, *Bombyx mori* nucleopolyhedrovirus (BmNPV) particles displaying *Neospora caninum* antigens (NcSAG1, NcSRS2 and NcMIC3) purified from the hemolymph or fat body of silkworm larvae were prepared to vaccinate mice against *N. caninum*. Each antigen was expressed on the surface of BmNPV particles through glycoprotein 64 (GP64) transmembrane and cytoplasmic domains. Antigen-specific antibody production was induced in mice by immunization with each recombinant BmNPV particle. NcMIC3-displaying BmNPV particles purified from the fat body induced a lower antibody titer than particles purified from the hemolymph. Antigen-specific IgG2a was predominantly produced in mice by immunization with NcSAG1-displaying BmNPV particles compared to IgG1, and induction of IFN-γ was dominant, indicating that antigen-displaying BmNPV particles can elicit a Th1 immune response in mice. Semi-quantitative PCR analysis revealed that immunization with each antigen-displaying BmNPV particle partially protected mice from cerebral *N. caninum* infection. These results suggest that antigen-displaying BmNPV particles can provide an alternative method of controlling neosporosis in cattle and represent a new generation of *N. caninum* vaccines.

Keywords: *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid・antigen displaying baculovirus・silkworm・*Neospora caninum*・vaccine

Introduction

Neospora caninum is classified in the phylum Apicomplexa as a protozoan parasite and causes neosporosis in cattle worldwide. The main symptom of neosporosis in cattle is abortion [1, 2]. The canids infected with *N. caninum* shed unsporulated oocysts in the feces, and sporulated oocysts are ingested by cattle, and tachyzoites transformed from sporozoites subsequently invade the gut wall, which lead to neosporosis in cattle. In addition, cysts formed from bradyzoites that are differentiated from tachyzoites lead to vertical transmission of *N. caninum* and the birth of congenitally *N. caninum*-infected calves. These cysts *N. caninum* have profound impacts on the economic performance of dairy and beef industries [3].

Initial research focused on diagnosis of the disease, but control and prevention of infection and abortion due to *N. caninum* have been the goals of recent studies [4]. Inactivated vaccine NeoGuard^{TM} used to be commercially available, but is now is not used because of moderate effect in field trials [5]. Recently, a new vaccine against *N. caninum* composed of dense granule protein 7 (NcGRA7) [6, 7], produces antibodies to NcGRA7 and induces IFN-γ production to protect cattle infected with *N. caninum* [6]. NcGRA7 was identified as a 17 kDa immunodominant antigen of *N. caninum* tachyzoites and expressed as a secretory protein in tachyzoites and bradyzoites [8, 9].

Surface antigen 1 (NcSAG1) and SAG1-related sequence 2 (SRS2) are expressed on the surface of *N. caninum* tachyzoites and vaccination trials using these antigens have been performed [10, 11]. In particular, SRS2 has been

suggested as an effective vaccine candidate following successful trial with lipoproteins in cattle to induce T-cell activation and IFN-γ production [12, 13]. In addition, microneme proteins (MICs) have also been selected as vaccine candidates [4, 14]. NcMIC1 and NcMIC3 were shown to prevent cerebral *N. caninum* infection in mice, although similar experiments have not been performed in cattle [15, 16].

To protect from parasite infection, baculoviruses have been utilized recently as vaccines. *Plasmodium yoelii* 19 kDa carboxyl terminus of merozoite surface protein 1 ($PyMSP1_{19}$) was displayed on the surface of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which induced production of a high titer of $PyMSP1_{19}$-specific antibody by intranasal immunization [17]. Recently AcMNPV displaying vesicular stomatitis virus G protein (VSV-G) and SAG1 protected mice from *T. gondii* infection [18]. These results show that baculoviruses are promising tools to prevent and protect against parasite infection because of their innate adjuvant capacity to induce humoral immune responses.

In this study, NcSAG1, NcSRS2 and NcMIC3 from *N. caninum* were displayed on the surface of *Bombyx mori* nucleopolyhedrovirus (BmNPV) particles. These BmNPV particles were produced in silkworm larvae using the BmNPV bacmid system [19] and injected into mice to induce antigen-specific antibody and humoral immune responses. The effects of these viruses as vaccines on cerebral *N. caninum* infection were investigated after a *N. caninum* challenge.

Materials and Methods

Materials

N. caninum Nc-Liverpool isolate (Nc-Liv; ATCC No. 50845), *N. caninum* Nc-1 genomic DNA and Vero cells (ATCC No. CCL-81) were purchased from American Type Culture Collection (Rockville, MD, USA). Oligonucleotides were purchased from Operon Biotechnology (Tokyo, Japan). Restriction enzymes and modification enzymes were purchased from Nippon gene (Tokyo, Japan), Takara-bio (Shiga, Japan), and Toyobo (Osaka, Japan). Other reagents were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma Aldrich Japan (Tokyo, Japan).

Construction of recombinant BmNPV bacmids

To display each antigen on the surface of BmNPV, antigen-GP64 fusion protein genes were constructed. The BmNPV GP64 gene lacking its putative signal sequence was amplified by PCR using pFB/GP64 F and pFB/GP64 R primers (Table 1). The gene coding GP64 transmembrane and cytoplasmic domains (31 amino acids at the C-terminus) was also amplified using pFB/Gp64 TM F and pFB/GP64 R primers (Table 1). Each amplified fragment was inserted at the *Hin*d III site in a pFastbac 1 vector (Life Technologies Japan, Osaka, Japan), and pFB/GP64 FL and pFB/GP64 TM, full-length GP64, and GP64 transmembrane and cytoplasmic domains were constructed.

NcSAG1 (Genbank: AF132217) and NcSRS2 (Genbank: JQ410454) were

amplified by PCR using recombinant bacmid DNA constructed previously [20] and pFB/Bx signal F, pFB/SAG1 R and pFB/SRS2 R primers (Table 1). Amplified NcSAG1 and NcSRS2 genes have the Bombyxin signal peptide sequence from *B. mori* (Bx signal peptide sequence), FLAG tag sequence and human rhinovirus (HRV) 3C protease recognition sequence at the N-terminus instead of its native signal sequence. Here, NcSAG1 and NcSRS2 GPI anchorage sequences were removed. The NcMIC3 gene (Genbank: XM_003880575) lacking the putative signal peptide sequence was amplified by PCR using MIC3-F and MIC3-R primers (Table 1) and genomic *N. caninum* Nc-1 DNA as a template. Amplified NcMIC3 was inserted into the *Kpn* I-*Not* I site in a pET52b vector. The NcMIC3 gene containing a Bx signal sequence, FLAG tag sequence and HRC 3C protease recognition sequence was amplified by PCR using CACC-Bx-FLAG-HRV3C F and MIC3-R primers (Table 1) and the constructed vector as a template. The amplified MIC3 gene was inserted into a pENTR/D/TOPO vector (Life Technologies, Japan). To fuse with the GP64 gene, MIC3 containing the Bx signal sequence, FLAG tag sequence and HRC 3C protease recognition sequence was amplified by PCR using pFB/Bx signal F and pFB/MIC3 R primers and the constructed pENTR vector as a template.

