Neospora caninum: Application of apical membrane antigen 1 encapsulated in the oligomannose-coated liposomes for reduction of offspring mortality from infection in BALB/c mice

Running head: Vaccine effects of NcAMA1 liposomes

Title: Application of *Neospora caninum* apical membrane antigen 1 encapsulated in the oligomannose-coated liposomes for reduction of offspring mortality from *N. caninum* infection in BALB/c mice

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Abstract

Liposomes coated with neoglycolipids constructed with mannopentaose and dipalmitoylphosphatidylethanolamine (Man3-DPPE), referred to as M3-DPPE liposomes, have been shown to induce cellular immunity against antigens encapsulated therein. To evaluate whether these M3-DPPE liposomes have an adjuvant capacity against *Neospora caninum* infection, a novel immunization method utilizing soluble *N. caninum* apical membrane antigen 1 (NcAMA1) encapsulated in the M3-DPPE liposomes (M3-NcAMA1) was employed. The intent was to reduce offspring mortality from *N. caninum* infection in susceptible, pregnant BALB/c mice. The results revealed that BALB/c mice developed IgG antibodies specific to *N. caninum*. A significant amount of interferon (IFN)-γ production was detected in culture supernatants of NcAMA1 protein- or *N. caninum* lysate-stimulated spleen cells obtained from the mice one week after the third immunization with M3-NcAMA1. This suggested that the T helper-type 1 (Th1) immune response was induced in the mice. The parasite burden in the dams' brain tissue was decreased in M3-NcAMA1-immunized mice. Moreover, the survival rate of offspring increased significantly in mice immunized with M3-NcAMA1. Taken together, the results demonstrated that a parasite-specific Th1 immune response was successfully induced in the pregnant mice immunized with M3-NcAMA1. Thus, an effective reduction of offspring mortality from *N. caninum* infection was triggered.

***Neospora caninum* / apical membrane antigen 1 / oligomannose-coated liposomes**

1. INTRODUCTION

The apicomplexan parasite *Neospora caninum* is an important pathogen known to cause abortions in dairy and beef cattle; in addition, it causes neuromuscular disease in dogs and other animals [1, 7, 8]. Transplacental transmission is considered a primary route of *N. caninum* transmission in cattle [10], and abortion or stillbirth is the only clinical sign of *N. caninum* infection [9]. Therefore, prevention of vertical transmission is crucial for limiting economic losses in cattle caused by neosporosis.

Vertical transmission is the primary mode of transfer of this parasite from mother to fetus via the placenta during successive pregnancies [1, 5, 29]. Transplacental transmission has been induced experimentally in cattle, dogs, sheep, goats, monkeys, cats, and mice and occurs naturally in many hosts [7]. In previous studies, a high frequency of vertical transmission in BALB/c mice infected with *N. caninum* was observed when the mice had been inoculated during pregnancy [21, 34, 36], but the precise mechanism of vertical transmission was poorly understood. Long and Baszler [30] hypothesized that the induction of a maternal type 1 response against *N. caninum* could prevent vertical transmission and demonstrated that modulation of type 2 cytokines by administering anti-interleukin-4 (anti-IL-4) monoclonal antibodies before pregnancy can reduce the frequency of vertical transmission of *N. caninum*. Kano et al. [21] reported that transplacental transmission may be due to the reactivation of the parasite or down-regulation of protective immunity in the mice. Additionally, *N. caninum* is an obligate intracellular parasite, and cell-mediated immunity plays a major role in

protection. The critical components of the immune response for combating infection in cattle are interferon gamma (IFN-γ) and CD4 T cells [17, 46]. Therefore, *N. caninum* vaccines that can induce cellular immune responses and prevent vertical transmission are crucial for the control of neosporosis.

Until now, many studies have focused on the prevention of vertical transmission of *N. caninum*. In affording protection against *N. caninum* infection and vertical transmission of the parasite in mice or cattle, immunization with live tachyzoites is shown to be far superior to immunization with tachyzoite lysates [16, 26, 28, 35, 45, 46]. Vaccination with irradiated tachyzoites was able to completely protect against lethal challenge but provided only partial protection against vertical transmission [38]. Recently, several recombinant vaccines have been evaluated and may be considered as potential candidates against vertical transmission of *N. caninum*. A vaccinia virus vaccine expressing SRS2 provided 83% protection [34]. Immunization of mice with plasmid DNA coding for NcGRA7 or NcsHSP33 confers partial protection against vertical transmission of *N. caninum* [27]. Vaccination of mice with a GRA7 DNA vaccine, combined with cytosine-phospho-guanine (CpG) as an adjuvant, reduced vertical transmission by 87% [20]. More recently, the recombinant *Brucella abortus* strain RB51 vaccines, expressing *N. caninum* dense granule 2 (GRA2) and GRA6, were able to induce significant protection against vertical transmission of *N. caninum*, drastically reducing the parasite burden in offspring and reducing offspring mortality. The RB51 expressing microneme antigen 1 (MIC1) and MIC3 was also able to reduce vertical transmission [38].

