

1 **Running head:** Vaccine effects of NcAMA1 liposomes

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

5 **Authors**

6 Houshuang Zhang¹, Yoshifumi Nishikawa¹, Yuzuru Ikehara², Naoya Kojima³,
7 Naoaki Yokoyama¹, Xuenan Xuan^{1*}

8 **Addresses**

9 ¹ National Research Center for Protozoan Diseases, Obihiro University of
10 Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555,
11 Japan

12 ² Research Center for Glycoscience, National Institute of Advanced Industrial
13 Science and Technology, Tsukuba, Ibaraki 305-8568, Japan

14 ³ Institute of Glycoscience, Tokai University, Hiratsuka, Kanagawa 259-1292,
15 Japan

16 ***Corresponding Author:** Xuenan Xuan

17 National Research Center for Protozoan Diseases, Obihiro University of
18 Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555,
19 Japan

20 Tel.: +81-155-49-5648 Fax: +81-155-49-5643

21 E-mail: gen@obihiro.ac.jp

22

1 **Abstract**

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

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

1 **1. INTRODUCTION**

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

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

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

5 Until now, many studies have focused on the prevention of vertical transmission
6 of *N. caninum*. In affording protection against *N. caninum* infection and vertical
7 transmission of the parasite in mice or cattle, immunization with live tachyzoites
8 is shown to be far superior to immunization with tachyzoite lysates [16, 26, 28, 35,
9 45, 46]. Vaccination with irradiated tachyzoites was able to completely protect
10 against lethal challenge but provided only partial protection against vertical
11 transmission [38]. Recently, several recombinant vaccines have been evaluated
12 and may be considered as potential candidates against vertical transmission of *N.*
13 *caninum*. A vaccinia virus vaccine expressing SRS2 provided 83% protection [34].
14 Immunization of mice with plasmid DNA coding for NcGRA7 or NcsHSP33
15 confers partial protection against vertical transmission of *N. caninum* [27].
16 Vaccination of mice with a GRA7 DNA vaccine, combined with cytosine-
17 phospho-guanine (CpG) as an adjuvant, reduced vertical transmission by 87%
18 [20]. More recently, the recombinant *Brucella abortus* strain RB51 vaccines,
19 expressing *N. caninum* dense granule 2 (GRA2) and GRA6, were able to induce
20 significant protection against vertical transmission of *N. caninum*, drastically
21 reducing the parasite burden in offspring and reducing offspring mortality. The
22 RB51 expressing microneme antigen 1 (MIC1) and MIC3 was also able to reduce
23 vertical transmission [38].

1 Various antigen-delivery systems were used in development of recombinant
2 vaccines. In a previous study, when administered as a construct in a recombinant
3 vaccinia virus delivery system, NcSRS2 provoked cellular immune responses and
4 antibody production at an early stage of *N. caninum* infection during pregnancy
5 [34]. Moreover, Jenkins et al. [20] achieved improved protection with an NC-
6 GRA7 DNA vaccine through the addition of CpG to improve the Th1 response.
7 Similarly, the strong Th1 responses induced by the *B. abortus* strain RB51
8 vaccines, expressing GRA2 and GRA6, were able to induce significant protection
9 against vertical transmission [38]. Furthermore, Shimizu et al. [40] identified that
10 intraperitoneal immunization of Oligomannose-coated liposomes (OMLs)
11 entrapped with a soluble leishmanial antigen in BALB/c mice induces an antigen-
12 specific Th1 immune response and protects against subsequent infection with
13 *Leishmania major*. Therefore, selection of an effective delivery system is
14 important for the induction of appropriate protective immune responses and the
15 induction of long-lasting immunity by administering antigens.

