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Abbreviations: BmNPV, *Bombyx mori* nucleopolyhedrovirus; GRA, dense granule protein; MIC, microneme protein; NcPFN, *Neospora caninum* profilin; NcROP, *Neospora caninum* rhoptry protein; NcSAG1, *Neospora caninum* surface antigen 1; NcSRS2, *Neospora caninum* SAG1-related sequence 2; RSV-LP, Rous sarcoma virus-like particles; SAG, surface antigen; SRS, SAG1 related sequences; and VLP, virus-like particle.

1. Introduction

Neosporosis as caused by *Neospora caninum* (Family: Sarcocystidae) has emerged as a severe disorder around the world in animals such as cattle and dogs [1,2]. When originally recognized in Norway in 1984, *N. caninum* was mistakenly classified as *Toxoplasma gondii* due to their similarities in both morphology and life cycle [3]. Like other apicomplexan protozoan parasites, *N. caninum* has three principal stages, tachyzoites, bradyzoites and sporozoites [4]. Through a horizontal transmission route, it is transmitted from its definitive hosts, canids (domestic dogs), and their oocysts are spread in feces to other intermediate ruminant hosts such as cattle, sheep, etc. [5,6]. This parasite can also be vertically transmitted to the fetus in pregnant individuals, where it is associated with mid-gestation abortion and/or neonatal mortality in cattle, causing over $1.3 billion in losses annually [7]. In terms of neosporosis treatments, however, no effective products are currently available to combat the replication of and infection by *N. caninum*. Although some compounds, such as sulfonamides and pyrimethamine, have been used experimentally to treat neosporosis in animal models, side effects such as thrombocytopenia and/or leukopenia may develop as well [8–11].

Low-virulence or attenuated tachyzoites at a sublethal dose or those treated with γ-irradiation reportedly demonstrated superior efficacy in cattle and other small laboratory animal models [12,13]. However, live vaccines have disadvantages such as high costs as well as potentially high risks of inducing chronic infections in the hosts, causing them to be carriers. Subunit vaccines are considered very safe because they contain no live components. However, the basic *Neospora* mechanism of pathogenesis and the host-immune responses are still elusive, so it is challenging to determine which antigen or antigen combinations should be used [14]. Hence, it is crucial to establish a platform for screening favorable antigens and developing a potential vaccine or vaccine cocktail to prevent neosporosis efficiently. Recently, several recombinant subunit

vaccines containing tachyzoite surface and dense granule antigens (NcSAGs/NcGRAs) have been recognized as candidates that confer partial protection by targeting *N. caninum* in murine models, stimulating T cell activation and/or IFN-γ production [15,16]. Among these antigens, the major surface antigen (SAG, e.g., NcSAG1), SAG1-related sequences (SRS, e.g., NcSRS2), microneme protein (MIC, e.g., NcMIC1/3), dense granule protein (GRA, e.g., NcGRA7), profilin (NcPFN), and rhoptry protein (NcROP) demonstrated moderate restrictions against *N. caninum* infection [15–22].

Virus-like-particles (VLPs) are structurally similar to their original viruses and retain the power of infection under no proliferation due to the absence of genetic materials. It is known that the unique VLP approach has already been employed in the forms of vaccines, antigens and drug transporters to raise significant specific immune responses or to target specific diseases [23]. Previously, we showed that an enveloped VLP from a truncated Gag protein construct of Rous sarcoma virus (aa 1-577, termed as RSV-LPs-Gag577) was produced efficiently using the silkworm-bacmid expression system [24,25]. Moreover, RSV-LPs could also be employed as protein surface display vehicles, as in the human prorenin receptor (hPRR) and NcSRS2 with a C-terminal transmembrane (TM) domain-containing region of baculovirus GP64 [24,25].

When using the RSV-LP as a carrier together with a coexpressed NcSRS2 membrane protein cargo, NcSRS2-displayed particles were identified in the purified RSV-LPs through transmission electron microscopy (TEM), which further confirmed the usability of the RSV-LP as an antigen display platform [25]. In following up on this concept and method in the current study, we intend to investigate if multiple antigen displays could be created by co-infections of several recombinant BmNPVs. Based on the results, a successful codisplay of NcSAG1 and NcSRS2 was confirmed by either separation via sucrose gradient density or affinity chromatography method. The antigen-displayed VLPs were further employed as vaccinations in *Neospora*-sensitive gerbils, *Meriones unguiculatus*, to evaluate the anti-*N. caninum* effects [26]. We demonstrated that the

antigen-displaying-RSV-LPs-immunized gerbils produced antigen-specific antibodies, which was correlated with the relatively the lower parasite load from immunized gerbils brain tissues after infections of *N. caninum*, suggesting that silkworm-expressed VLP vaccines are potentially promising candidates against *N. caninum* infections.

2. Materials and Methods

2.1. Construction of the recombinant Bombyx mori nucleopolyhedrovirus (rBmNPV) bacmid

The rBmNPV bacmids were generated according to our previous reports. Both NcSAG1 and NcSRS2 are adapted for the silkworm-baculovirus expression system. To ensure the membrane localizations, the constructs for NcSAG1 (aa 23–313, signal peptide deleted) and NcSRS2 (aa 1–376, full length) contained an extra N-terminal signal peptide from silkworm bombyxin (Bx), a FLAG tag, and a C-terminal GP64 region, as previously reported [25,27]. All the sequences were confirmed by sequencing, and the resulting pFastBac1 plasmids were further used to produce the recombinant BmNPVs [28].