Each amplified antigen gene was inserted into the *Eco*R I-*Sal* I site in pFB/GP64 FL and pFB/GP64 TM. Each resulting plasmid was transformed into an *Escherichia coli* BmDH10Bac strain [19] to construct recombinant BmNPV bacmids. BmNPV/SAG1-GP64FL, BmNPV/SRS2-GP64FL, BmNPV/MIG3-GP64FL, BmNPV/SAG1-GP64TM, BmNPV/SRS2-GP64TM and BmNPV/MIG3-GP64TM were constructed. Antigen-GP64FL and -GP64TM

denote that the antigen was fused to full-length GP64 and the transmembrane and cytoplasmic domains of GP64, respectively.

Production of recombinant BmNPV particles

Approximately 10 μg of extracted BmNPV bacmid (containing a helper plasmid) was mixed with one-tenth volume of DMRIE-C reagent (Life Technologies, Japan), and injected (~50 μl) into a fifth instar silkworm. Injected larvae were reared for 6–7 days with Silkmate S2 (Nohsan Corporation, Yokohama, Japan), and hemolymph and fat body were collected. Hemolymph and fat body were used to produce recombinant BmNPV particles and confirm antigen-GP64 fusion protein expression. Collected hemolymph was diluted to 1×10^5 pfu with phosphate-buffered saline (PBS, pH 7.4). The diluted hemolymph was injected again into fifth instar silkworms and reared for 4 days. Hemolymph and fat body were collected to purify recombinant BmNPV particles.

Purification of recombinant BmNPV particles and measurement of virus titer by semi-quantitative real-time PCR

For NcSAG1- or NcSRS2-displaying BmNPV particles, hemolymph was loaded onto a 20% sucrose cushion and centrifuged at 112,000 × g at 4°C for 1 h to collect recombinant BmNPV particles. The pellet was suspended in PBS by sonication and loaded onto a 20–60% sucrose density gradient and centrifuged at 122,000 × g at 4°C for 3 h. A white band was collected as the viral solution and centrifuged again at 112,000 × g at 4°C for 1 h to collect recombinant BmNPV particles. The pellet was suspended in PBS by sonication and dialyzed against

PBS using a 300 kDa cut-off dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA, USA) to remove free antigens that were not displayed and only attached to the surface of BmNPV particles.

NcMIC3-displaying BmNPV particles were purified from fat body homogenate. Fat body suspended in PBS was disrupted by sonication. The homogenate was centrifuged at 30,000 × g at 4°C for 20 min to remove debris. From the supernatant, NcMIC3-displaying BmNPV particles were purified by the same method as NcSAG1- and NcSRS2-displaying BmNPV particles described above.

Recombinant BmNPV particle titers were measured by semi-quantitative real-time PCR [21]. Primers sets are shown in Table 1.

SDS-PAGE and western blot

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using either 10% (w/v) or 12% (w/v) acrylamide that was subsequently subjected to western blotting. After SDS-PAGE, proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). The membrane were blocked in 5% (w/v) skimmed milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST), the membrane was incubated for 1 h in either 1:10000 diluted mouse anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) or 1:4000 diluted rabbit anti-BmNPV GP64 polyclonal antibody (Biogate, Gifu, Japan). The membrane was washed with TBST and incubated for 1 h in 1:20000 diluted anti-mouse or anti-rabbit IgG antibody labeled with

horseradish peroxidase (GE Healthcare, Buckinghamshire, UK). Detection was performed using ECL Plus Western blotting reagent (GE Healthcare). Specific bands were detected on a Fluor-S MAX MultiImager (Bio-Rad).

Enzyme-linked immunosorbent assay

The solution containing antigen-displaying BmNPV particles (1×10^7 pfu) was placed into a 96-well plate and incubated at 37°C for 1 h to immobilize particles. The supernatant was removed and background was blocked using 2% (w/v) skimmed milk in TBST for 1 h. The skimmed milk solution was removed and mouse anti-FLAG M2 antibody (Sigma-Aldrich) diluted 2000-fold with 2%(w/v) skimmed milk in TBST was added to each well. After incubation at room temperature for 1 h, the antibody solution was removed and each well was washed with TBS. Anti-mouse IgG antibody-HRP (GE Healthcare) diluted 2000-fold with TBST was added to each well and incubated at room temperature for 1 h. Wells were washed with TBST followed by HRP reaction. One hundred microliters of substrate (0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine in 100 mM sodium acetate, pH 6.0, with 0.2% (v/v) of 30% hydrogen peroxide) was added to each well and left at room temperature for development of blue coloration. The reaction was stopped by addition of 50 µl 1N H_2SO_4 solution and resulting color was measured at an optical density (OD) of 450 nm.

In the other method, 100 µl of 2 µg/ml mouse anti-DDDDK tag polyclonal antibody (Medical & Biological Laboratories, Nagoya, Japan) in 10 mM carbohydrate buffer (pH 9.6) was added to each well in a 96-well plate, followed

by incubation at 4°C overnight to immobilize the antibody. The supernatant was removed and each well was blocked as mentioned above. The skimmed milk solution was removed and each well was washed with PBS. Next, 1 $\times 10^7$ pfu recombinant BmNPV particles were added to each well and incubated with shaking at room temperature for 1 h. The virus solution was removed and each well was washed with TBST. Serum from mice immunized with BmNPV diluted 200-fold with TBST was added to each well and incubated at room temperature for 1 h. After washing each well with TBST, anti-mouse IgG antibody conjugated with HRP (GE Healthcare) diluted 2000-fold was added into each well and incubated at room temperature for 1 h. Each well was washed with TBST, followed by the HRP reaction as described above.

To confirm IgG subclasses, 100 ng of each purified antigen was immobilized in wells in a 96-well plate blocked using 2% skimmed milk in TBST. After washing each well with TBST, serum from antigen-immunized mice was added to each well. HRP-conjugated anti-mouse IgG1 and IgG2a antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) were used as a secondary antibody.

Immunization of mice with recombinant BmNPV particles

All mice used in the present study were treated under the guiding principles for the care and use of research animals promulgated by the Nippon Veterinary and Life Science University, Japan. Female BALB/c mice (6 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed under conventional day/night conditions. At the age of 7 weeks, mice were randomly distributed into 6 experimental groups of 12 mice each. Groups 1–5 were

242 inoculated with the immunogens BmNPV/SAG1-GP64TM,
243 BmNPV/SRS2-GP64TM, BmNPV/MIC3-GP64TM, BmNPV only or PBS in
244 Freund's incomplete adjuvant (FIA, adjuvant control group). Mice in Group 6
245 received PBS alone (infection control group). One hundred microliters of
246 recombinant BmNPV particles (1×10^8 pfu) were injected into the mice (BALB/c,
247 7 weeks, female) intramuscularly. Injection was performed three times every two
248 weeks for immunization. Whole blood was collected two weeks after the last
249 injection, and serum was prepared by centrifugation at 1,000 × g.