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

1 liposomes [15]. Up to now, liposomes have been widely used as an antigen
2 transport system in vaccines against viruses [13], bacteria [19], fungi [39], tumors
3 [22], and protozoa [24, 40]. These studies suggested that there was no detectable
4 toxicity and no liposome-caused skin damage at the injection site. Therefore,
5 liposomes could be suitable for use as a safe adjuvant in the induction of a Th1
6 immune response and to elicit a protective Th1 immune response against several
7 infections. A previous study indicated that the liposomes coated with a
8 neoglycolipid composed of mannotriose (Man3) and
9 dipalmitoylphosphatidylethanolamine (DPPE) (referred to as Man3-DPPE
10 liposomes) were the best compounds for macrophage uptake. They were
11 incorporated into peritoneal macrophages and triggered the induction of a Th1
12 immune response. The encased antigen was effectively presented by both MHC
13 class I and class II molecules [15]. Consequently, these findings led us to
14 hypothesize that immunization with soluble *N. caninum* antigens encapsulated in
15 liposomes coated with Man3-DPPE could reduce the offspring mortality from *N.*
16 *caninum* infection in BALB/c mice.

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

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

3

4 **2. MATERIALS AND METHODS**

5 **2.1. Parasite culture and purification**

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

9 **2.2. Cloning, expression, and purification of the recombinant NcAMA1** 10 **(rNcAMA1)**

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

20 **2.3. Preparation of Man3-DPPE liposomes**

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

15 **2.4. Immunization, mating, and challenge of mice**

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

1 NcAMA1-GST encapsulated in Man3-DPPE liposomes (M3-NcAMA1) (~3.3 µg)
2 in 100 µl PBS. These were administered three times at intervals of one week. One
3 week after the final immunization, female and male mice were kept in the same
4 cage until the female mouse presented a sperm-plug. At 10 days of pregnancy all
5 pregnant dams were challenged intraperitoneally with 1×10^5 *N. caninum*
6 tachyzoites. The number of live or stillborn offspring and any deaths during the
7 30-day observation period were recorded. At the end all dams were sacrificed and
8 brain samples were collected for PCR analysis.

9 10 **2.5. Antibody isotype assay**

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

21 **2.6. *In vitro* spleen cell proliferation**

22 One week after the final immunization, 3 mice from each group were sacrificed.
23 Spleen cells were suspended and hemolyzed in a lysing buffer (0.83% NH₄Cl,

1 0.01M Tris-HCl, pH 7.2). After washing with PBS, cells in a complete RPMI-
2 1640 medium (Sigma, USA) supplemented with 5% FBS were dispensed in
3 triplicate into a 96-well culture plate (5×10^5 cells in each well). The spleen cells
4 were stimulated with 2 $\mu\text{g}/\text{ml}$ of Concanavalin A (ConA), 50 $\mu\text{g}/\text{ml}$ of *N. caninum*
5 tachyzoite lysates or NcAMA1 (10 or 40 $\mu\text{g}/\text{ml}$), and 50 μM 2-mercaptoethanol
6 (2-ME) and incubated at 37 °C for 48 h in a humidified 5% CO₂ incubator. As a
7 control heterogeneous protein, 10 $\mu\text{g}/\text{ml}$ of the purified recombinant protein of *N.*
8 *caninum* dense granule protein 7 (NcGRA7) fused with GST [12] was also used.
9 The supernatants of the spleen cell cultures were collected at 48 h and kept at -
10 80 °C until assaying of the cytokines. Cytokines (IFN- γ and IL-4) in the culture
11 supernatant were quantified using ELISA kits (IFN- γ and IL-4, Endogen, USA) in
12 accordance with the manufacturer's instructions.

13 **2.7. DNA extraction from brain tissue of dams and the conventional PCR** 14 **analysis**

15
16 For DNA preparation, brain tissue samples were weighed and homogenized in
17 an extraction buffer (0.1 M Tris-HCl pH 9.0, 1% SDS, 0.1 M NaCl, 1 mM EDTA)
18 and 1 mg/ml of proteinase K at 55°C overnight. The DNA was purified by
19 phenol-chloroform extraction and ethanol precipitation. The DNA concentration
20 was adjusted to 100 $\mu\text{g}/\text{ml}$ for each brain and used as a template DNA for PCR
21 analysis. The DNA amplified by PCR was performed as described by Yamage et
22 al. [47]. The PCR products were visualized by electrophoresis in agarose gels.