2.2. Expression of RSV-LPs in silkworm larvae and Bm5 cells

The *B. mori* Bm5 cells (Thermo Scientific, CA, USA) were maintained in Sf-900 II SFM medium (Life Technologies, NY, USA) with 10% fetal bovine serum (Life Technologies, NY, USA) at 27°C. The fifth instar silkworm larvae were purchased from Ehime Sansyu Inc. (Ehime, Japan) and reared on an artificial diet, Silkmate S2 (Yokohama, Japan), in an incubator (MLR-351H, Sanyo, Tokyo, Japan) at 65% humidity and 27°C. On the third day of the fifth instar, each silkworm was injected with 40 μl of recombinant bacmid DNA solutions containing 10 μg of

rBmNPV bacmid DNA and 10% (v/v) DMRIE-C reagent (Invitrogen, CA, USA) in phosphate-buffered saline (PBS, pH 7.5) using a 1 mL syringe with a needle (Terumo, Japan). At 7 d post-injection (dpi), the hemolymph was harvested in Falcon tubes (NJ, USA) containing 2 mM phenylthiourea to inhibit the melanization of the hemolymph. Larval hemolymphs were stacked in a –80°C freezer until they could be used to infect silkworms. For expression in cultured silkworm Bm5 cells, monolayers of Bm5 cells in 75-cm^2 flasks (Trueline, Tokyo, Japan) were infected with recombinant viruses at a multiplicity of infection (MOI) of 5. Both the infected cells and culture media were then harvested at 5 dpi, followed by a western blot analysis.

2.3. Purification and verification of antigens displaying VLPs

The purification of VLPs displaying *Neospora* antigens was performed according to our previous protocols, with modifications [24,25]. In brief, every 8 mL volume of hemolymph collected from baculovirus-infected silkworm larvae (5 dpi) was clarified by centrifugation at 5000 g for 30 min to remove the hemocytes, cell debris, and aggregates. The supernatant was then concentrated in a 20% sucrose solution via ultracentrifugation at 122,000 g (CS120GXII; Hitachi Koki, Tokyo, Japan) for 6 h. The precipitation was subsequently dissolved in PBS and subjected to separation by density through ultracentrifugation at 122,000 g in a 20%–60% sucrose cushion, followed by western blotting to verify the locations of the VLP and antigens. Regarding the FLAG-tag affinity purification, the pooled protein samples containing VLP antigens were applied to the column containing Anti-DDDDK agarose gel (Medical & Biological Laboratory, Nagoya, Japan). The column was washed and eluted with an elution buffer (0.17 M glycine buffer, pH 2.3). All the samples were further validated for RSV-gag and antigens using a specific antibody by western blot.

2.4. SDS-PAGE and western blot

To detect the expression of their VLPs and recombinant antigens, the hemolymph and fat body tissues from infected silkworm larvae were harvested and treated as described previously [27]. In brief, 10 mL of hemolymph sample with protease inhibitors (Roche, Branford, USA) was centrifuged at 8,000 g at 4°C for 30 min to remove the debris. The fat bodies were extracted in lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Triton X-100, and protease inhibitors), followed by sonication and clarification through centrifugation at 14,000 g at 4°C for 30 min. The supernatants containing the target RSV-LP-antigen complexes were diluted in PBS, further subjected to 10% (w/v) SDS-PAGE and then visualized with Coomassie Brilliant Blue R-250. The protein concentration was determined using a BCA protein assay kit (Thermo Scientific, USA). For the western blot, the proteins from the SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked for 1 h in TBST buffer (20 mM Tris-HCl pH 7.6, 500 mM NaCl, and 0.1% w/v Tween-20) with 5% w/v skim milk (Wako, Japan) followed by incubation with an anti-DDDDK tag antibody (1:5000, MBL, Tokyo, Japan) or rabbit anti-RSV-gag serum (1:5000) [24] for 1 h, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse (1:10000, GE Healthcare Japan) or anti-rabbit IgG secondary antibodies (1:10000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. Specific bands were visualized using ECL prime Western blot reagents (Millipore, Milford, MA).

2.5. Gerbil immunizations and infections

The elution fractions containing RSV-LP-antigen proteins were dialyzed against PBS buffer at 4°C overnight, and the concentrations of roughly purified VLPs were determined using a standard BCA protein kit (Pierce, USA). In terms of immunizations, 6-week-old gerbils,

Meriones unguiculatus, were divided into 4 groups (PBS, RSV-LP only, RSV-LP-NcSRS2, or RSV-LP-NcSRS2/NcSAG1, with 3 gerbils per group) and PBS, or approximately 100 μg of proteins, and they were immunized subcutaneously every two weeks three times. After the third immunization, blood samples were collected for further antibody production analysis by indirect enzyme-linked immunosorbent assay (ELISA). The immunized gerbils were then challenged with *N. caninum* (Strain Liverpool) via intramuscular injection at a dose of 1×10^5 at the 5^{th} week. The gerbils were closely observed in relation to their body weights and survival rates during the entire process.

2.6. Indirect enzyme-linked immunosorbent assay (ELISA)

The indirect ELISA protocol was adopted as previously reported [29], with minor modifications. In brief, 100 ng of purified recombinant NcSAG1 or NcSRS2 from our previous study [30] was coated onto a 96-well ELISA plate (NUNC, Langenselbold, Germany) at 4°C overnight. Subsequently, the plate was washed using a plate washer (Model 1575, ImmunoWash, Bio-Rad) containing PBST (PBS containing 0.05% Tween 20) and blocked with 2% skim milk in PBST for 2 h, followed by binding for 2 h with diluted serum samples from PBS, RSV-LP, RSV-LP-NcSRS2, or RSV-LP-NcSAG1/NcSRS2-immunized gerbils. An HRP-conjugated secondary antibody against gerbil IgG (1:10000, Bioss Inc. Massachusetts, USA) was then added to each well and incubated for an hour. After the plates were washed with PBST, 100 μL of substrate [0.2 mg/ml 3,3,5,5,-tetramethylbenzidine (TMBZ, Wako) in 100 mM sodium acetate (pH 6.0) with 0.2% (v/v) 30% hydrogen peroxide (H_2O_2)] was added to develop a blue color at room temperature. Then, 50 μl of 10% (w/v) H_2SO_4 solution was used to stop the reaction, and the absorbance was measured at 450 nm using a microplate reader (Model 680, Bio-Rad).