250 Preparation and indirect immunofluorescence detection of *N. caninum*

251 Vero cells were cultivated in T-25 flasks at confluence using MEM medium (Life
252 Technologies Co., Tokyo, Japan) supplemented with 50 U/ml
253 penicillin-streptomycin (Life Technologies) and 5% (v/v) horse serum (Life
254 Technologies). Medium was replaced with MEM medium supplemented with 50
255 U/ml penicillin-streptomycin and 1% (v/v) horse serum and cells were cultivated
256 for two weeks after adding *N. caninum*. Cells were collected and disrupted by
257 flashing through a 26S needle (Fisher Scientific UK Ltd., Loughborough, UK)
258 several times. The homogenate was filtered through a 5 μm filter (Adevantec,
259 Tokyo, Japan) to remove debris, and the filtrate was used as the parasite
260 solution.

261 Parasites suspended in PBS were immobilized onto an APS-coated glass
262 slide (Matsunami Glass Ind., Osaka, Japan) and washed three times with PBS.
263 Parasites were fixed with 4% (v/v) paraformaldehyde and blocked with 4% (v/v)

BSA in PBS supplemented with 25 mM NH_4Cl. After washing with PBS three times, serum from immunized mice, which was diluted 200-fold with 2% (w/v) BSA in PBS, was added to glass slides and incubated at room temperature for 1 h. The slides were washed three times with PBS, and parasites were incubated at room temperature for 1 h with anti-mouse IgG conjugated with Alexa Fluor 594 diluted 200-fold (Jackson ImmunoResearch, Laboratories, West Grove, PA, USA). In addition, parasites were incubated with 1 μg/ml DAPI to stain the nucleus. Fluorescence was observed using confocal laser scanning microscopy (LSM700, Carl Zeiss Japan, Tokyo, Japan).

Measurement of cytokines

Cytokine levels were measured in splenocytes seeded at 1×10^6 cells per well in Hybridoma-SFM medium (Invitrogen-Gibco BRL, Gaithersburg, MD, USA) containing 10 % (w/v) FCS with or without 10 μg/ml of each recombinant antigen for cells isolated from the vaccinated groups or Concanavalin A (ConA; Sigma-Aldrich Corporation, St. Louis, MO, USA) for both control groups in 24-well microplates (Corning Incorporated, Corning, NY, USA). Microplates were kept at 37 °C for 48 h in a 5% (v/v) CO_2 atmosphere. After stimulation, interferon (IFN)-γ and interleukin (IL)-4 levels in the culture supernatant were measured using IFN-γ and IL-4 enzyme-linked immunoassay (ELISA) kits (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions (assay range: 37 to 3700 pg/ml, sensitivity: <10 pg/ml). The amount of secreted cytokine was calculated using standard cytokine calibration curves run on the same immunoplate.

N. caninum exposure in mice and semi-quantitative real-time PCR using *N. caninum* DNA from immunized mouse brain

Mice were immunized with 1×10^8 pfu (100 μl) of each BmNPV particle diluted in PBS intramuscularly three times every two weeks. After immunization, 2×10^6 *N. caninum* Nc-Liv isolate was injected intraperitoneally into immunized mice and reared for 5 weeks. Blood and brains were collected and serum was prepared from the blood.

DNA was extracted from the brain using the DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan). Semi-quantitative real-time PCR was performed using *N. caninum*-specific primers (Table 1) and 2×Full Velocity SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). Seven hundred nanograms of extracted DNA were used as a template. PCR conditions were as followed: 1st cycle 95°C 5 min, 2nd cycle 95°C 10 s, 60°C 30 s, 80 cycles. The DNA fragment amplified by semi-quantitative PCR was verified as a single band by melting curve analysis and agarose gel electrophoresis.

Results and Discussion

Construction of recombinant BmNPV bacmids

Recombinant BmNPV bacmids were constructed according to the protocol described. Each antigen was fused at the C-terminus with full-length GP64 or transmembrane and cytoplasmic domains for display on the surface of BmNPV. NcSAG1and NcSRS2 have signal peptide sequences at the N-terminus and a

GPI anchorage sequence at the C-terminus respectively and was replaced with the Bombyxin Bx signal peptide sequence from *B. mori* as described in a previous study [20]. GPI anchorage sequence was removed to be fused with GP64. The native signal peptide sequence in NcMIC3 was replaced with the bx signal peptide sequence. Constructs of the expressed proteins in this study are shown in Fig. 1.

Antigen display on the BmNPV surface

Purified BmNPV/SAG1-GP64FL and BmNPV/SAG1-GP64TM particles were detected by western blot using an anti-FLAG M2 antibody and anti-BmNPV GP64 polyclonal antibody, respectively (Fig. 1A and B). SRS2-GP64TM and MIC3-GP64TM were detected in purified antigen-displaying BmNPV particles by western blot using an anti-FLAG M2 antibody (Fig. 1C and D).

Both SAG1 fusion proteins were detected by ELISA using purified BmNPV/SAG1-GP64FL and BmNPV/SAG1-GP64TM particles and compared to BmNPV particles (no display). A 7-fold higher level of SAG1-GP64TM was displayed than SAG1-GP64FL based on A450 OD values (Fig. 2A). GP64TM fused-SRS2 and -MIC3 on the surface of BmNPV particles were also confirmed by ELISA (Fig. 2B). SRS2 and MIC3 were displayed on the surface of BmNPV particles using the transmembrane and cytoplasmic domains rather than full-length GP64. SRS2-GP64TM levels were 30% lower than SAG1-GP64TM in each purified BmNPV sample (Fig. 2B), indicating that SAG1 tends to be displayed more efficiently compared to SRS2. In a previous study, a 4.6-fold

higher amount of SAG1 was purified than SRS2 [20]. These results suggest that the efficiency of antigen display on the particle surface may be related to expression levels of the antigen.

MIC3-displayed BmNPV particles were not purified from hemolymph, but from fat body homogenate, meaning that MIG3-GP64TM cannot be displayed on the surface of BmNPV particles efficiently. The purified BmNPV/MIG3-GP64TM particles have not complete envelopes but only incomplete envelopes, because they could not be secreted into hemolymph. Purified BmNPV samples from fat body still contained both MIC3-GP64TM protein and GP64 even after dialysis using a 300 kDa cut-off membrane to remove free MIC3 and MIC3-GP64TM proteins. This indicates that BmNPV/MIC3-GP64TM particles purified from fat body homogenate have an incomplete envelope that retains MIC3-GP64TM protein and GP64. Free MIC3-GP64 could exist in purified BmNPV/MIC3-GP64TM particles because MIC3-GP64TM expression level in purified recombinant BmNPV samples was higher than SAG1-GP64TM (Fig. 1A and D). The amount of SAG1-GP64TM displayed on the surface of 1×10^8 pfu BmNPV/SAG1-GP64TM was 48.6 ng as determined by sandwich ELISA using an anti-DYKDDDDK tag polyclonal antibody and serum from mice immunized with BmNPV (data not shown).

Immunization with antigen-displaying BmNPV particles and antigen-specific antibody production in immunized mice

Mice were immunized with 1×10^8 pfu (100 μl) of each purified BmNPV particle three times, and antigen-specific antibody production was measured by ELISA

(Fig. 3). All three types of BmNPV particles induced each antigen-specific antibody. BmNPV/SAG1-GP64TM induced the highest antigen-specific antibody titer (Fig. 3B). The MIC3-specific antibody titer in serum from mice immunized with BmNPV/MIC3-GP64TM was 10–100-fold lower compared to other titers (Fig. 3D). This indicated that BmNPV particles purified from fat body homogenate are not effective in inducing antigen-specific antibody production.