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25 **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

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

9 **3.3. Litter numbers and survival rates of offspring in vaccinated and** 10 **challenged mice**

11
12 One week after the last immunization, mice were mated and challenged with
13 *N. caninum* tachyzoites (1×10^5) at 10 days of gestation. The litter number and
14 survival rate of offspring in all treatment groups are summarized in Table I. The
15 number of offspring in the M3-NcAMA1-immunized group was not statistically
16 different from that of other groups. These results indicated that immunization with
17 M3-NcAMA1 has no effect on the birth rate. Moreover, with an offspring survival
18 rate of 60% the mice immunized with M3-NcAMA1 showed specific protection,
19 whereas the mice immunized with the NcAMA1 alone, M3-GST, or PBS showed
20 lower survival rates (8%, 18.6%, and 17.5%, respectively). As shown in Table I
21 and Figure 3, the differences were statistically significant. These results suggested
22 that M3-NcAMA1 immunization induced a systemic immune response that
23 reduces offspring mortality from *N. caninum* infection in mice.

24 **3.4. Detection of *N. caninum* DNA in brain tissue of dams**

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

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9
10 **4. DISCUSSION**

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

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

1 Therefore, in order to investigate whether s.c. immunization with M3-NcAMA1
2 also induces antigen-specific anti-*N. caninum* immunity *in vivo*, BALB/c mice
3 were immunized subcutaneously with PBS, M3-GST, uncoated NcAMA1, or M3-
4 NcAMA1, and boosted twice. The results revealed no detectable toxicity and no
5 skin damage caused by the M3-DPPE liposomes at the injection site. Moreover,
6 the M3-DPPE liposome-based vaccines have two advantages. First, the amount of
7 immunization is small (maximum is 4 µg/mouse each time). Second, the M3-
8 DPPE liposomes were taken up specifically through macrophages at 1-week
9 interval immunization, and it accelerated the production of antibody response. In
10 this study, we used s.c. injection of OMLs. The time-course analysis and the side
11 effects induced by s.c. injection of M3-NcAMA1 should be further investigated to
12 assure their safe clinical application.

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

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

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

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

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

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

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

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

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Acknowledgments.

We thank Dr. J.P. Dubey (United States Department of Agriculture, Agriculture Research Service, Livestock and Poultry Sciences Institute, Parasite Biology and Epidemiology Laboratory) for the gift of *Neospora caninum*, NC-1 isolate. This work was supported by a Grant-in-Aid for Young Scientists (Start-up) from the Japan Society for the Promotion of Science (18880003) and the grant for the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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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%)
NcAMA1	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 ($P < 0.01$).