2.7 Genomic DNA extraction and semi-quantitative real-time PCR (qPCR)

To investigate the parasite load in infected gerbils, the genomic DNAs were isolated from the brain tissues (15 mg/sample) using DNeasy Blood & Tissue Kit (Cat. No. 69506, Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The concentration of the extracted genomic DNA was quantified by NanoDrop (2000c, ThermoFisher Scientific, Tokyo, Japan). Subsequently, the load of *N. caninum* from cerebral tissue (50 ng/sample) was determined using real-time PCR with THUNDERBIRD SYBR qPCR Mix (Toyobo, Tokyo, Japan) with primer set Nc-F (5'-gtgagaggtgggatacg-3') and Nc-R (5'-gtccgcttgctcccta-3'). The statistical analysis was conducted using Prism 7 (GraphPad Software Inc., San Diego CA, USA). All values represent the mean ± standard error of the mean (SEM) for three replications. Significant differences were evaluated using one-way ANOVA.

3. Results

3.1. Expression verification of antigens and RSV-gag in infected silkworms

As proposed in Fig. 1A and B, some but not all proteins (antigens) with a baculovirus GP64 TM anchor region (Fig. 1A) were expressed as membrane proteins in the silkworm cells. When an immature structure from an enveloped virus, such as BmNPV and RSV, buds from cells into the extracellular environment, it uses the host cell membrane as virus envelope material. As a result, those budded viral particles are decorated with many natural host substrates including membrane proteins and lipid compounds. Thus, it is reasonable to conclude that a higher number of possible protein displays could occur on the surface of the envelope virus when a membrane protein is overexpressed on the infected host cells. This concept has already been frequently employed as one of the major methods for displaying a subunit antigen in baculovirus [31] or other enveloped VLPs [32].

As demonstrated in Fig. 1C, the protein expression of each infection and co-infection (equal amounts, 1:1 or 1:1:1) of rBmNPVs in cultured Bm5 cells was verified by western blot using antibodies against the FLAG-tag and the RSV-gag, respectively. Consistent with our previous results [24], NcSAG1 or NcSRS2 expression was observed intracellularly during either double or triple co-infections, suggesting the success of the rBmNPV co-infections in the silkworms. Although low expression levels for both antigens were noted extracellularly (Fig. 1C, right panel), when the collected medium was concentrated in the 20% sucrose gradient by ultracentrifugation, specific bands for NcSAG1 and NcSRS2 emerged (Fig. 1C, lower panel), indicating the possibility of generating an antigen display or codisplay on the surface of BmNPV or RSV-LP.

3.2. Purification of antigens displaying RSV-LPs from hemolymph

To confirm the display of antigens and purify the resulting RSV-LPs, a 20%–60% sucrose cushion (Fig. 2A) was prepared to separate the RSV-LP from crude silkworm hemolymph following single or double antigen co-infections. After ultracentrifugation, the western blot results clearly showed that the expressed NcSAG1/NcSRS2 and RSV-gag proteins are colocalized in fractions #5 to #7 as demonstrated in Fig. 2B–D, implying that these fractions might have RSV-LPs that are displaying one or both antigens together. We also noticed that the overall display level, but not the expression level of NcSRS2, was relatively lower than the NcSAG1 in the concentrated hemolymph, although we have tried to increase the PFU of rBmNPV-NcSRS2 used for infection (data not shown).

3.3. Validation of antigens displaying RSV-LPs using FLAG affinity chromatography

To validate the true display status, we conducted a FLAG affinity purification for the pooled protein sample range from fraction Nos. 5 to 7 (Fig. 2A) and confirmed their binding (Fig. 2B–D). Efficient displays are thought to allow for the pull-down of FLAG-tagged antigens together with RSV-LP (Fig. 3A). The expression of each target before purification (as loading samples) was also verified by western blot, as shown in Fig. 3B. Compared to the single NcSRS2-displaying RSV-LPs, it is clear that the presentation of NcSRS2 was less abundant in double display VLPs (Fig. 3B, left panel). After purification, specific RSV-gag bands were identified in the eluted protein samples (pulled down samples), which further supported the successful display of antigens on the RSV-LP surface.