Indirect immunofluorescence staining of *N. caninum* was performed using serum from mice immunized with BmNPV/SAG1-GP64TM or BmNPV/SRS2-GP64TM (Supplementary Fig. 1). Both sera samples stained the peripheral region of *N. caninum,* including the positive control (anti-*N. caninum* antibody), indicating recognition of each antigen on the surface of the parasite and antigen-specific and anti-*N. caninum* antibodies.

Immunoglobulin and cytokine production in immunized mice

IgG subclasses in sera from mice immunized with BmNPV/SAG1-GP64FL or BmNPV/SAG1-GP64TM was investigated (Fig. 4). More IgG2a was detected in sera from mice immunized with BmNPV/SAG1-GP64FL or BmNPV/SAG1-GP64TM compared to that of IgG1. In addition, levels of secreted IFN-γ□ and IL-4 were measured by stimulation with each antigen or ConA in splenocytes from mice immunized with each recombinant BmNPV particle (Fig. 5). IFN-γ levels were significantly increased in splenocytes from mice immunized with BmNPV/SAG1-GP64TM or BmNPV/SRS2-GP64TM particles compared to splenocytes from mice immunized with BmNPV/MIC3-GP64TM or PBS,

375 although IL-4 levels were almost the same in all four samples. These results
376 indicate that the Th1 immune response dominated compared to the Th2 immune
377 response when mice were immunized with BmNPV/SAG1-GP64TM or
378 BmNPV/SRS2-GP64TM particles. Difference in immune response between
379 BmNPV/MIC3-GP64TM and BmNPV/SRS2-GP64TM might result from the
380 expression from fat body and from hemolymph, respectively. It was previously
381 reported that AcMNPV particles elicited IFN-γ production in mammalian cells,
382 leading to anti-viral activity [22]. Moreover, AcMNPV particles with antigens also
383 have strong adjuvant activity to induce humoral and cellular immune responses
384 to antigens [23]. AcMNPV displaying four types of hemagglutinin (HA) from
385 several influenza viruses induced high levels of IFN-γ production and a
386 HA-specific $CD8^+$ cell response, resulting in 100% protection of mice against a
387 lethal influenza virus challenge [24]. Induction of IgG2a production by
388 immunization with each recombinant BmNPV particles corresponds to the
389 previous report in which baculovirus particles elicited a Th1 immune response in
390 mice. Intranasal immunization of baculovirus particles induced Th1/Th2 type
391 immunity and mucosal IgA production in previous studies [17, 25].

392 *N. caninum* challenge in immunized mice

393 After immunization with BmNPV/SAG1-GP64TM or BmNPV/SRS2-GP64TM,
394 mice were infected with 2×10^6 *N. caninum*. The mice did not show obvious
395 clinical signs after vaccination or the challenge infection. Five weeks after the *N.*
396 *caninum* challenge, the cerebral parasite burden was measured by
397 semi-quantitative real-time PCR. The *N. caninum* burden was significantly lower

using recombinant BmNPV particles than using FIA and PBS (Fig. 6), indicating that each antigen-displaying BmNPV particle (especially SAG1) can suppress *N. caninum* infection in mice. However, BmNPV particles (no display) were also able to suppress *N. caninum* infection in mice to the same extent as each antigen-displaying BmNPV particle. Optimization of immunization of recombinant BmNPV particles has to be investigated to maximize the immunity of recombinant BmNPV particles. Baculovirus particles can induce humoral and cellular responses in mice due to their strong inherent adjuvant activity [23, 26].

In this study, BmNPV particles displaying antigens (NcSAG1, NcSRS2, MIC3) fused with transmembrane and cytoplasmic domains of GP64 were purified from silkworm larval hemolymph. Immunization of mice with these purified recombinant BmNPV particles elicited antigen-specific IgG2a and IFN-γ production, except for MIC3-displaying BmNPV particles, which induced low level of MIC-specific antibodies and did not elicit IFN-γ□ production. In addition, the cerebral *N. caninum* burden decreased in mice immunized with each antigen-displaying BmNPV particle following a *N. caninum* challenge in mice. These results suggest that BmNPV particles displaying the *N. caninum* antigen could protect against parasitic infection. However, we did not test prevention of vertical *N. caninum* transmission in pregnant mice in this study. Vertical transmission of parasite is one of the most important factors causing *N. caninum* infection. Congenitally infected cattle leads to reproductive loss because abortion and dead fetuses often occur. The Th1 immune response appears to be involved in protection against *N. caninum* [7]. In addition, *N. caninum*-specific antibodies are contributing factors that protect against this parasite by inhibiting

entry in host cells [27, 28]. These indicate that both Th1 and Th2 immune responses are necessary to effectively protect and prevent cattle from neosporosis. Here using a mice model, antigen-displaying BmNPV particles purified from silkworm larvae showed potential as a vaccine against *N. caninum* due to the production of *N. caninum*-specific antibodies and IFN-γ. However, this BmNPV system should be improved to induce IL-4 production and a Th2 immune response. Recently, baculoviruses have been improved as dual vectors for antigen expression and display [29]. These baculoviruses display antigens on the surface and have the antigen gene under control of the mammalian promoter to function in mammalian cells, indicating their potential as both subunit and DNA vaccines. The most prominent example is human malaria vaccines that use baculoviruses. AcMNPV displaying the *Plasmodium falciparum* circumsporozite (CS) protein-GP64 fusion protein with the CS protein gene controlled by the CMV promoter was constructed [30]. AcMNPV induced a high titer of CS protein-specific antibody and CS-specific $CD4^+$ and $CD8^+$ T cell responses. Baculovirus can prevent and protect cattle from *N. caninum*.

Conflict of Interest

The authors declare no Conflict of Interest.

Supplementary Fig. 1. Indirect immunofluorescence detection of *N. caninum* using serum from mice immunized with recombinant BmNPV particles. *N. caninum* was treated with serum and stained with DAPI and anti-mouse IgG conjugated with Alexa Fluor 594 as a secondary antibody. Figures with merged images of DIC, DAPI staining and Alexa Fluor 594 are shown. Bars indicate 5 μm. (A) No display, (B) positive control, (C) SAG1-GP64TM, and (D)

445 SRS2-GP64TM.

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Table 1

Primers

Name	5’-3’
pFB/GP64 F	TATAAGCTTATGGCGGAGCACTGCAAC
pFB/GP64 R	TATAAGCTTTTAATATTGTCTACTATTA
pFB/GP64 TM F	TATAAGCTTTTCATGTTTGGTCATGTA
pFB/Bx-signal F	ATAGAATTCATGTGGTGGAGGCTTTG
pFB/SAG1 R	ATAGTCGACCCCGCGACGCCAGCCGCTA
pFB/SRS2 R	ATAGTCGACCCGTACGCAAAGATTGCCGT
MIC3-F	TAT GGTACC GA CGTGGCGGGGCGTCCGCTC
MIC3-R	TAT GCGGCCGC TTATCGAGCCGTTCCGCAT
CACC-Bx-FLAG-HRV3C-F	CACCATGAAGATACTCCTTGCTATTGCATTAAT
	GTTGTCAACAGTAATGTGGGTGTCAACAGACT
	ACAAGGATGACGATGACAAGGGTGCACTTGA
	AGTCCTCTTTCAG
pFB/MIC3 R	ATAGTCGACCCTCGAGCCGTTCCGCATT
Bm ie-1-F	CCCGTAACGGACCTTGTGCTT
Bm ie-1-R	TTATCGAGATTTATTTACATACAACAAG
NC-F	GTGAGAGGTGGGATACG
NC-R	GTCCGCTTGCTCCCTA

Underlined sequences indicate restriction enzyme sites used to clone PCR-amplified genes into vectors.

