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Evaluation of recombinant *Neospora caninum* antigens purified from silkworm larvae for the protection of *N. caninum* infection in mice

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ABSTRACT

Three antigens (NcSAG1, NcSRS2 and NcMIC3) from *Neospora caninum* (silkworm) larvae were expressed using the BmNPV bacmid system and purified from the hemolymph. From 20 silkworm larvae, 1.5, 1.2 and 1.4 mg of purified recombinant NcSAG1, NcSRS2 and NcMIC3 were obtained, respectively. When each purified recombinant antigen was immunized with Freund's incomplete adjuvant (FIA) to mice, recombinant NcSAG1 induced a Th2 immune response in immunized mice and produced a SAG1-specific antibody. In the experiment where NcSAG1-immunized mice were challenged with *N. caninum*, the cerebral *N. caninum* burden was significantly reduced compared with that of either the FIA- or PBS-immunized mice. Recombinant NcSRS2 or NcMIC3 induced both Th1 and Th2 immune responses, but NcMIC3-immunization did not induce significant production of NcMIC3-specific antibodies. These results suggest that the silkworm can produce recombinant antigens of *N. caninum*, which can be used as a recombinant vaccine against *N. caninum*.

[Key words: *N. caninum*, antigen, subunit vaccine, BmNPV bacmid, silkworm]

INTRODUCTION

Neospora caninum, an intracellular apicomplexan parasite that is closely related to *Toxoplasma gondii*, causes abortion, stillbirth and congenital infection in cattle (1,2). This parasite can transmit to cattle by oral ingestion of oocysts excreted by a canid host (horizontal transmission) and from an infected cow to its fetus (vertical transmission). Neosporosis is a major problem for the livestock industry because of calf mortality and loss of milk production. The elimination of infected cattle from the herd by culling is a safe method to control the disease, but this method requires the identification of infected animals in the herd. No chemotherapeutic agent is available commercially. Vaccine treatment is favorable to prevent this infection in cattle because there is no risk of long lasting residues in the milk and meat. As vaccine candidates, live vaccines, inactivated parasite vaccines and native or recombinant subunit vaccines have been investigated (3).

Immunization with live parasite has provided promising results to protect against fetal death in cattle. In the case of toxoplasmosis, a live vaccine (Toxovac™) for sheep composed of a non-persistent strain *T. gondii* S48 is commercially available in Europe and New Zealand (4). Immunization with a naturally isolated less-virulent parasite, *N. caninum* Nowra, protected adult cattle and their fetuses from *N. caninum* infection (5,6). Other live attenuated parasites were also isolated (7). However, immunization with live parasite vaccines has serious disadvantages from a safety point of view, in addition to high production costs and unfavorable product stability. Inactivated parasites are regarded as vaccine candidates. Protection against fetal infection in cattle by killed parasites was observed, but the abortion rate was still approximately 50% (8).

Additionally, killed parasite vaccines are not as cost-effective as live attenuated vaccines.

Subunit vaccines are a promising candidate for the prevention of *N. caninum* infection in cattle as they are safer and less costly. CoxAbic® is composed of affinity-purified gametocyte antigens from *Eimeria maxima* and is commercially available as a native subunit vaccine against coccidiosis in hens (9). However, no recombinant subunit vaccine against *N. caninum* is commercially available, but this type of vaccine is currently being investigated to protect cattle from parasite infection (3,10). As a recombinant subunit vaccine, proteins on the surface of parasites, proteins in micronemes, rhoptry proteins, dense granule proteins and proteins in the parasitophorous vacuole membrane of *N. caninum* have been investigated (3).

In this study, *N. caninum* surface antigen 1 (NcSAG1), SAG1-related sequence 2 (NcSRS2) and microneme protein 3 (NcMIC3) were expressed into the hemolymph of silkworm larvae and purified. NcSAG1 and NcSRS2 are two major surface antigens of *N. caninum* tachyzoites (11,12). The invasion by tachyzoites into hosts begins via the interaction between these antigens and the membrane of host cells (13). Therefore, these antigens are recognized as a promising recombinant subunit vaccine candidate (14,15). In addition, MIC proteins also seem to interact with the receptors of host cells (16) and have been investigated as a vaccine candidate as well (17,18). Each recombinant antigen purified from silkworm hemolymph was immunized into mice, and the prevention of *N. caninum* cerebral infection in mice was evaluated by *N. caninum* real-time PCR.