3.4. Antigen-displayed RSV-LPs induced promising anti-Neospora effects

Our previous study confirmed that specific antigenicity could be induced in a BALB/c mouse model immunized by silkworm-produced RSV-LPs with effective displays of native NcSRS2 [25]. Here, we chose the *Neospora*-sensitive Mongolian gerbil (*M. unguiculatus*) model (acute infection [26]) for immunization and asked whether the RSV-LPs codisplaying NcSAG1 and NcSRS2 could enhance the antigenicity and show improved efficacy against *N. canine* infection. After three rounds of immunization (Fig. 4A, four different groups: PBS, VLP only, VLP-NcSRS2, and VLP-NcSAG1/NcSRS2, with 100 μg of total proteins for each gerbil/immunization), there were no significant changes among all the test groups in terms of body weights during vaccinations, while a dramatic decrease was observed in either the PBS or VLP groups after challenging them with 1×10^5 *Neospora* tachyzoites (Fig. 4B). Consistently, the two gerbil groups immunized with antigen-displaying RSV-LPs had relatively lower loads of *N. caninum* in brain tissues then the control groups post-infection, indicating that the immunized antigen-displayed RSV-LPs are effective to prevent cerebral infections (Fig. 4C). To confirm the production of antibodies against NcSAG1/NcSRS2, the blood samples collected before the challenge were used for the antibody titration, during ELISA, of NcSAG1 and NcSRS2 against silkworm-expressed FLAG-NcSAG1 or FLAG-NcSRA2, respectively. In this study, the dilution rate of the gerbil serum is shown in Log2, and the absorbance (optical density) was measured at 450 nm after sufficient color development. As indicated in Fig. 4D and E, compared to the controls, serum samples from the antigen-displayed RSV-LP-injected gerbils demonstrated specific antibody binding to the purified antigens. Since the NcSAG1 or NcSRA2 used for the plate coating were purified from the silkworm hosts and might be contaminated with silkworm endogenous substances, serum samples from VLP without antigen therefore exhibited a higher absorbance compared to the PBS control. In addition, a slightly higher absorbance is shown when using serum from gerbils immunized with VLP-NcSRS2 compared to those with VLP-only because both antigens coated onto the ELISA plates were expressed as a fusion protein with the

same tag, the FLAG tag. Unfortunately, no enhancement against infections was observed in the VLP-NcSAG1/NcSRS2 double antigen-displaying group.

4. Discussion

Recently, antigen- or drug-displaying VLPs have been employed extensively as transporters to target diseases, and they show advanced specificity and efficacy in various scenarios [32,33]. We established a unique silkworm/bacmid-RSV-antigen-displaying platform to decorate the envelope of RSV-LPs with the desired antigens [24,25]. Previously, the sufficient display of the transmembrane human prorenin receptor-displaying on the RSV-LP surface was verified, and it showed functional binding to human prorenin [24]. Likewise, as an antigen-delivery system, a monovalent NcSRS2-displaying RSV-LP has been produced, and it induced specific antibody productions from the immunized mice [25]. Taken together, our results implied that it is feasible to use the silkworm-RSV-antigen-displaying platform for targeting various diseases. In the current study, we wished to display the bivalent antigens of *N. caninum* on RSV-LPs produced from the silkworm-bacmid protein expression system. The results from both the sucrose density analysis and FLAG affinity purification reveal that NcSAG1 and NcSRS2 are displayed together on RSV-LPs. To our knowledge, this is the first successful effort to obtain a VLP, either enveloped or nonenveloped, that presents two different antigens from *N. caninum*. Compared with the *E. coli* prokaryotic protein expression system, silkworm-bacmid system enables the production of an enveloped VLP with antigen-decorated. Our platform also offers the potential to display an antigen cocktail as needed or designed to achieve a higher antigenicity and efficacy. However, it is well-known that in baculovirus expression vector systems, the co-infection of baculoviruses usually leads to low expression levels and imbalance ratios for each point of interest, which creates a burden when attempting to control the display of each antigen in our display system [34]. In this study, the displayed antigens, NcSRS2 in particular, are dramatically

decreased when the silkworm larvae are co-infected with three different baculoviruses. We consider this obstacle as the key reason that we failed to show a stacking effect in RSV-LP-NcSAG1/NcSRS2-immunized gerbils. The coinfection of recombinant baculoviruses is the first choice to express complexed VLPs because many VLPs, regardless of the types of viruses, were expressed in the baculovirus expression vector system using the coinfection strategy [34]. One of the solutions is to assemble all the expression cassettes into a single recombinant baculovirus (an all-in-one recombinant baculovirus) with consideration of the promotors and of optimizing the direction for each gene [35–37]. During future studies, we will continue to improve our system to enhance the efficiency of antigen displays.

It is very challenging to develop subunit vaccines for protozoan parasites with satisfactory efficacy due to the complexity of the infection stages. Therefore, it is essential to select a dominant candidate with an appropriate antigenicity for antibody production. The antigens, NcSAG1 and NcSRS2, have already been investigated as major surface antigens from tachyzoites [15,16,30]. The NcSRS2 has been characterized as one of the effective vaccines, and it has been shown to activate T cells and elevate the production of IFN-γ in cattle [38, 39]. Previously, we successfully produced recombinant NcSAG1 and NcSRS2 proteins from the silkworm-bacmid system and induced antigen-specific antibodies against NcSAG1 and NcSRS2 in immunized mice [30]. Furthermore, NcSAG1- and NcSRS2-displaying BmNPV particles can elicit a Th1 immune response in mice, and particularly, the NcSAG1-displaying BmNPV particle could significantly suppress *N. caninum* infection in mice [40]. Currently, antigen-displaying RSV-LPs, both NcSAG1-monovalent and NcSAG1/NcSRS2-bivalent ones, are known to protect cerebral infections and induce antigen-specific antibodies in the gerbil acute infection models, although we could not provide other positive evidence, such as the proliferation of parasites in other tissues, due to the swift death of the animals after acute infection. Unfortunately, no stacking effects were observed in the VLP-NcSAG1/NcSRS2 bivalent antigen-displaying group. As discussed above,

it is possible that the less abundant display of NcSRS2 attenuated the overall efficacy of the bivalent VLPs derived from co-infections of three recombinant baculoviruses. It may be necessary to optimize the antigen display further and to consider other antigen combinations, e.g., NcSRS2/NcGAR7 [41]. Moreover, other novel antigen candidates, such as toxofilin, could also be selected based on the results from the prime model apicomplexan, *T. gondii* [42,43]. Apparently, it is also worth trying other polyvalent vaccines for neosporosis or other parasitic syndromes in a high-throughput manner due to the plasticity of the silkworm/bacmid-RSV-antigen-displaying platform.