Figure legends

Fig. 1 Detection of each antigen-GP64 fusion protein in BmNPV particles purified from hemolymph or fat body by western blot. Recombinant BmNPV particles purified from silkworm larval hemolymph were applied to SDS-PAGE and antigen-GP64 fusion proteins were detected by western blot using a mouse anti-FLAG M2 antibody or rabbit anti-BmNPV GP64 polyclonal antibody. TM indicates the GP64 transmembrane and cytoplasmic domains from BmNPV. (A) BmNPV/SAG1-GP64FL, (B) BmNPV/SAG1-GP64TM, (C) BmNPV/SRS2-GP64TM, and (D) BmNPV/MIC3-GP64TM.

Fig. 2 Detection of each antigen on the surface of recombinant BmNPV particles by ELISA. (A) BmNPV/SAG1-GP64FL and BmNPV/SAG1-GP64TM, (B) BmNPV/SAG1-GP64TM, BmNPV/SRS2-GP64TM and BmNPV/MIC3-GP64TM. BmNPV, which has a polyhedrin gene and no foreign genes, was used as a negative control (“No display”).

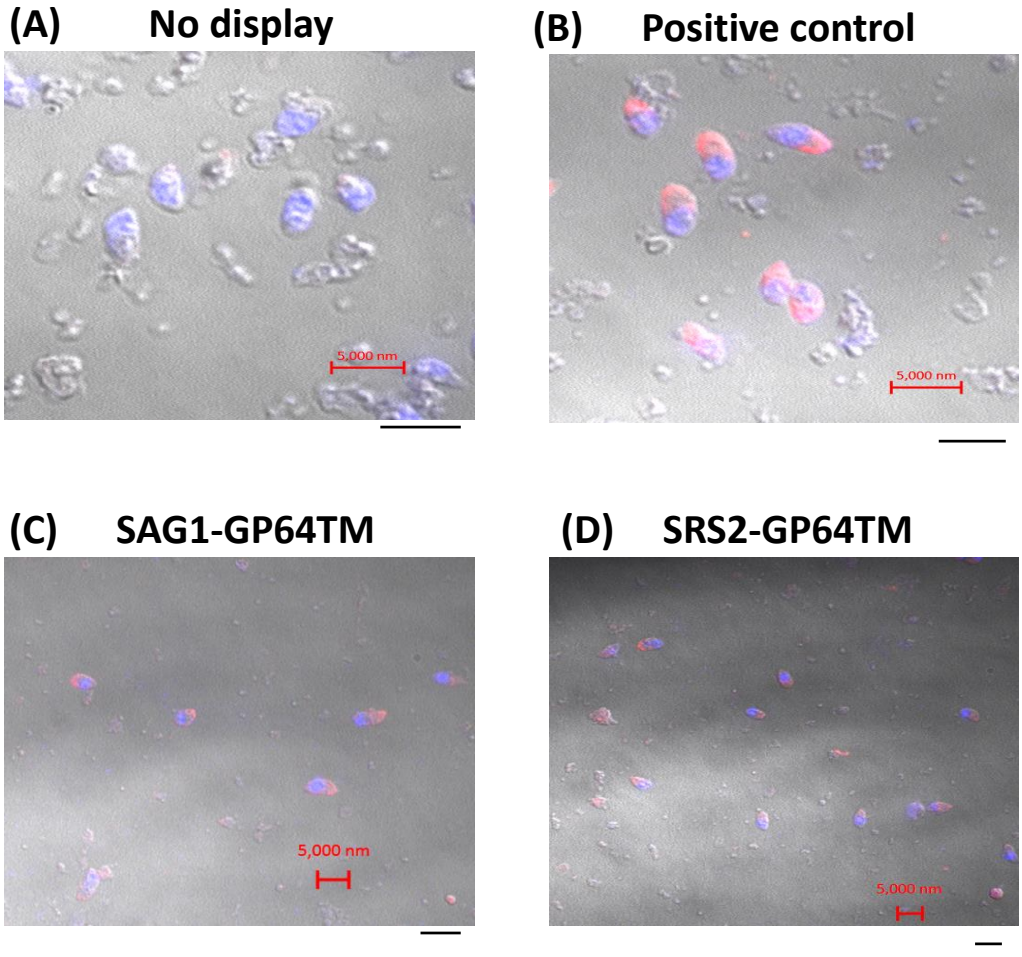
Fig. 3 Reactivity of antigen-specific antibodies elicited by immunization of mice with recombinant BmNPV particles. Each antigen was immobilized on the plate and antibody titer was determined by ELISA. Four mice were immunized with each recombinant BmNPV particle. White circles: No display BmNPV; black symbols: antigen-displaying BmNPVs (n = 4). (A) BmNPV/SAG1-GP64FL, (B) BmNPV/SAG1-GP64TM, (C) BmNPV/SRS2-GP64TM, and (D) BmNPV/MIC3-GP64TM.

Fig. 4 IgG subclasses in serum from mice immunized with recombinant BmNPV

particles. HRP-conjugated anti-mouse IgG1 (gray bars) and IgG2a antibodies (black bars) were used to determine IgG subclasses by ELISA.

Fig. 5 The amount of IFN-γ (A) and IL-4 (B) secreted into the culture medium from mice splenocytes immunized with each recombinant BmNPV particle. Splenocytes were isolated from mice immunized with each antigen-displaying BmNPV particle and stimulated with each antigen for 48 h. Secreted IFN-γ and IL-4 were quantified using mouse IFN-γ and mouse IL-4 ELISA kits, respectively. * $P < 0.05$, ** $P < 0.01$.

Fig. 6 Quantification of cerebral *N. caninum* burden in groups of mice (n = 5–7) immunized with recombinant BmNPV particles using semi-quantitative real-time PCR. DNA was extracted from the brain of mice using a DNeasy Blood and Tissue kit. Semi-quantitative real-time PCR was performed using extracted DNA (125 ng) and *N. caninum*-specific primers. BV SAG: BmNPV/SAG1-GP64TM, BV SRS2: BmNPV/SRS2-GP64TM, BV MIC3: BmNPV/MIC3-GP64TM, no display: BmNPV. * $P < 0.05$, ** $P < 0.01$.