MATERIALS AND METHODS

Materials 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 Chemical Ind. Ltd. (Osaka, Japan) and Sigma Aldrich Japan (Tokyo, Japan). Oligonucleotides were purchased from Operon Biotechnology (Tokyo, Japan). *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). Female BALB/c mice (6 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan)

Construction of recombinant BmNPV bacmids For NcSAG1 expression in silkworm larvae, BmNPV CP^- Chi^--bx-FLAGHRV3C-SAG1 bacmid was constructed as described previously (19). In the case of NcSRS2, the *srs2* gene (54-376 aa, NcSRSNC) lacking an N-terminal signal peptide-coding sequence and C-terminal domain was amplified by PCR using a primer set (Table 1). The amplified truncated *srs2* gene was replaced with the full srs2 gene in recombinant pDEST8 constructed as previously described (19). The constructed recombinant pDEST8 vector in this study has a truncated *srs2* gene connected with the signal sequence of bombyxin from *B. mori*. This constructed recombinant pDEST8 vector was transformed into *Escherichia coli* BmDH10Bac CP^-Chi^- (20), and the BmNPV CP^- Chi^--bx-FLAGSRS2NC bacmid was obtained.

The NcMIC3 gene lacking an N-terminal signal peptide-coding sequence was

amplified by a primer set (Table 1). The amplified gene was inserted into the pET52b vector at the *Kpn*I-*Not*I site. Next, each gene was amplified by PCR using a CACC-bx-FLAG-HRV3C primer and the NcMIC3 reverse primer (Table 1). The amplified gene was composed of the bx signal peptide sequence, a FLAG peptide sequence, a human rhinovirus 3C protease cleavage site sequence, and each protein coding sequence. Each amplified gene was inserted into a pENTR/D/TOPO (Life Technologies Japan, Tokyo, Japan) vector by the TOPO reaction. The *mic3* gene was moved from the constructed pENTR vector to pDEST8 by the GATEWAY LR reaction (Life Technologies Japan). The constructed pDEST vector was transformed into *E. coli* BmDH10Bac $CP^{-}Chi^{-}$ and the BmNPV CP^{-} Chi^{-}-bx-FLAGMIC3 bacmid was obtained. All antigens were expressed as a fusion protein with the bx signal peptide, a FLAG peptide, and a human rhinovirus 3C protease cleavage site.

Expression of recombinant *N. caninum*-antigens and purification Each recombinant BmNPV bacmid DNA injection into silkworm larvae and rearing silkworm larvae was performed according to the previous report (21). Hemolymph was collected from silkworm larvae by cutting the prolegs, and 1-phenyl-2-thiourea was added into the collected hemolymph at 5 mM to prevent melanization. Collected hemolymph was centrifuged at 10000 × g for 15 min to remove hemocytes and debris, and its supernatant was used as the hemolymph sample.

To purify expressed recombinant *N. caninum*-antigens, 1 ml of anti-FLAG M2 antibody agarose (Sigma Aldrich Japan) was packed in an empty column and equilibrated with Tris-buffered saline (TBS; pH 7.5). Hemolymph was diluted 5-fold with TBS and loaded onto the anti-FLAG M2 antibody agarose column. The column

was washed with 10 ml of TBS after loading the hemolymph and proteins were eluted with 8 ml of glycine-HCl buffer (pH 3.5). Every fraction of the 1 ml eluent was collected.

SDS-PAGE and Western blot Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% or 12% acrylamide gels. After SDS-PAGE, proteins were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). After blocking in 5% skimmed milk in TBS containing 0.1% Tween 20 (TBST), the membrane was incubated for 1 h in 1:10000 mouse anti-FLAG M2 antibody (Sigma-Aldrich Japan). The membrane was washed with TBST and incubated for 1 h in 1:20000 anti-mouse IgG antibody labeled with horseradish peroxidase (GE Healthcare, Buckinghamshire, UK). Detection was performed with ECL Plus Western blotting reagent (GE Healthcare). Specific protein bands were detected by Fluor-S MAX MultiImager (Bio-Rad, Hercules, CA, USA).