In summary, the current study has shown the usability and feasibility of the silkworm/bacmid-RSV-antigen-displaying platform in producing bivalent vaccines against neosporosis in a gerbil model. The antigen-displaying RSV-LPs demonstrate promising efficacy in producing specific antibodies and extending subject life-span post-*N. caninum* infection. To the best of our knowledge, this is the first study to present an RSV-LP vaccine displaying bivalent antigens in neosporosis. There is still room for improvement in the antigen display to obtain polyvalent vaccines. Further studies should be aimed at optimizing the expression and display of each antigen in a balanced manner, and it will be highly interesting to try other polyvalent RSV-LP vaccines to achieve the best protection against neosporosis.

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Conflict of interest

The authors declare no conflict of interest.

Contributor statement

JX made substantial contributions to this work and JX, RH and HS expressed the antigen-displaying RSV-LPs in silkworm and Bm5 cells. KE takes responsibility for the animal experiments. JX and TK were involved in the study conceptualization and design. EYP validated the experimental data and supervised this work. All the authors were involved in making critical revisions to the manuscript. All the authors have approved the final manuscript.

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Figure legend

Fig. 1. Schematic diagram of the experimental design of the current study. (**A**) Construction of plasmids used for recombinant baculoviruses. BxSP: a signal peptide from the silkworm bombyxin protein; FLAG: DYKDDDDK epitope for affinity purification; GP64TM: C-terminal transmembrane domain of baculoviral GP64 protein; MA/CA/NC: matrix/capsid/nucleocapsid protein of *Rous sarcoma* virus (RSV) gag (Group-specific antigen, aa 1–577). (**B**) With co-infections of recombinant baculoviruses of different antigens or RSV Gag proteins, the expressed antigens with C-terminal TM are processed as membrane proteins anchored and displayed on the cell membrane, while the Gag proteins assemble automatically inside the cytoplasm and form the VLP, which afterward secrets into the culture medium of Bm cells or silkworm larval hemolymph. The resulting baculovirus or RSV-LP could be decorated with antigen(s) with better immunogenicity than antigen protein only when used as vaccines. (**C**) Verification of expression levels and patterns from the indicated combination of co-infections. Protein samples from infected cultured silkworm cells or medium (with or without ultracentrifuge in 20% sucrose solution) were employed to be examined with different antibodies, anti-DDDDK for antigens (NcSAG1 and NcSRS2), and anti-RSV-gag for VLPs respectively. Arrows indicate the specific protein bands for each protein.

Fig. 2. Verification and purification of RSV-LP displaying NcSAG1/NcSRS2 by the sucrose gradient centrifugation. (**A**) The experimental flow of the purification process of this study. Briefly, the collected silkworm hemolymph was concentrated in the 20% sucrose solution and then separated in 20%–60% sucrose gradients by ultracentrifuge. Subsequently, aliquots of each 500 μl were subjected to western blot to verify the fractions with expressions of both NcSAG1/NcSRS2 (**B**, **C**, **D**, upper panels, anti-DDDDK) and RSV-gag (**B**, **C**, **D**, lower panel,

anti-RSV-gag). Blue, red, and black arrows indicate the positive expression of NcSAG1, NcSRS2, and RSV-gag, respectively. The asterisks (*) indicate the fractions with the colocalizations of RSV-LP and antigens.

Fig. 3. Verification of RSV-LP displaying NcSAG1/NcSRS2 by Flag tag affinity purification. Following our concept that if display exists, RSV-LP should be retained along with the Flag tag-labeled NcSAG1/NcSRS2 during Flag tag affinity chromatography (**A**). Western blot analysis of protein samples from purifications of silkworm hemolymph (20% sucrose-concentrated) with co-infection of single antigen (NcSRS2) or double antigens (NcSAG1 and NcSRS2) with RSV-Gag (**B**). Blue, red, and black arrows indicate the positive expression of NcSAG1, NcSRS2, and RSV-gag, respectively. The asterisk (*) indicates the unspecific band.

Fig. 4. Evaluation of antigens-displayed RSV-LPs employed for vaccination against *Neospora* infection in a Mongolian gerbil model. (**A**) Time-schedule of gerbil vaccination, bleeding, *Neospora* challenge, and sacrifice. The prime and boost immunization strategies for four different groups (PBS, VLP only, VLP-NcSRS2, and VLP-NcSAG1/NcSRS2) were as shown. Briefly, gerbils were boosted in 2 and 4 week and challenged with *Neospora* via intramuscular injection at a dose of 1×10^5 in the 5th week. Mice were closely observed and investigated for their body weight (**B**) and parasite load in brain tissues by quantitative-PCR (**C**). Significant differences were evaluated using one-way ANOVA (* $p<0.05$). The blood samples were collected before the challenge, which were further utilized for the antibody titration (ELISA) of NcSAG1 (**D**) and NcSRS2 (**E**), respectively. In this study, silkworm-expressed Flag-NcSAG1 or Flag-NcSRA2 were employed as antigens. The dilution rate of the gerbil serum was shown in Log2 and the absorbance (optical density) was measured at 450 nm after sufficient color development. Three gerbils were used and investigated in each group.