Various antigen-delivery systems were used in development of recombinant vaccines. In a previous study, when administered as a construct in a recombinant vaccinia virus delivery system, NcSRS2 provoked cellular immune responses and antibody production at an early stage of *N. caninum* infection during pregnancy [34]. Moreover, Jenkins et al. [20] achieved improved protection with an NC-GRA7 DNA vaccine through the addition of CpG to improve the Th1 response. Similarly, the strong Th1 responses induced by the *B. abortus* strain RB51 vaccines, expressing GRA2 and GRA6, were able to induce significant protection against vertical transmission [38]. Furthermore, Shimizu et al. [40] identified that intraperitoneal immunization of Oligomannose-coated liposomes (OMLs) entrapped with a soluble leishmanial antigen in BALB/c mice induces an antigen-specific Th1 immune response and protects against subsequent infection with *Leishmania major*. Therefore, selection of an effective delivery system is important for the induction of appropriate protective immune responses and the induction of long-lasting immunity by administering antigens.

OMLs can be incorporated into F4/80-positive cells (most probably macrophages) or intraperitoneal CD11b-positive DC and induce a protective response when injected into the peritoneal cavity [14, 40]. Moreover, OMLs may activate peritoneal macrophages (PEMs) to up-regulate the expression of co-stimulatory molecules and preferentially secrete IL-12, which would result in the activation of T-lymphocytes (both CD4-positive and CD8-positive T cells) [43]. Furthermore, OMLs employed in effective antigen-delivery could induce both Th subsets and cytotoxic T-lymphocyte (CTL) against antigens encapsulated in the

liposomes [15]. Up to now, liposomes have been widely used as an antigen transport system in vaccines against viruses [13], bacteria [19], fungi [39], tumors [22], and protozoa [24, 40]. These studies suggested that there was no detectable toxicity and no liposome-caused skin damage at the injection site. Therefore, liposomes could be suitable for use as a safe adjuvant in the induction of a Th1 immune response and to elicit a protective Th1 immune response against several infections. A previous study indicated that the liposomes coated with a neoglycolipid composed of mannotriose (Man3) and dipalmitoylphosphatidylethanolamine (DPPE) (referred to as Man3-DPPE liposomes) were the best compounds for macrophage uptake. They were incorporated into peritoneal macrophages and triggered the induction of a Th1 immune response. The encased antigen was effectively presented by both MHC class I and class II molecules [15]. Consequently, these findings led us to hypothesize that immunization with soluble *N. caninum* antigens encapsulated in liposomes coated with Man3-DPPE could reduce the offspring mortality from *N. caninum* infection in BALB/c mice.

In this study, the *N. caninum* apical membrane antigen 1 (NcAMA1) encapsulated in Man3-DPPE liposomes (referred to as M3-NcAMA1) was explored as a new vaccine candidate. Subcutaneous (s.c.) immunization of M3-NcAMA1 for the induction of anti-parasite immunity was evaluated, and the profile of proinflammatory cytokines (the production of IFN-γ as the type 1 cytokine, and IL-4 as the type 2 cytokine) was investigated. We aim to clarify

whether M3-NcAMA1 can elicit a protective immune response against *N. caninum* infection and reduce the offspring mortality in pregnant BALB/c mice.

2. MATERIALS AND METHODS

2.1. Parasite culture and purification

The *N. caninum* (Nc-1 strain) was maintained, purified, and counted as previously described [49]. The *N. caninum* tachyzoites were resuspended in an MEM medium for the challenge test.

2.2. Cloning, expression, and purification of the recombinant NcAMA1 (rNcAMA1)

The cloning, expression, and purification of rNcAMA1 were performed as previously described [48]. The purity of proteins was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [25]. The endotoxin of the purified proteins was removed through Detoxi-Gel^{TM} Endotoxin Removing Gel (Thermo Scientific, USA) according to the manufacturer's instructions. The concentration of proteins was determined with a BCA protein assay kit (Thermo Scientific, USA). The proteins were sterilized by filtration through a 0.20-μm filter (Pall, USA) and stored at -80℃ until use.

2.3. Preparation of Man3-DPPE liposomes

Man3-DPPE liposomes were prepared as previously described [40]. Briefly, a chloroform/methanol (2:1, v/v) solution containing 1.5 μmol of DPPC, 1.5 μmol of cholesterol, and 0.15 μmol of Man3-DPPE was added to a conical flask and rotary evaporated to prepare a lipid film containing neoglycolipids. Procedures for protein-encapsulating of OMLs were performed as described previously [40]. Briefly, 180 μl of GST or NcAMA1 (500 μg/ml) was added to the dried lipid film, and multilamellar vesicles were prepared by intense vortex dispersion. The multilamellar vesicles were extruded 5 times through 1-μm-pore polycarbonate membrane (Whatman, USA). Liposome-encapsulated NcAMA1 was separated from free NcAMA1 proteins by three successive rounds of washing in PBS with centrifugation (12,000 rpm, 30 min, 4℃). The amounts of encapsulated proteins were measured using a modified Lowry protein assay kit (Thermo Scientific, USA) in the presence of 0.3% (w/v) SDS using bovine serum albumin (BSA) as the standard.