Immunization of recombinant *N. caninum*-antigens in mice and challenge of *N. caninum* tachyzoites into immunized mice All mice used in the present study were treated under the guiding principles for the care and use of research animals as outlined by the Nippon Veterinary and Life Science University, Japan. Female BALB/c mice were 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 immunized intramuscularly with 10 μg of each purified recombinant *N. caninum*-antigen or PBS in Freund's incomplete adjuvant (FIA, adjuvant control group). Mice in Group 6 received PBS alone (infection control group). Immunization

was performed three times every two weeks. After immunization, 3.84×10^6 of *N. caninum* Nc-Liverpool was injected intraperitoneally into immunized mice and they were reared for 5 weeks. Blood and brains were collected and serum was prepared by centrifuging the blood at 1,000 × g.

Enzyme-linked immunosorbent assay (ELISA) The crude extract of *N. caninum* (250 ng of protein/well) were immobilized onto a 96-well plate by incubation at 37°C for 1 h. The supernatant was removed and 2% skimmed milk in TBST was added into each well and incubated for 1 h. The skimmed milk solution was removed and 100 μl of diluted mice serum 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 5000-fold with TBST was added to each well and incubated at room temperature for 1 h. Each well was washed with TBST and then the HRP reaction was carried out. 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 incubated at room temperature until a blue color developed in each well. The HRP reaction was stopped by the addition of 50 μl 1N H_2SO_4 solution and the absorbance of each well was measured at 450 nm.

To confirm IgG subclasses, the crude extract of *N. caninum* (250 ng of protein/well) 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 secondary antibodies. The

168 HRP reaction was the same as that used in the ELISA method described above.

169 **Indirect immunofluorescence detection of *N. caninum*** Vero cells were
170 cultivated in T-25 flasks at confluence using MEM medium (Life Technologies Japan)
171 supplemented with 50 U/ml penicillin-streptomycin (Life Technologies Japan) and 5%
172 horse serum (Life Technologies Japan). Medium was replaced with MEM medium
173 supplemented with 50 U/ml penicillin-streptomycin and 1% horse serum and cells
174 were cultivated for two weeks after *N. caninum* infection. Cells were collected and
175 disrupted by flushing through a 26 s needle several times. The cell homogenate was
176 filtered through a 5 μm-filter to remove debris, and the filtrate was used as a parasite
177 solution.

178 For indirect immunofluorescence detection of *N. caninum, N. caninum* cells
179 suspended in PBS were immobilized onto an APS-coated glass slide (Matsunami Glass
180 Ind., Osaka, Japan) and washed three times with PBS. Parasites were fixed with 4%
181 paraformaldehyde and blocked with 4% BSA in PBS supplemented with 25 mM
182 NH_4Cl. After washing with PBS three times, serum from immunized mice, which was
183 diluted 200-fold with 2% BSA in PBS, was added to glass slides and incubated at room
184 temperature for 1 h. The slides were washed three times with PBS, and parasites were
185 incubated at room temperature for 1 h with anti-mouse IgG conjugated with Alexa
186 Fluor 594 diluted 200-fold (Jackson ImmunoResearch, Laboratories, West Grove, PA,
187 USA). In addition, parasites were incubated with 1 μg/ml DAPI for nuclear staining.
188 Fluorescence was observed using a confocal laser scanning microscope (LSM700, Carl
189 Zeiss Japan, Tokyo, Japan).

Semi-quantitative real-time PCR using *N. caninum* DNA from immunized mouse brains DNA was extracted from the brains using the DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan). Semi-quantitative real-time PCR was performed using a *N. caninum*-specific primer set (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 follows: 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.