Fig. 1

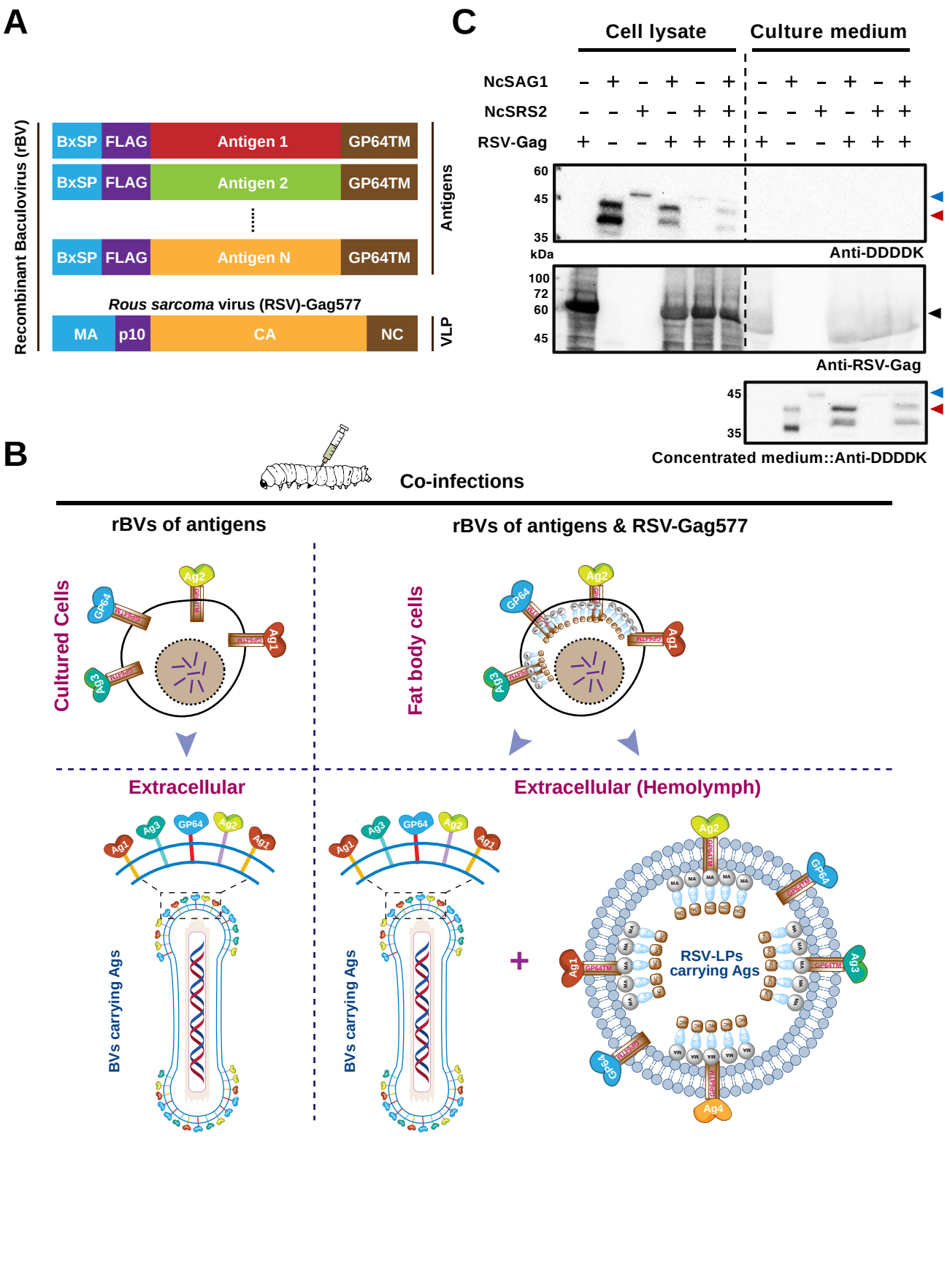

A
Recombinant Baculovirus (rBV)
BxSP
FLAG
Antigen 1
GP64TM
BxSP
FLAG
Antigen 2
GP64TM
BxSP
FLAG
Antigen N
GP64TM
Antigens
Rous sarcoma virus (RSV)-Gag577
MA
p10
CA
NC
VLP
C
Cell lysate
Culture medium
NcSAG1
NcSRS2
RSV-Gag
60
45
35
kDa
Anti-DDDDK
100
72
60
45
Anti-RSV-Gag
45
35
Concentrated medium::Anti-DDDDK
B
Co-infections
rBVs of antigens
rBVs of antigens & RSV-Gag577
Cultured Cells
Fat body cells
Ag1
Ag2
Ag3
GP64
Extracellular
Extracellular (Hemolymph)
BVs carrying Ags
RSV-LPs carrying Ags
Ag4