2.4. Immunization, mating, and challenge of mice

Eight-week-old specific pathogen-free female BALB/c mice (n=8 per group) were purchased from Clea (Japan). The mice were maintained under specific pathogen-free conditions. All animal experiments were conducted according to the Guidelines for the Care and Use of Research Animals provided by Obihiro University of Agriculture and Veterinary Medicine. Four groups of mice were immunized subcutaneously in the neck area with either: (i) 100 μl PBS, (ii) 40 nmol GST encapsulated in Man3-DPPE liposomes (M3-GST) (~1 μg) in 100 μl PBS, (iii) 40 nmol NcAMA1-GST (~3.3 μg) in 100 μl PBS, or (iv) 40 nmol

NcAMA1-GST encapsulated in Man3-DPPE liposomes (M3-NcAMA1) (~3.3 μg) in 100 μl PBS. These were administered three times at intervals of one week. One week after the final immunization, female and male mice were kept in the same cage until the female mouse presented a sperm-plug. At 10 days of pregnancy all pregnant dams were challenged intraperitoneally with 1×10^5 *N. caninum* tachyzoites. The number of live or stillborn offspring and any deaths during the 30-day observation period were recorded. At the end all dams were sacrificed and brain samples were collected for PCR analysis.

2.5. Antibody isotype assay

The sera were collected one week after each immunization for antibody titer detection. *N. caninum*-specific total immunoglobulin G (IgG), IgG1, and IgG2a levels in mouse serum were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, flat-bottom 96-well plates were pre-coated with 50 μl of 20 μg/ml of *N. caninum* lysates in a 50 mM carbonate–bicarbonate buffer (pH 9.6) overnight at 4℃. The levels of total IgG, IgG1, and IgG2a in the serum which were diluted by 100-fold with 3% skim milk in PBS were measured in the plates using a horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, or IgG2a (BETHYL, USA). Absorbance at 415 nm in each well was measured using MTP-500 micro plate reader (Corona Electric, Japan).

2.6. *In vitro* spleen cell proliferation

One week after the final immunization, 3 mice from each group were sacrificed. Spleen cells were suspended and hemolyzed in a lysing buffer (0.83% NH_4Cl,

0.01M Tris–HCl, pH 7.2). After washing with PBS, cells in a complete RPMI-1640 medium (Sigma, USA) supplemented with 5% FBS were dispensed in triplicate into a 96-well culture plate (5×10^5 cells in each well). The spleen cells were stimulated with 2 μg/ml of Concanavalin A (ConA), 50 μg/ml of *N. caninum* tachyzoite lysates or NcAMA1 (10 or 40 μg/ml), and 50 μM 2-mercaptoethanol (2-ME) and incubated at 37 ℃ for 48 h in a humidified 5% CO_2 incubator. As a control heterogeneous protein, 10 μg/ml of the purified recombinant protein of *N. caninum* dense granule protein 7 (NcGRA7) fused with GST [12] was also used. The supernatants of the spleen cell cultures were collected at 48 h and kept at -80 ℃ until assaying of the cytokines. Cytokines (IFN-γ and IL-4) in the culture supernatant were quantified using ELISA kits (IFN-γ and IL-4, Endogen, USA) in accordance with the manufacturer's instructions.

2.7. DNA extraction from brain tissue of dams and the conventional PCR analysis

For DNA preparation, brain tissue samples were weighed and homogenized in an extraction buffer (0.1 M Tris-HCl pH 9.0, 1% SDS, 0.1 M NaCl, 1 mM EDTA) and 1 mg/ml of proteinase K at 55°C overnight. The DNA was purified by phenol-chloroform extraction and ethanol precipitation. The DNA concentration was adjusted to 100 μg/ml for each brain and used as a template DNA for PCR analysis. The DNA amplified by PCR was performed as described by Yamage et al. [47]. The PCR products were visualized by electrophoresis in agarose gels.

3. RESULTS

3.1. Antibody responses induced in M3-NcAMA1-immunized group

BALB/c mice were subcutaneously immunized with the proteins that were encapsulated into liposomes, and no toxicity or inflammation was observed at the skin sites where the protein-coated liposomes had been injected (Data not shown). ELISA was performed to evaluate the induction of antibody response and measure the serum levels of NcAMA1-specific IgG, IgG1, and IgG2a. As shown in Figure 1, NcAMA1-specific total IgG, IgG1, and IgG2a antibody levels in M3-NcAMA1-immunized mice were significantly higher than that of the control mice (uncoated NcAMA1). Immunization with uncoated NcAMA1 protein displayed a negligible amount of IgG1 and IgG2a.