Measurement of cytokines Cytokine levels were measured in splenocytes seeded at 1×10^6 cells per well in Hybridoma-SFM medium (Life Technologies Japan) containing 10% FCS with or without 10 μg/ml of each recombinant *N. caninum* antigen or Concanavalin A (ConA; Sigma-Aldrich Japan, positive control) in 24-well microplates (Corning Incorporated, Corning, NY, USA). Microplates were kept at 37 °C for 48 h in a 5% CO_2 atmosphere. After stimulation, interferon-γ (IFN-γ) and interleukin 4 (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), respectively, according to the manufacturer's instructions (assay range: 37 to 3700 pg/ml, sensitivity: <10 pg/ml). The amount of secreted cytokines was calculated using a standard cytokine curve ran on the same immunoplate.

RESULTS AND DISCUSSION

Expression of NcSAG1, NcSRS2 and NcMIC3 in silkworm larvae and

purification NcSAG1 expression was carried out using the same construct (19) in silkworm larvae. NcSRS2 (54-376 aa) lacking its N-terminal signal peptide and C-terminal domain was expressed into silkworm hemolymph by fusion with the bx signal peptide at its N-terminus. MIC3 (26-362 aa), whose signal peptide was removed, was also expressed ito silkworm hemolymph by fusion with the bx signal peptide at its N-terminus. Each antigen was also fused with a FLAG peptide and purified from silkworm hemolymph by anti-FLAG M2 antibody agarose gel. All three antigens were detected by their estimated molecular weight (32 kDa for NcSAG1, 36 kDa for NcSRS2, and 41 kDa for NcMIC3, Fig. 1). In a previous paper, recombinant NcSAG1 expressed in silkworms was also observed at this estimated molecular weight (19). These results suggest that these antigens expressed in silkworms may be modified post-translationally, and in fact, NcSAG1, NcSRS2 and NcMIC3 have 4, 3 and 1 putative N-glycosylation sites, respectively. However, modification by N-glycosylation has not been investigated. Native NcSRS2 in *N. caninum* tachyzoites and recombinant NcSRS2 expressed in insect cells were observed at approximately 42 kDa (22,23). Recombinant NcSAG1 and NcSRS2 were observed as an almost single band, but recombinant NcMIC3 was observed with several minor bands. By Western blot analysis, these minor bands came from NcMIC3 (data not shown). From 20 silkworm larvae, 1.5, 1.2 and 1.4 mg of purified recombinant NcSAG1, NcSRS2 and NcMIC3 were obtained, respectively. Previously, 370 μg of NcSRS2 was obtained from 17 silkworm larvae (19). In this study, an approximately 2.7-fold higher yield of NcSRS2 was obtained than that of the previous paper. NcSRS2 has a glycophosphoinositol (GPI) anchor at its C-terminus (23) and a GPI anchor in the recombinant NcSRS2 was also observed in silkworm larvae (24). This suggests that the deletion of the C-terminal

domain coding its GPI anchor signal peptide enhances the expression of NcSRS2 in the hemolymph of silkworms because the expressed NcSRS2 might be more robustly extracellularly secreted into silkworm hemolymph.

Immunization of each *N. caninum* antigen to mice and challenge of *N. caninum* tachyzoites Serum of mice immunized by recombinant NcSAG1 or NcSRS2 emulsified with FIA showed reactivity to the crude extract of *N. caninum,* but mice immunized by recombinant NcMIC3 emulsified with FIA showed only slight reactivity to the *N. caninum* crude extract (Fig. 2A). Serum from mice immunized with recombinant NcMIC3 showed the reactivity to recombinant NcMIC3 (data not shown). In the case of FIA and PBS, antibodies to *N. caninum* were not detected in serum from mice even after the challenge of *N. caninum* tachyzoites. It indicates that *N. caninum* tachyzoite challenge hardly elicited antibodies to *N. caninum*, compared to recombinant NcSAG1 and NcSRS2 immunization and NcMIC3 is not favorable as a subunit vaccine candidate to *N. caninum* tachyzoites. In sera from mice immunized with recombinant NcSAG1 and NcSRS2, IgG1 production was predominant compared to IgG2a (Fig. 2B). To confirm the reactivity of antibodies in the serum from mice immunized by recombinant NcSAG1 or NcSRS2, *N. caninum* tachyzoites were stained using these sera via indirect immunofluorescence microscopy (Fig. 2C). Both sera from mice immunized by recombinant NcSAG1 or NcSRS2 stained *N. caninum*, indicating that the immunization of each antigen purified from silkworm hemolymph produced the antibodies to each antigen in mice. *N. caninum* tachyzoites were not significantly stained by serum from mice immunized by recombinant NcMIC3 (data not shown). *N. caninum* tachyzoites have micronemes which house several MIC

proteins (3). MIC3 also localizes at the apical tip within the *N. caninum* microneme (25). These results suggest that the produced NcMIC3-specific antibody has only a low reactivity to native NcMIC3 in *N. caninum* tachyzoites.