(A) No display
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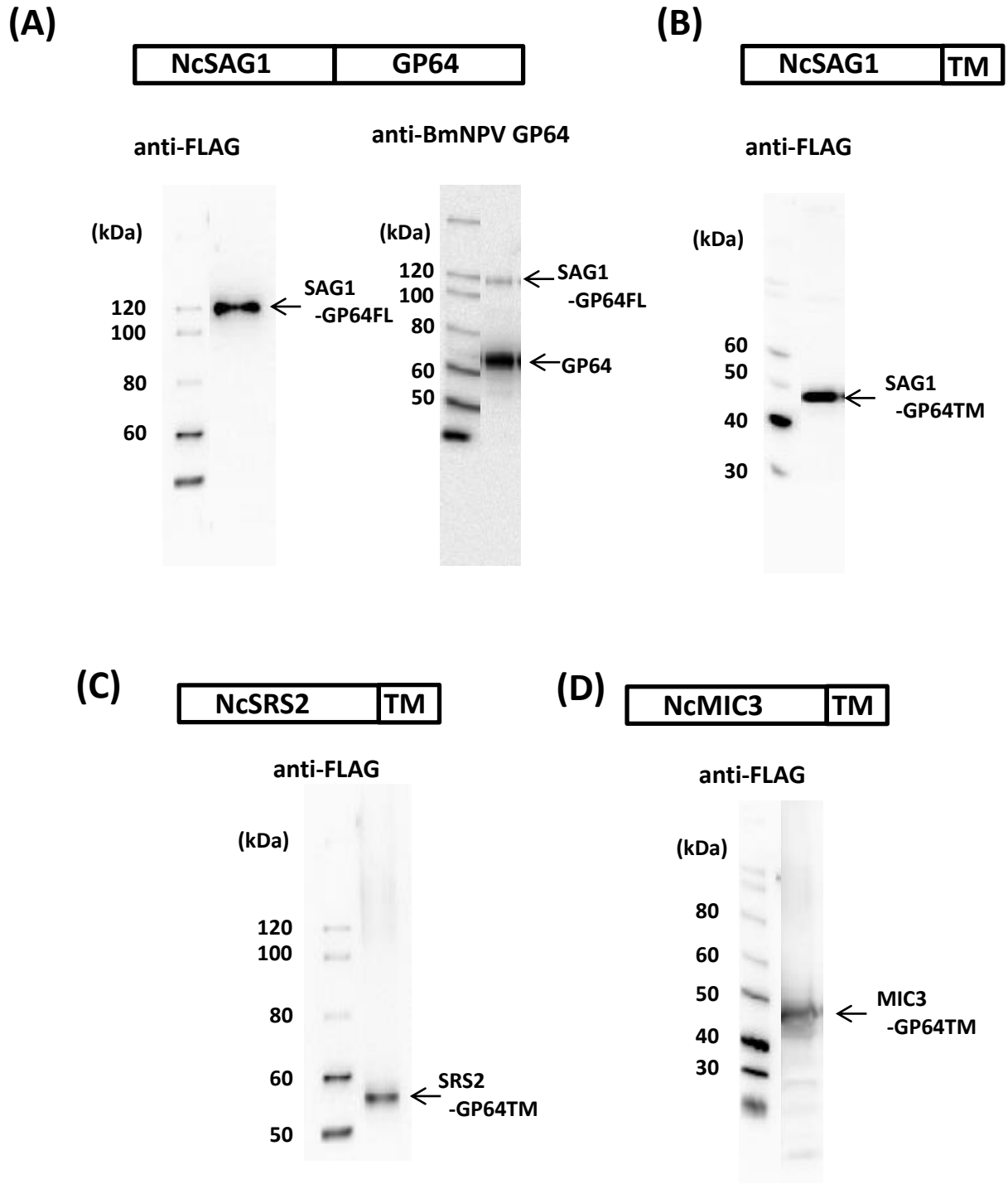
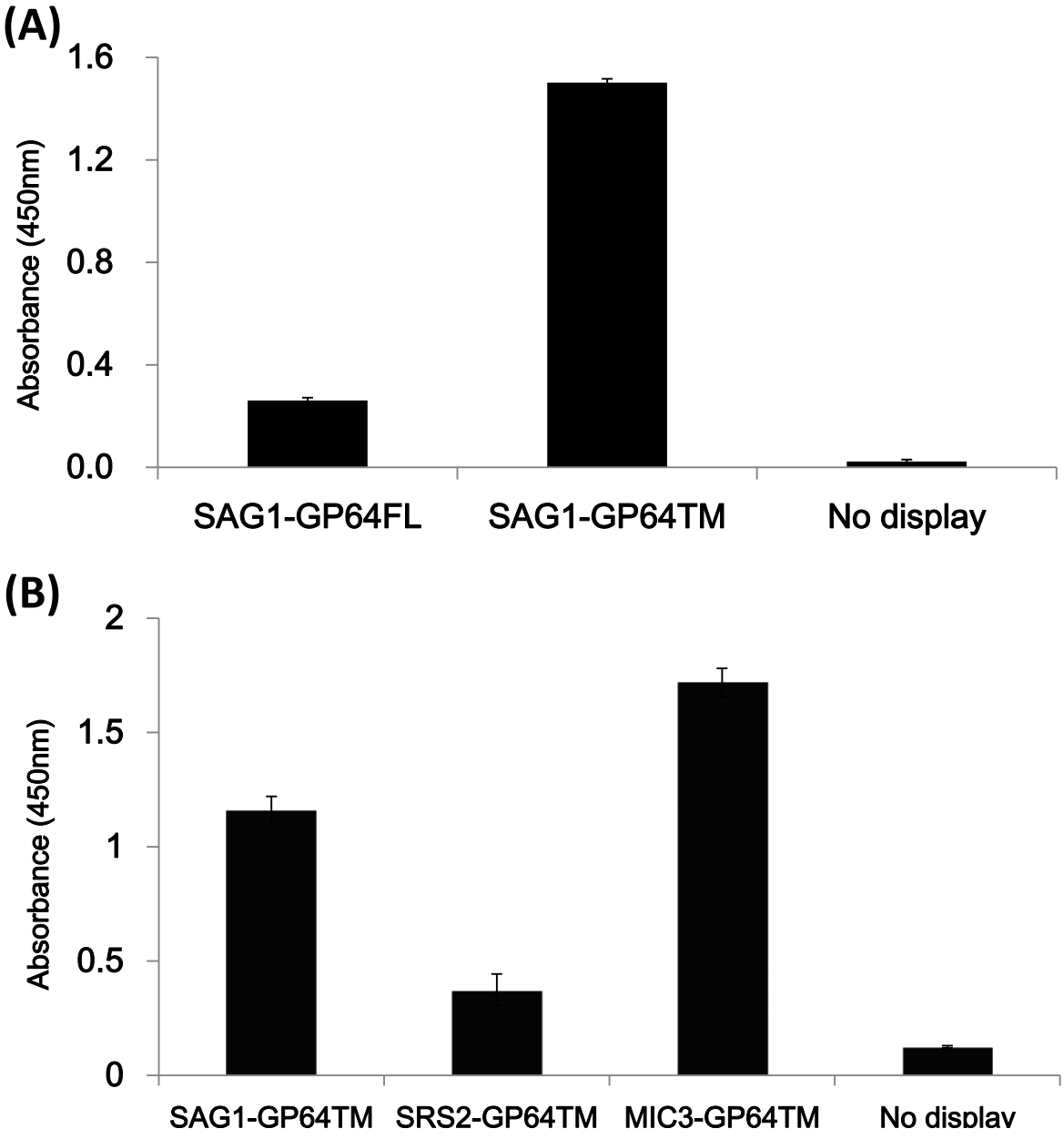
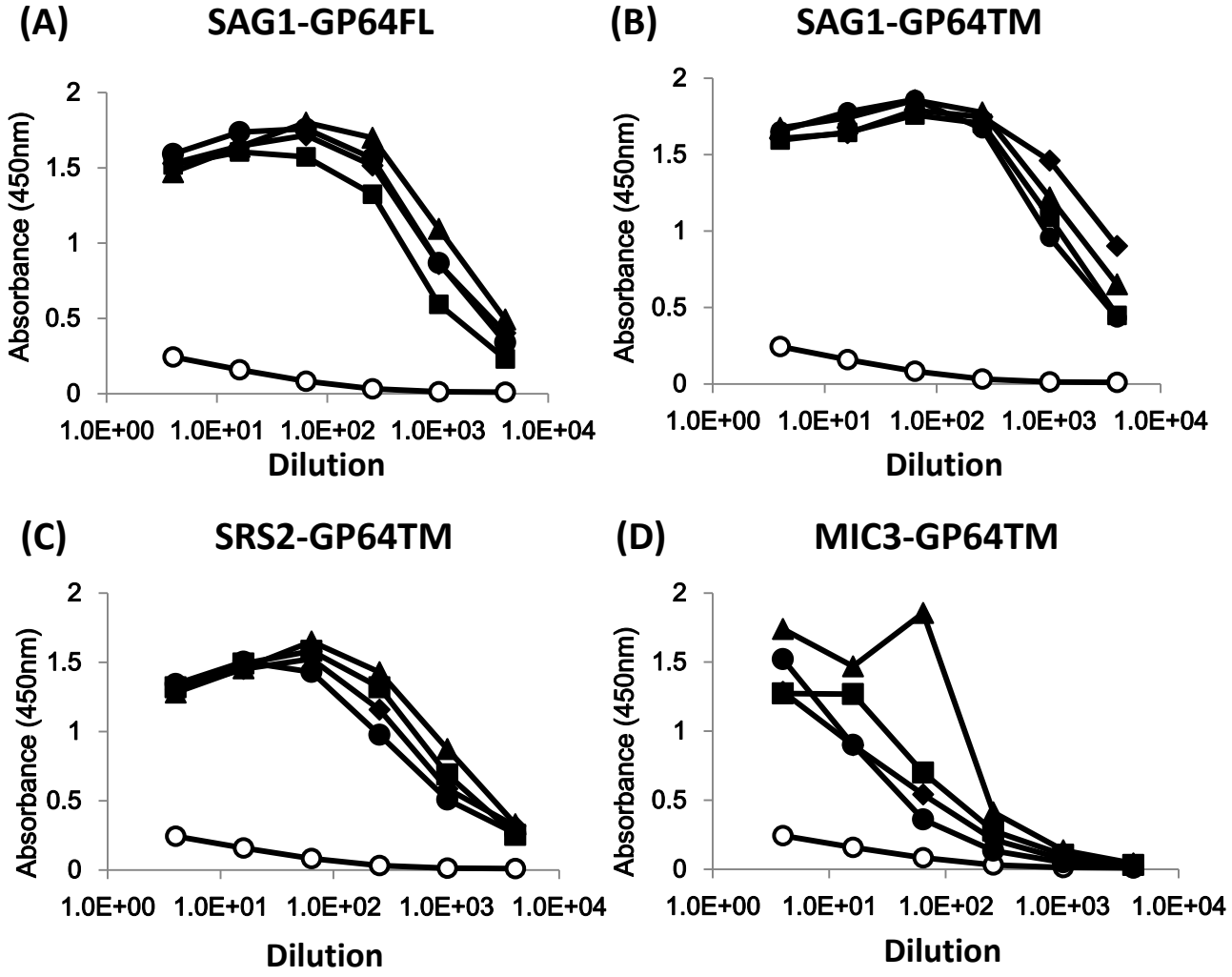