Fig. 2

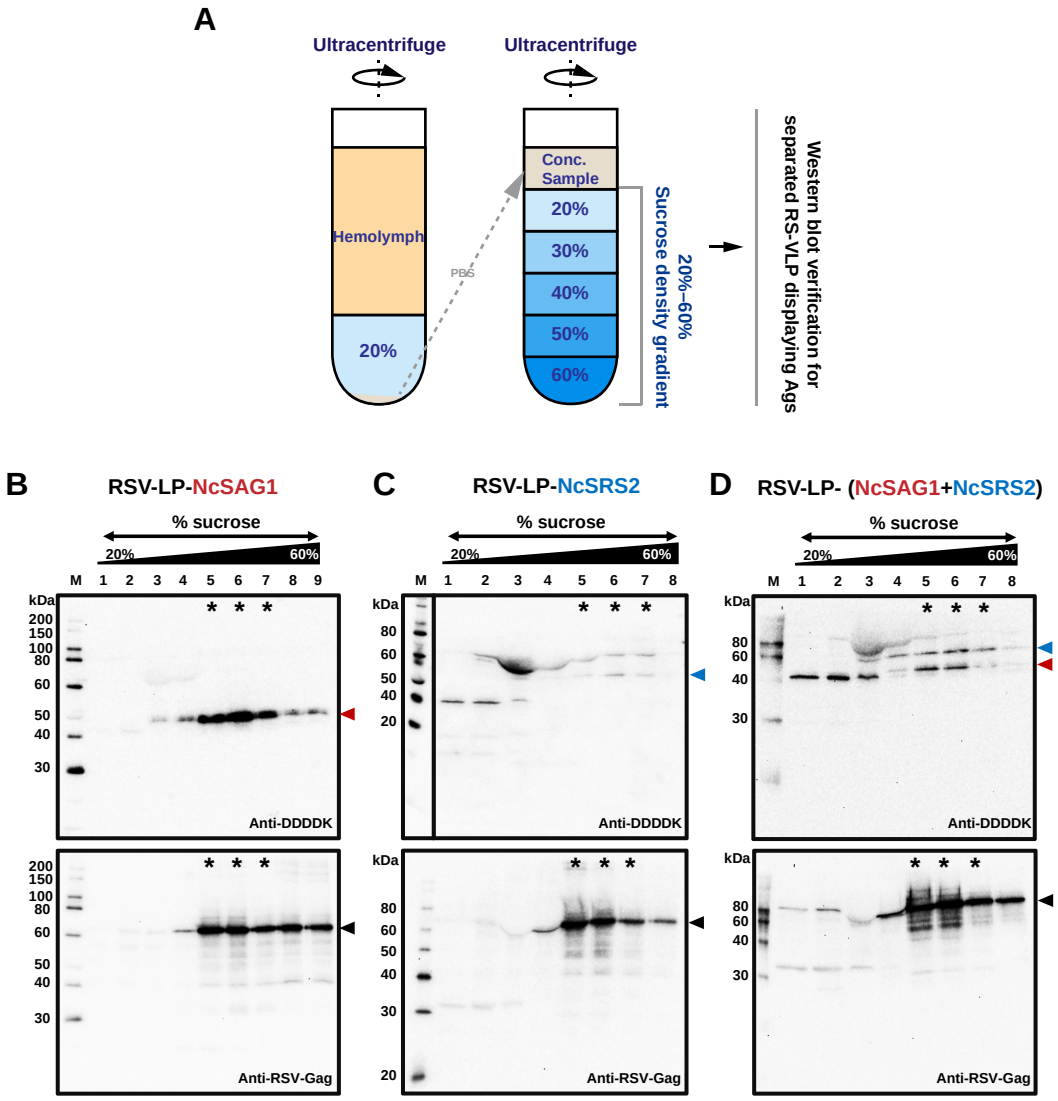
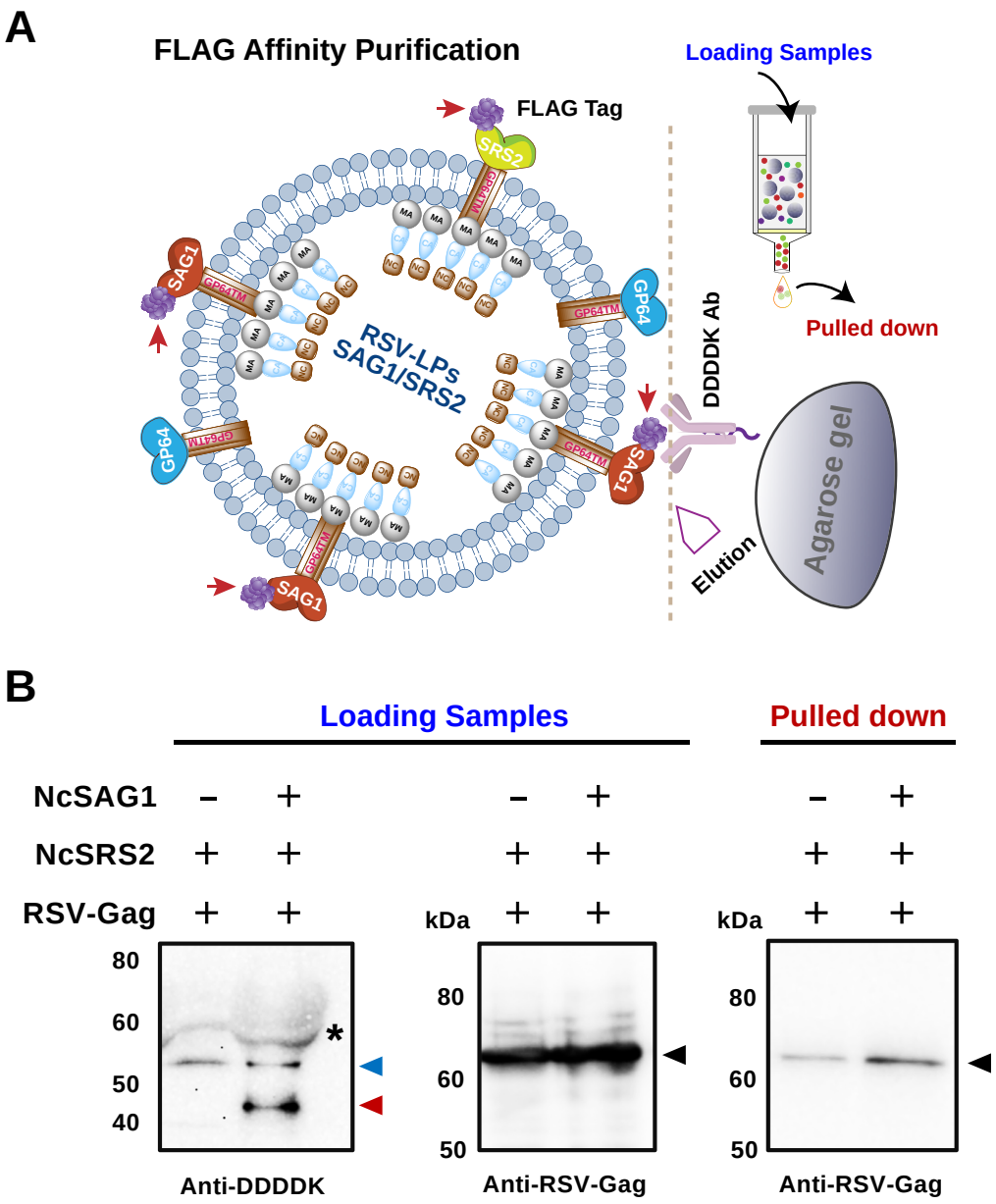