Challenge of *N. caninum* tachyzoites into immunized mice After challenge of *N. caninum* tachyzoites into mice immunized with each recombinant antigen, the cerebral *N. caninum* burden was measured by semi-quantitative real-time PCR using *N. caninum*-specific primers. The cerebral parasite burden in mice was decreased significantly by immunization with NcSAG1 compared with only FIA and PBS (Fig. 3).

Cytokines The levels of secreted IFN-γ and IL-4 in splenocytes from mice immunized with each recombinant antigen were measured by stimulation with each antigen or ConA. Splenocytes from mice immunized with recombinant NcSAG1 predominantly produced IL-4 and IgG1 (Fig. 2B), but those from mice immunized with recombinant NsSRS2 produced both IFN-γ and IL-4 (Fig. 4), and IgG1 and low level of IgG2a (Fig. 2B). These results indicate that recombinant NcSAG1 induced a Th2 immune response with FIA in mice, and recombinant NsSRS2, induced Th1 and Th2 immune responses. In the case of recombinant NcMIC3, productions of IFN-γ and IL-4 were confirmed (Fig. 4), but those of IgG1 and IgG2a did not. In this experiment, the immunization by recombinant NcMIC3 was very low reactivity compared to those of other antigens (Fig. 2A). This is the reason why the recombinant NcMIC3 produced low level of IgG subclass.

In case of an immunization with native NcSRS2 purified from *N. caninum*-infected

281 Vero cells elicited only a Th2 immune response with FIA and reduced the vertical
282 transmission of *N. caninum* in mice (22). In addition, native NcMIC3 also induced
283 only Th2-type IgG1 antibody production in mice (17). In non-pregnant mice, the Th2
284 immune response seems to be effective in preventing the cerebral *N. caninum* infection.
285 To prevent the vertical transmission of *N. caninum* in pregnant mice and cows, a
286 balanced Th1/Th2 immune response is needed (3). Antigens from *N. caninum* purified
287 from silkworm hemolymph elicited both a Th1 and Th2 immune response in
288 immunized mice, suggesting that recombinant antigens purified from silkworm
289 hemolymph are likely to be vaccine candidates that could prevent the horizontal and
290 vertical transmission of *N. caninum.*

291 Recombinant NcSAG1 or NcSRS2 expressed in *E. coli* significantly reduced the
292 cerebral neosporosis in mice by immunization (26). Recombinant NcMIC3 expressed
293 in *E. coli* induced an IgG1/IgG2a antibody response in mice with ribi adjuvant, but did
294 not IFN-γ, indicating Th2 immune response was predominant in cattle immunization
295 (27). Even though the parasite burden was reduced in brain tissues, recombinant
296 antigens expressed in *E. coli* with immune stimulating complexes failed to prevent the
297 vertical transmission of *N. caninum* in pregnant cattle by experimental intravenous
298 challenge (27). Further research should investigate the effectiveness of recombinant
299 antigens expressed in *E. coli* and silkworms as a recombinant subunit vaccine in both
300 mice and cattle. This study shows that it is possible to use silkworm larvae to produce
301 effective recombinant *N. caninum* antigens for this parasite infection in mice.
302 Simultaneous immunization with recombinant antigens of *N. caninum* with DNA
303 vaccines possessing the antigen genes of *N. caninum* may enhance the induction of

304 immune responses to *N. caninum* in mice (18,26). In addition, the Th1 immune
305 response can be induced by an oil-in-water emulsion with bitter ground extract (28).