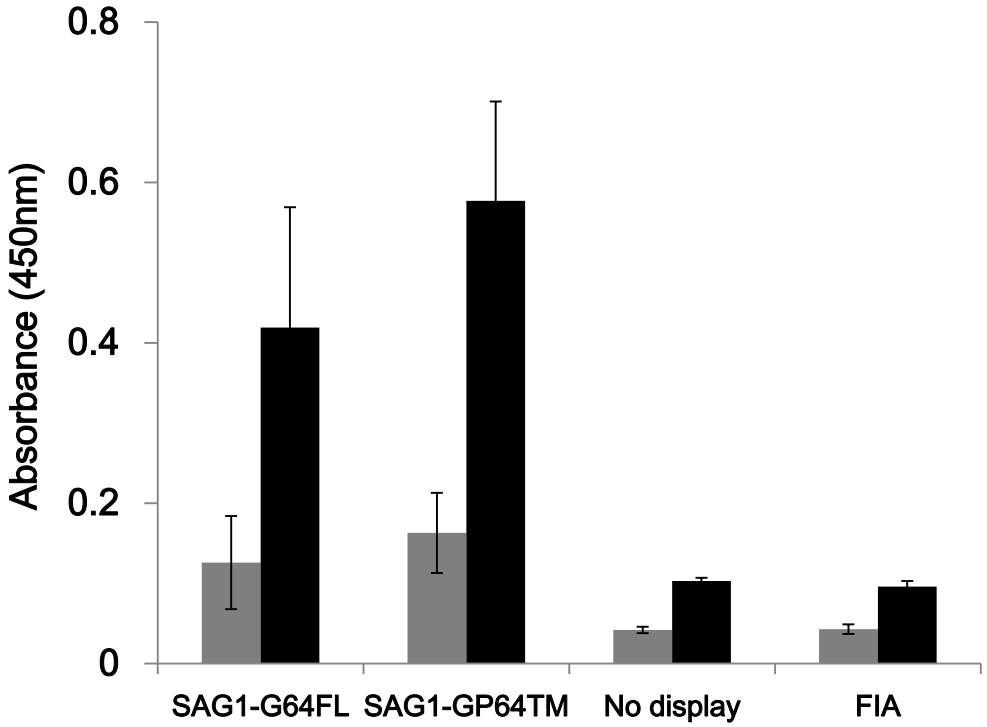
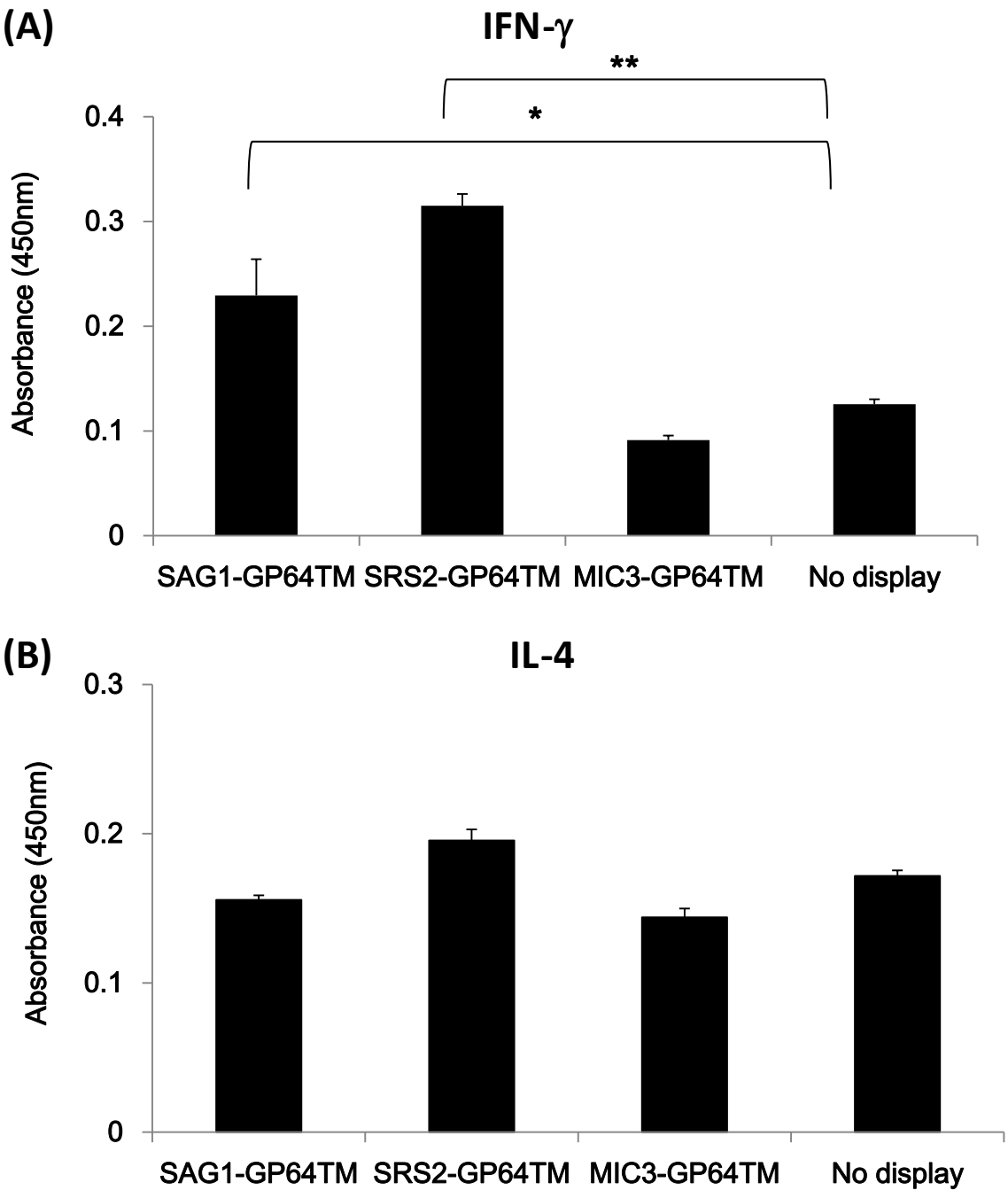
(B) Positive control
5,000 nm
(C) SAG1-GP64TM
5,000 nm
(D) SRS2-GP64TM
5,000 nm