3.2. Cytokine levels in culture supernatants from spleen cells of immunized mice

The levels of IFN-γ and IL-4 in culture supernatants from spleen cells of immunized mice stimulated with *N. caninum* lysates and NcGRA7 or NcAMA1 proteins were quantified by ELISA. Significant levels of IFN-γ were detected in the culture supernatants of NLA or NcAMA1 protein-stimulated spleen cells obtained from mice immunized with M3-NcAMA1, and low levels of IFN-γ were detected in spleen cells from mice that received NcAMA1. IFN-γ was not detectable in spleen cells from mice that received PBS or M3-GST (Figure 2). On the other hand, small amounts of IL-4 were produced in the culture supernatants of NcAMA1 protein-stimulated spleen cells obtained from mice immunized with M3-NcAMA1, but IL-4 was not detectable in spleen cells from mice that received PBS, M3-GST, or NcAMA1 (Figure 2). Moreover, there are no detectable IFN-γ

and IL-4 in the culture supernatants of NcGRA7 protein-stimulated spleen cells obtained from immunized mice. This suggests that M3-NcAMA1-immunized mice produced cytokines in an antigen-specific manner. These results indicated that s.c. immunization with M3-NcAMA1 induced both the parasite- and antigen-specific Th1 and Th2 immune responses in mice. All ConA controls induced significant levels of the respective cytokines while the medium controls did not (Figure 2).

3.3. Litter numbers and survival rates of offspring in vaccinated and challenged mice

One week after the last immunization, mice were mated and challenged with *N. caninum* tachyzoites (1×10^5) at 10 days of gestation. The litter number and survival rate of offspring in all treatment groups are summarized in Table I. The number of offspring in the M3-NcAMA1-immunized group was not statistically different from that of other groups. These results indicated that immunization with M3-NcAMA1 has no effect on the birth rate. Moreover, with an offspring survival rate of 60% the mice immunized with M3-NcAMA1 showed specific protection, whereas the mice immunized with the NcAMA1 alone, M3-GST, or PBS showed lower survival rates (8%, 18.6%, and 17.5%, respectively). As shown in Table I and Figure 3, the differences were statistically significant. These results suggested that M3-NcAMA1 immunization induced a systemic immune response that reduces offspring mortality from *N. caninum* infection in mice.

3.4. Detection of *N. caninum* DNA in brain tissue of dams

For detection of *N. caninum* infection in dams, DNA extracted from brain tissue homogenates was examined by *N. caninum*-specific conventional PCR. The rates of PCR positive results were 100% in treatment of the mice with PBS, M3-GST, or NcAMA1 alone, while the rate of PCR positive results was lowest (44.4%) in the M3-NcAMA1 group (Table II). Therefore, these results reveal that mice injected with M3-NcAMA1 had a significant reduction in the brain parasite burden in comparison with that of the control groups ($P < 0.01$).

4. DISCUSSION

Previous studies indicated that *Toxoplasma* AMA1 (TgAMA1) plays a critical role in host cell invasion, and DNA vaccination with the gene encoding TgAMA1 gene appears to generate a strong specific immune response and provide effective protection against toxoplasmosis [6, 31]. Moreover, we identified and characterized NcAMA1, indicating that the anti-NcAMA1 antibody inhibits host cell invasion by *N. caninum*, and suggesting that NcAMA1 might be a potential vaccine candidate to control *N. caninum* infection [48]. Therefore, recombinant NcAMA1 was chosen as vaccine candidate in the current study.

Intraperitoneal administration is one of the most frequently used parenteral routes in rodents. However, the intraperitoneal administration of liposomes is not a convenient method of vaccination for large animals. In addition, intraperitoneal administration is accompanied by a high risk of side effects such as catheter-related complications and abdominal pain [3]. Recently, s.c. immunization of an OML-based vaccine was shown to induce effective anti-tumor immunity [22].

Therefore, in order to investigate whether s.c. immunization with M3-NcAMA1 also induces antigen-specific anti-*N. caninum* immunity *in vivo*, BALB/c mice were immunized subcutaneously with PBS, M3-GST, uncoated NcAMA1, or M3-NcAMA1, and boosted twice. The results revealed no detectable toxicity and no skin damage caused by the M3-DPPE liposomes at the injection site. Moreover, the M3-DPPE liposome-based vaccines have two advantages. First, the amount of immunization is small (maximum is 4 μg/mouse each time). Second, the M3-DPPE liposomes were taken up specifically through macrophages at l-week interval immunization, and it accelerated the production of antibody response. In this study, we used s.c. injection of OMLs. The time-course analysis and the side effects induced by s.c. injection of M3-NcAMA1 should be further investigated to assure their safe clinical application.