306 In this study, three *N. caninum* antigens (NcSAG1, NcARA2 and NcMIC3) were
307 expressed in silkworm larvae using the BmNPV bacmid system and purified from
308 silkworm hemolymph. From 20 silkworm larvae, 1.5, 1.2 and 1.4 mg of recombinant
309 NcSAG1, NcSRS2 and NcMIC3 were obtained, respectively. The immunization of
310 mice with recombinant NcSAG1 induced a predominantly Th2 immune response and
311 significantly reduced the cerebral *N. caninum* infection. In addition, the immunization
312 of mice with recombinant NcSRS2 provoked Th1 and Th2 immune responses and
313 reduced the cerebral *N. caninum* infection although not significantly. Silkworm larvae
314 can produce recombinant antigens of *N. caninum*, which can be used as a recombinant
315 subunit vaccine against *N. caninum* infection.

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421 antigens, NcBAG1, NcBSR4, NcMAG1, and NcSAG4, in a mouse model of
422 acute parasitic infection. Parasitol. Res., **112**, 655-663 (2013).
423

424 **TABLE 1.** Primers

	5’– 3’
NcSRS2NC-F	TATGGTACCGAGCGCCGTTCAAGTCGGAA
NcSRS2NC-R	TATGAGCTCTTATCCTCTTAACACGGGGGA
NcMIC3-F	TATGGTACCGATTGACTGTTCAGAAA
NcMIC3-R	TATGCGGCCGCTTATCGAGCCGTTCCGCAT
CACC-bx-FLAG	CACCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAA
-HRV3C	CAGTAATGTGGGTGTCAACAGACTACAAGGATGACGATGA
	CAAGGGTGCACTTGAAGTCCTCTTTCAG
NC-F	GTGAGAGGTGGGATACG
NC-R	GTCCGCTTGCTCCCTA

425

426 **Figure legends**

427 **FIG. 1.** SDS-PAGE of recombinant *N. caninum* antigens purified from silkworm
428 hemolymph. Each recombinant antigen was expressed in silkworm larvae and purified
429 from silkworm hemolymph using anti-FLAG M2 antibody agarose. SDS-PAGE gels
430 were stained with Coomassie Brilliant Blue.

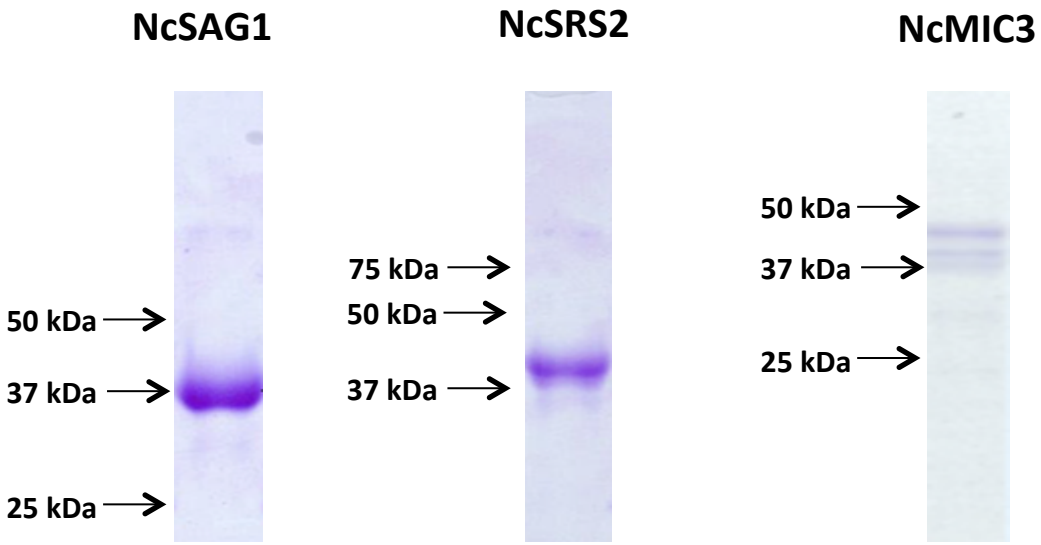
431 **FIG. 2.** (A) Antibody responses in mice immunized with each antigen before (white
432 bars) and after (grey bars) the *N. caninum* challenge. Female BALB/c mice were
433 immunized intramuscularly with 10 μg of each purified recombinant antigen or PBS in
434 Freund's incomplete adjuvant three times every two weeks. After immunization, $3.84 \times$
435 10^6 of *N. caninum* Nc-Liv was injected intraperitoneally into immunized mice and
436 reared for 5 weeks. (B) IgG subclass measurement of serum from mice immunized
437 with each recombinant antigen. (C) Indirect immunofluorescent microscopy of *N.*
438 *caninum* tachyzoites using the serum of mice immunized with recombinant NcSAG1
439 or NcSRS2. The nuclei of *N. caninum* tachyzoites were stained with DAPI. Scale bars
440 indicate 5 μm.