A
Ultracentrifuge
Ultracentrifuge
Hemolymph
20%
PBS
Conc. Sample
20%
30%
40%
50%
60%
20%–60%
Sucrose density gradient
Western blot verification for separated RS-VLP displaying Ags
B
RSV-LP-NcSAG1
C
RSV-LP-NcSRS2
D
RSV-LP- (NcSAG1+NcSRS2)
% sucrose
20%
60%
M 1 2 3 4 5 6 7 8 9
M 1 2 3 4 5 6 7 8
kDa
Anti-DDDDK
Anti-RSV-Gag

Fig. 3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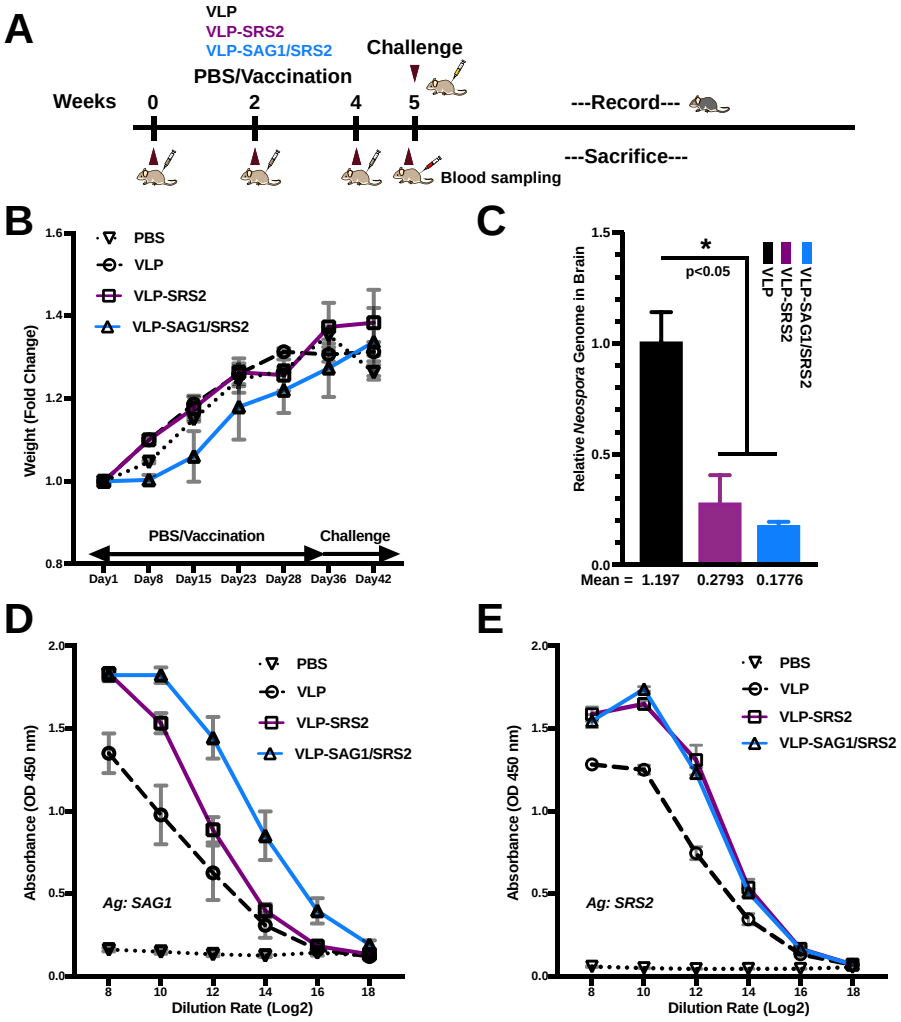
FLAG Affinity Purification
Loading Samples
FLAG Tag
SRS2
SAG1
GP64
GP64TM
MA
NC
RSV-LPs
SAG1/SRS2
DDDDK Ab
Pulled down
Agarose gel
Elution
B
Loading Samples
Pulled down
NcSAG1
NcSRS2
RSV-Gag
kDa
80
60
50
40
*
Anti-DDDDK
Anti-RSV-Gag
Anti-RSV-Gag

Fig. 4


A
VLP
VLP-SRS2
VLP-SAG1/SRS2
Challenge
PBS/Vaccination
Weeks
0
2
4
5
---Record---
---Sacrifice---
Blood sampling
B
PBS
VLP
VLP-SRS2
VLP-SAG1/SRS2
Weight (Fold Change)
PBS/Vaccination
Challenge
Day1
Day8
Day15
Day23
Day28
Day36
Day42
C
Relative Neospora Genome in Brain
*
p<0.05
VLP
VLP-SRS2
VLP-SAG1/SRS2
Mean = 1.197 0.2793 0.1776
D
Absorbance (OD 450 nm)
Ag: SAG1
Dilution Rate (Log2)
E
Absorbance (OD 450 nm)
Ag: SRS2
Dilution Rate (Log2)