In this study, measurements of antibody levels were carried out using *N. caninum* lysates rather than recombinant antigens in order to ensure that the recombinant antigens were expressed in the right conformation and that the immune responses were induced specifically against the native *N. caninum* antigens. In Figure 1, immunization of susceptible BALB/c mice with M3-NcAMA1 significantly increased the antibody levels (both *N. caninum*-specific IgG1 and IgG2a isotype antibodies were detected) in comparison to the control group of mice that received PBS, M3-GST, or uncoated NcAMA1. This suggests that the mice have already obtained an immune response by s.c. injection of M3-NcAMA1. Moreover, the high level of IgG1 antibodies was detected in M3-NcAMA1-immunized mice. A previous study indicated that a high level of

production of IgG1 Ab to the parasite is important for clearance of *N. caninum* at the early stage of infection and that the T cell response plays a crucial role in protection against the intracellular infection at a later stage [32, 33]. Therefore, it appears that the M3-NcAMA1 performed better as a vaccination at the early stage of *N. caninum* infection. Furthermore, both IgG1 and IgG2a isotype antibodies were detected in the serum of M3-NcAMA1-immunized mice (Figure 1), suggesting that both Th1 and Th2-like immune response may be induced.

A recent study involving vaccination with live tachyzoites and tachyzoite lysates suggests that antigen-specific T cells (especially those that secrete IFN-γ), but not antibodies, are crucial for protection against *N. caninum*-induced fetal death in cattle [45]. Previous studies indicated that a Th1 type immune response in the maternal immune system during pregnancy could result in the overproduction of IFN-γ, IL-12, tumour necrosis factor α (TNF-α), and nitric oxide (NO). These are strong enough to reduce vertical transmission of *N. caninum* in vaccinated dams [2, 37]. The advantage of OML-mediated immunization is Th1-skewing of the cytokine profiles [41]. To examine whether the Th1 immune response induced in the immunized mice could be maintained throughout the course of *N. caninum* infection, the production of Th1 cytokines (IFN-γ) and Th2 cytokines (IL-4) was measured in culture supernatants of NcAMA1 protein or *N. caninum* lysate-stimulated spleen cells obtained from mice 1 week after the final immunization. *In vitro* stimulation of spleen cells obtained from mice immunized with M3-NcAMA1 elicited an effective NcAMA1-specific Th1 immune response, producing significantly higher levels of IFN-γ in comparison with spleen cells

from mice treated with other groups (those treated with PBS, M3-GST, or NcAMA1 alone) ($P < 0.005$) and small amounts of IL-4 ($P < 0.005$) (Figure 2). Mice administered NcAMA1 alone produced low levels of both IFN-γ and IL-4 because the mice were administered only 40 nmol protein, which could not activate the production of cytokines in mice (Figure 2). Thus, s.c. administration of M3-NcAMA1 induced a strong Th1 immune response with significant IFN-γ production and a Th2 immune response with IL-4 production, and protection against subsequent *N. caninum* vertical transmission.

In this study, we found higher levels of IFN-γ in the supernatants of spleen cell cultures obtained from mice immunized with M3-NcAMA1 (Figure 2). However, a previous study indicated that expression of pro-inflammatory cytokines, such as IFN-γ and TNF-α, is down-regulated while regulatory cytokines such as IL-10, TGF-b, and IL-4 are up-regulated during pregnancy in cattle and mice [4, 11, 18]. Therefore, the strong Th1 responses induced by vaccination, which can be capable of inducing abortion and/or foetal resorption, are incompatible with successful pregnancy [38]. However, the down-regulation of Th1 responses during pregnancy may be reflected in an increase in frequency of vertical transmission [2, 42, 44]. Conversely, if a dominant Th2 response occurs, increased IL-4 levels will support the development of the foetus. These cytokines may not, however, adequately control a parasitic infection, leading in turn to an increased parasite burden in the dams and increased vertical transmission rates [4, 11, 23]. These results suggested that a suitable balance in the production of Th1/Th2-type cytokines is crucial for the control of vertical transmission of *N. caninum.* In this

study, a systemic elevation of both Th1 and Th2 cytokines was found in M3-NcAMA1-immunized mice. These results implied that the M3-NcAMA1 vaccine might be suited to both successful pregnancy and reduced parasite infection. It is possible that the higher levels of IFN-γ and IL-4 in the systemic cytokine milieu may differ from the local cytokine environment of the gravid uterus, and this needs to be investigated by further study.

As described in a previous study, dams infected with *N. caninum* at 10 days of gestation showed a high rate of parasite transmission to their offspring [26]. In determining the tissue-parasite load, *N. caninum* DNA was detected in brain tissue of dams by conventional PCR. The results revealed that M3-NcAMA1 was able to substantially reduce the parasite burden in the dams (Table II). As shown in Table I and Figure 3, the survival rate of offspring from mice immunized with M3-NcAMA1 is the highest after 30 days when the pregnant mice were challenged with 1×10^5 *N. caninum*. On the other hand, the survival rate was low in PBS, M3-GST, or uncoated NcAMA1-immunized mice. Therefore, immunization of mice with PBS, M3-GST, or uncoated NcAMA1 did not reduce the offspring mortality. These results indicated that M3-NcAMA1-mediated immunization can decrease the parasite burden in the dams and reduce offspring mortality.