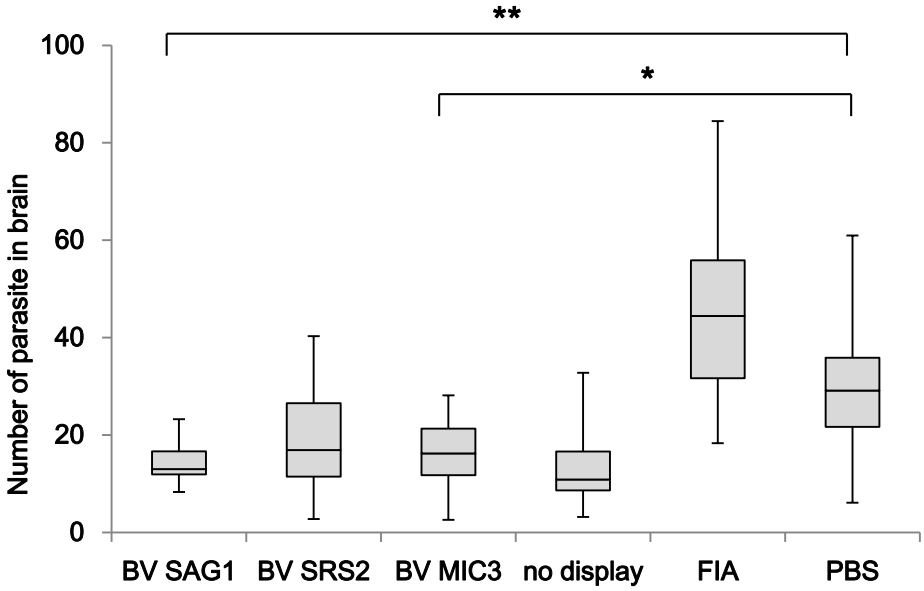

(A)
NcSAG1
GP64
anti-FLAG
anti-BmNPV GP64
(kDa)
120
100
80
60
SAG1
-GP64FL
(kDa)
120
100
80
60
50
SAG1
-GP64FL
GP64
(B)
NcSAG1
TM
anti-FLAG
(kDa)
60
50
40
30
SAG1
-GP64TM
(C)
NcSRS2
TM
anti-FLAG
(kDa)
120
100
80
60
50
SRS2
-GP64TM
(D)
NcMIC3
TM
anti-FLAG
(kDa)
80
60
50
40
30
MIC3
-GP64TM


(A)
Absorbance (450nm)
1.6
1.2
0.8
0.4
0.0
SAG1-GP64FL
SAG1-GP64TM
No display
(B)
Absorbance (450nm)
2
1.5
1
0.5
0
SAG1-GP64TM
SRS2-GP64TM
MIC3-GP64TM
No display


(A)
SAG1-GP64FL
Absorbance (450nm)
2
1.5
1
0.5
0
1.0E+00
1.0E+01
1.0E+02
1.0E+03
1.0E+04
Dilution
(B)
SAG1-GP64TM
Absorbance (450nm)
2
1.5
1
0.5
0
1.0E+00
1.0E+01
1.0E+02
1.0E+03
1.0E+04
Dilution
(C)
SRS2-GP64TM
Absorbance (450nm)
2
1.5
1
0.5
0
1.0E+00
1.0E+01
1.0E+02
1.0E+03
1.0E+04
Dilution
(D)
MIC3-GP64TM
Absorbance (450nm)
2
1.5
1
0.5
0
1.0E+00
1.0E+01
1.0E+02
1.0E+03
1.0E+04
Dilution


0.8
0.6
0.4
0.2
0
Absorbance (450nm)
SAG1-G64FL
SAG1-GP64TM
No display
FIA


(A)
IFN-γ
**
*
0.4
0.3
0.2
0.1
0
Absorbance (450nm)
SAG1-GP64TM
SRS2-GP64TM
MIC3-GP64TM
No display
(B)
IL-4
0.3
0.2
0.1
0
Absorbance (450nm)
SAG1-GP64TM
SRS2-GP64TM
MIC3-GP64TM
No display


**
*
100
80
60
40
20
0
Number of parasite in brain
BV SAG1
BV SRS2
BV MIC3
no display
FIA
PBS