441 **FIG. 3.** Quantification of cerebral *N. caninum* amount in mice ($n = 5$) immunized with
442 each recombinant antigen using semi-quantitative real-time PCR. DNA was extracted
443 from the brain of mice using a DNeasy Blood and Tissue kit. Semi-quantitative
444 real-time PCR was performed using extracted DNA (500 ng) and *N. caninum*-specific
445 primers. Significance differences between two groups were observed statistically by
446 Student's *t*-test. ($*p < 0.05$).

447 **FIG. 4.** IFN-γ and IL-4 production by splenocytes of mice immunized with each
448 recombinant antigen. Splenocytes were isolated from mice immunized with each
449 antigen and stimulated with each antigen for 48 h. The secreted amount of IFN-γ and

IL-4 was quantified using mouse IFN-γ and mouse IL-4 ELISA kits, respectively. Significance differences between two groups were observed statistically by Student's *t*-test. (**p*< 0.05, ***p*< 0.01). White and black bars indicate each cytokine production without the stimulation by each antigen (white bars) and with the stimulation by each antigen (black bars).

FIG. 1., Kato et al.



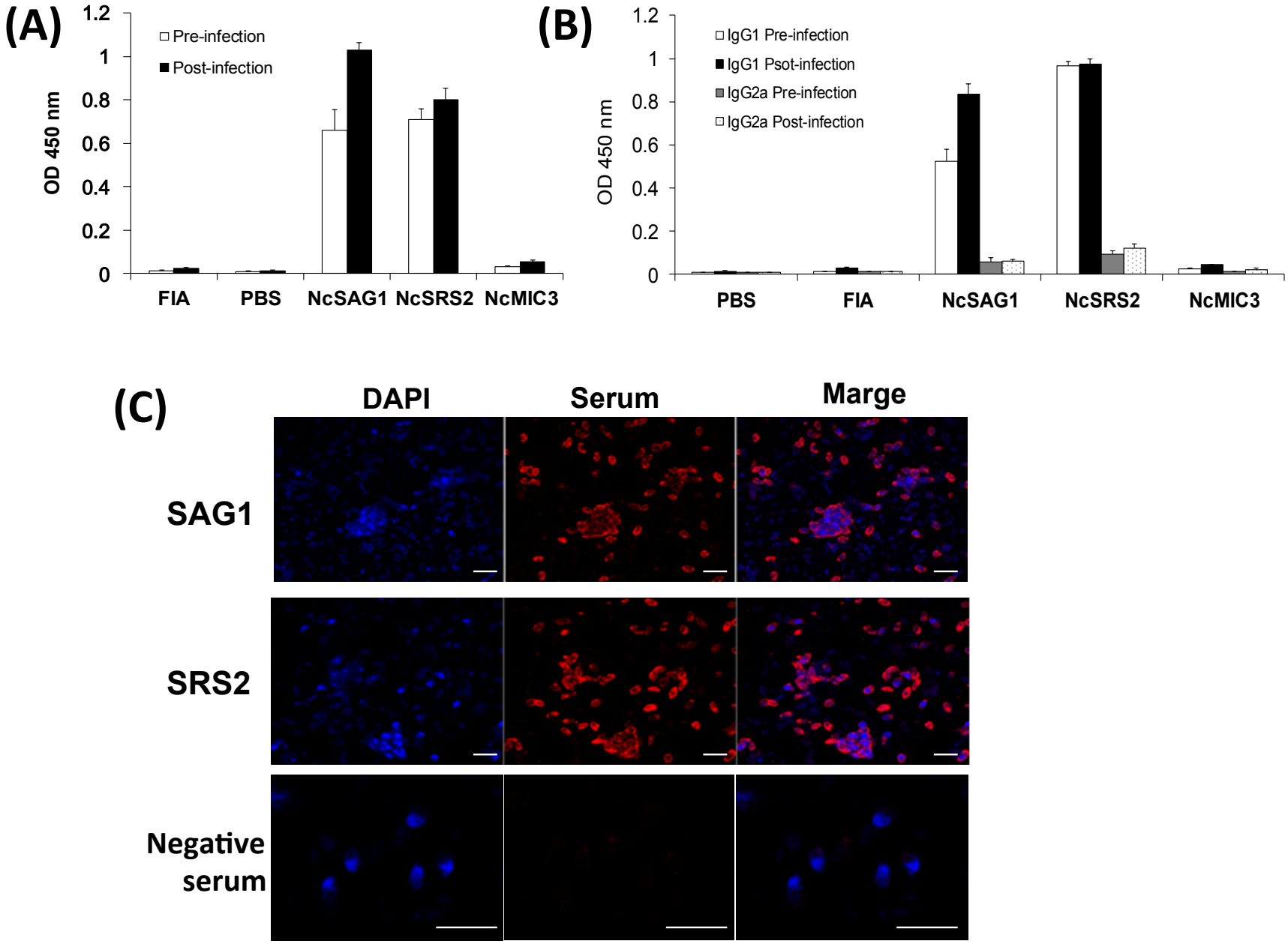

(A)
OD 450 nm
1.2
1
0.8
0.6
0.4
0.2
0
Pre-infection
Post-infection
FIA
PBS
NcSAG1
NcSRS2
NcMIC3
(B)
OD 450 nm
IgG1 Pre-infection
IgG1 Psot-infection
IgG2a Pre-infection
IgG2a Post-infection
PBS
FIA
NcSAG1
NcSRS2
NcMIC3
(C)
DAPI
Serum
Marge
SAG1
SRS2
Negative serum

FIG. 3., Kato et al.

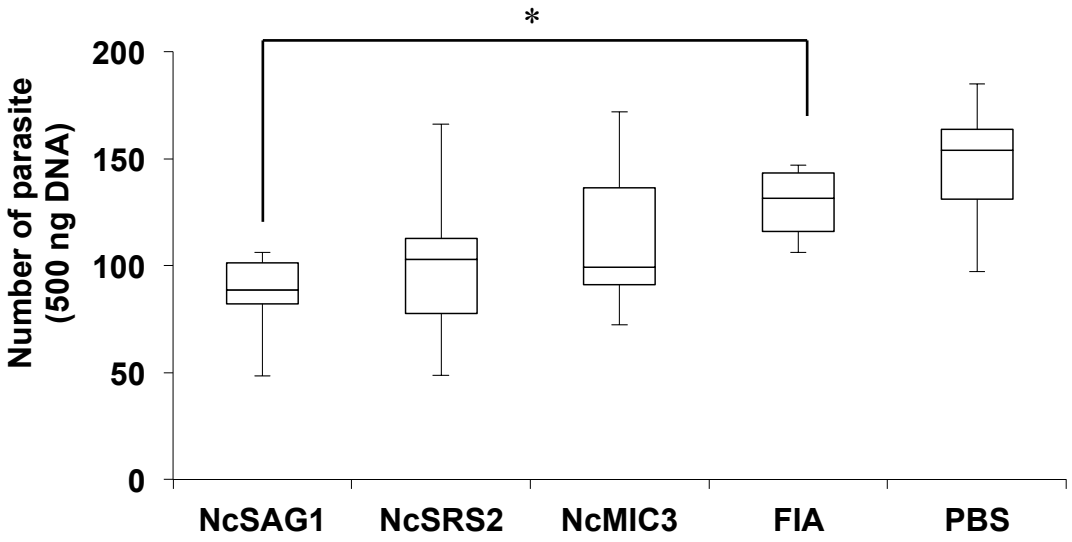


FIG. 4., Kato et al.