In conclusion, our results demonstrated that NcAMA1 proteins delivered by adjuvant OMLs via s.c. administration have potential for clinical use in anti-*N. caninum* vaccination in efforts to control neosporosis. Consequently, further evaluation of the usefulness of M3-NcAMA1 as a potential vaccine to control vertical transmission of *N. caninum* will be also required.

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Table I. Litter sizes and survival rates in control mice and vaccinated mice

Group	Trial	No. litters	Mean no. offspring / litter	No. of survival offspring / no. of offspring in litter	Total no. of survival offspring / total no. of offspring (%)
PBS	Trial 1	4	6.8	2/3, 2/7, 0/8, 2/9	6 / 27 (22.2%)
	Trial 2	5	7.2	1/5, 0/8, 0/10, 4/8, 0/5	5/36 (13.4%)
	Total	9	7.0		11/63 (17.5%)
M3-GST	Trial 1	4	6.8	0/7, 3/6, 1/9, 3/5	7/27 (25.9%)
	Trial 2	5	6.4	0/7, 3/5, 0/7, 1/8, 0/5	4/32 (12.5%)
	Total	9	6.6		11/59 (18.6%)
NcAMA1	Trial 2	4	6.3	2/9, 0/7, 0/6, 0/3	2/25 (8%)
M3-NcAMA1	Trial 1	4	6.0	4/6, 6/8, 1/3, 4/7	15/24 (62.5%)
	Trial 2	5	5.2	2/7, 3/4, 1/3, 3/6, 6/6	15/26 (57.7%)
	Total	9	5.6		30/50 (60%) *****

Female mice were immunized subcutaneously with NcAMA1 encapsulated in Man3-DPPE liposomes (M3-NcAMA1), GST encapsulated in Man3-DPPE liposomes (M3-GST), NcAMA1 in PBS, or PBS three times at intervals of one week. One week after the final immunization, female and male mouse were kept in the same cage until the female mouse presented a sperm-plug. All pregnant dams were challenged intraperitoneally with 1×10^5 *N. caninum* tachyzoites on 10 days of gestation. The number of live and dead offspring during the 30-day observation period was recorded. * Significant different from the PBS group as determined by chi-square analysis ($P<0.01$).

Table II. Detection of *N. caninum* infection in brain tissue of dams by PCR

Group	Trial	No. of positive/total dams (%)
PBS	Trial 1	4/4 (100%)
	Trial 2	5/5 (100%)
	Total	9/9 (100%)
M3-GST	Trial 1	4/4 (100%)
	Trial 2	5/5 (100%)
	Total	9/9 (100%)
NcAMA1	Trial 2	4/4 (100%)
M3-NcAMA1	Trial 1	2/4 (50%)
	Trial 2	2/5 (40%)
	Total	4/9 (44.4%) *

Female mice were immunized subcutaneously with PBS, M3-GST, NcAMA1 or M3-NcAMA1 three times at intervals of one week. One week after the final immunization, female and male mouse were kept in the same cage until the female mouse presented a sperm-plug. All pregnant dams were challenged intraperitoneally with 1×10^5 *N. caninum* tachyzoites on 10 days of gestation. DNA was extracted from brain tissue of the dams on 40 days after the infection, and then was analyzed by PCR. * Significant different from the PBS group as determined by chi-square analysis ($P<0.01$).

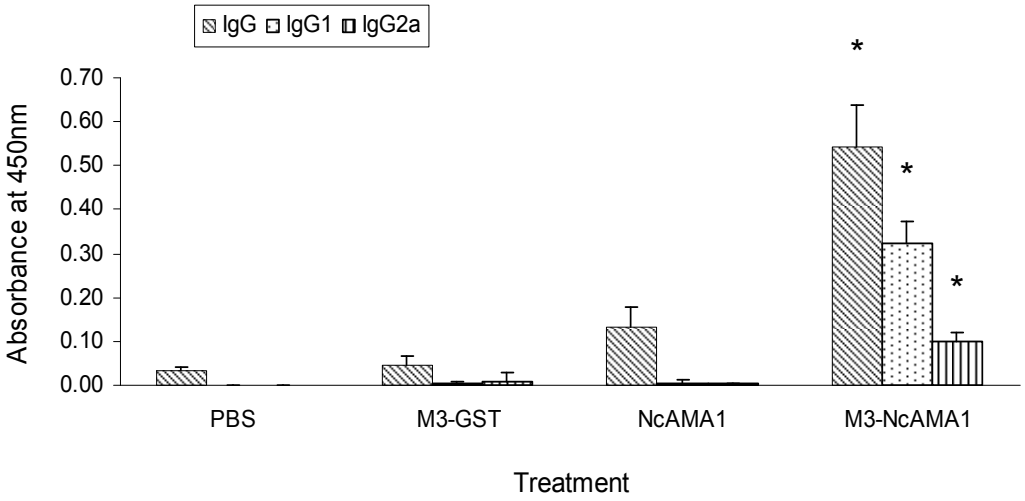
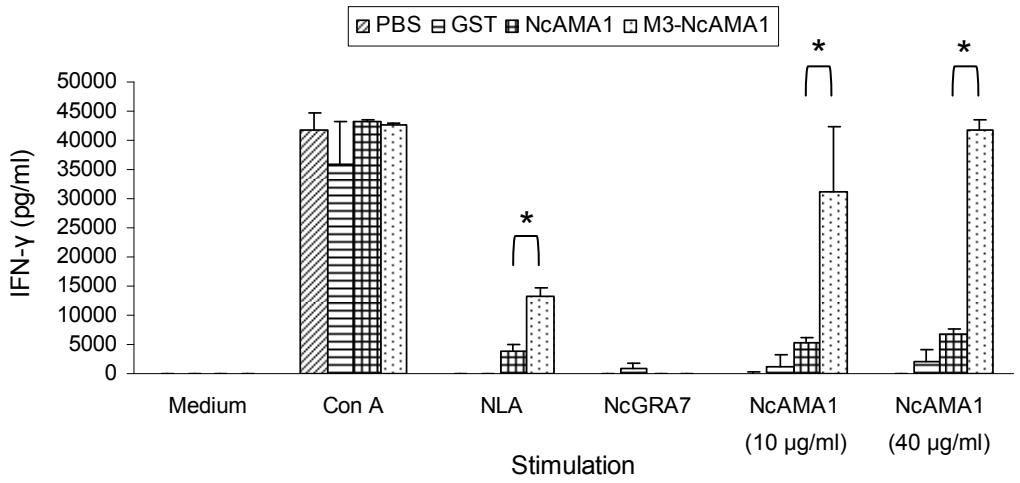


Figure 1. ELISA detection of *N. caninum*-specific antibody responses in vaccinated mice after the last immunization. Eight mice per group were immunized subcutaneously with PBS, GST encapsulated in Man3-coated liposomes (M3-GST), NcAMA1, or NcAMA1-GST encapsulated in Man3-coated liposome (M3-NcAMA1) three times at one-week intervals. NLA were used as antigen for measurement of the parasite-specific total IgG, IgG1, and IgG2a antibodies in sera. The values are expressed as the absorbance at 415 nm. Each bar represents the mean ± standard deviation (SD) of five mice per group. Results are representative of two independent experiments. Statistically significant differences were observed between the groups immunized with M3-NcAMA1 and other groups (*P<0.01).



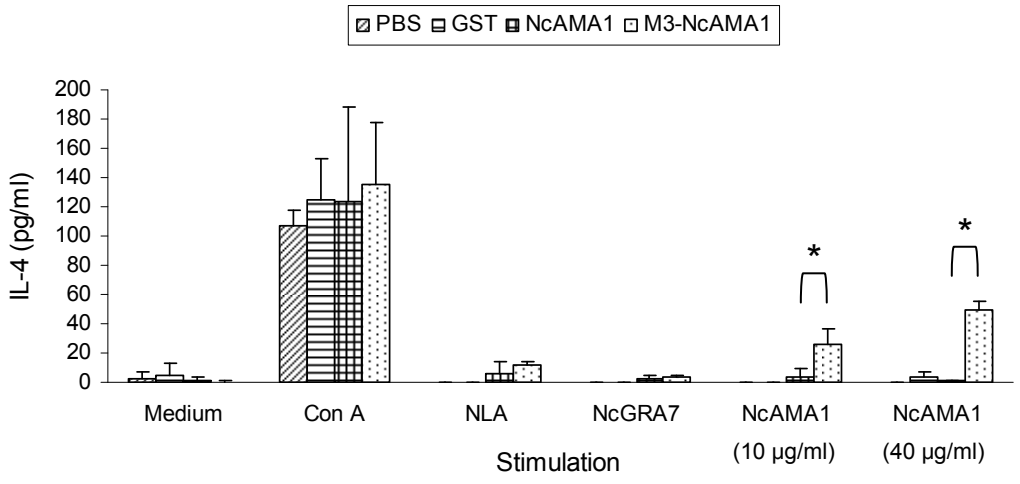


Figure 2. Productions of IFN-γ and IL-4 in culture supernatants of spleen cells. Three mice per group were immunized subcutaneously with PBS, M3-GST, NcAMA1, or M3-NcAMA1 three times at intervals of one week and then sacrificed one week after the last immunization. Single cell suspensions were prepared from the spleens of individual mice in the groups and cultured in

triplicate for 48 h in the presence of 2 μg/ml ConA, 50 μg/ml NLA, 10 μg/ml NcGRA7, 10 μg/ml NcAMA1, or 40 μg/ml NcAMA1 or without any stimulator. The IFN-γ and IL-4 secreted in culture supernatants were assayed by ELISA. Each bar represents the mean ± SD of three mice per group. Results are representative of two independent experiments. Statistically significant differences were observed between groups of NcAMA1 and M3-NcAMA1 (*P<0.01).

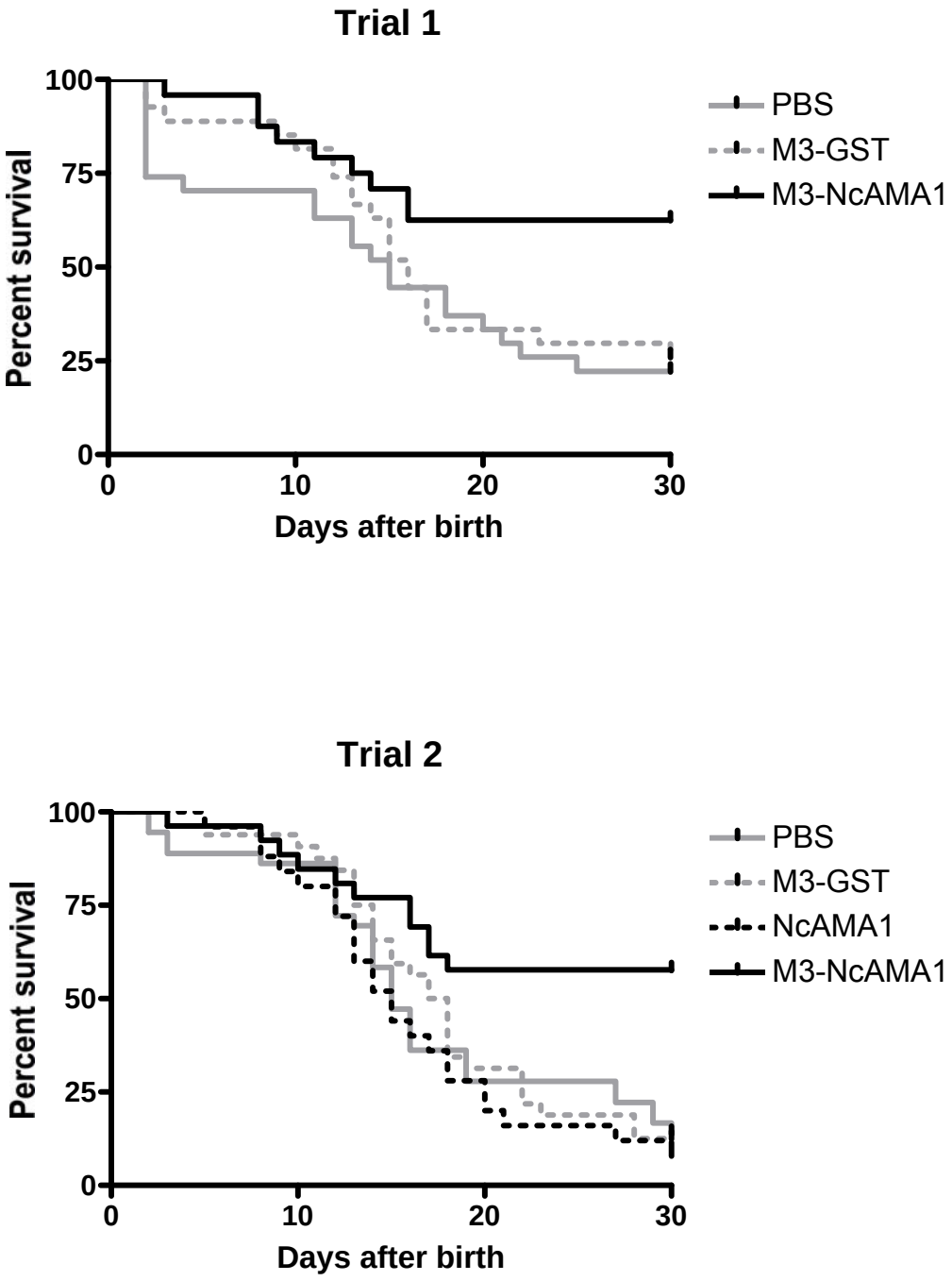


Figure 3. Survival rates of offspring in trials 1 and 2. Female mice were immunized subcutaneously with PBS, M3-GST, NcAMA1, or M3-NcAMA1 three

times at intervals of one week. One week after the final immunization, female and male mice were kept in the same cage until the female mouse presented a sperm-plug. All pregnant dams were challenged intraperitoneally with 1×10^5 *N. caninum* tachyzoites at 10 days of gestation. The number of live and dead offspring during the 30-day observation period was recorded.