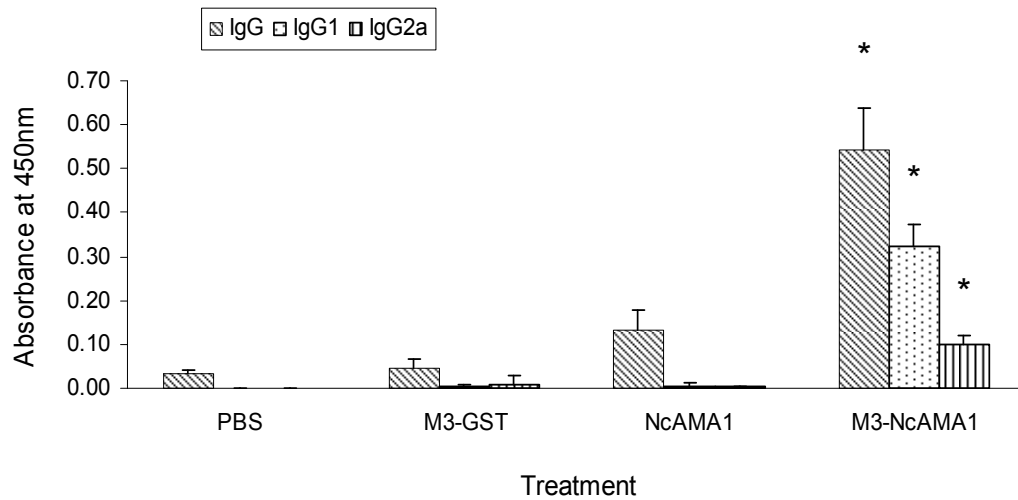
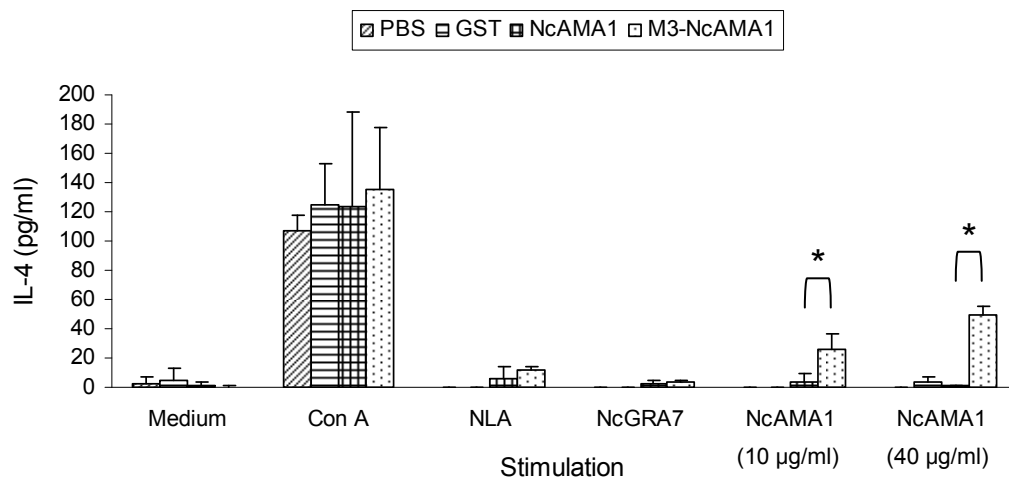
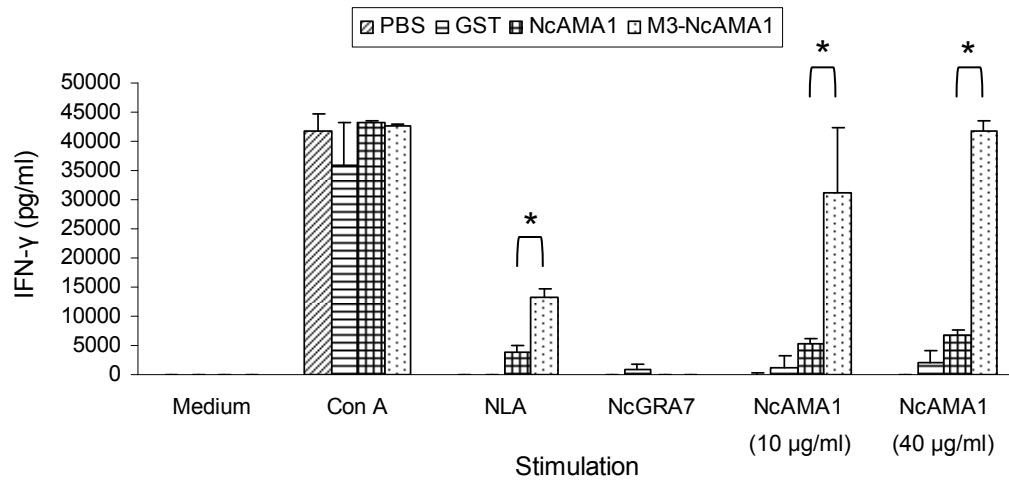


Figure 1. ELISA detection of *N. caninum*-specific antibody responses in vaccinated mice after the last immunization. Eight mice per group were immunized subcutaneously with PBS, GST encapsulated in Man3-coated liposomes (M3-GST), NcAMA1, or NcAMA1-GST encapsulated in Man3-coated liposome (M3-NcAMA1) 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 \pm 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).

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Figure 2. Productions of IFN- γ and IL-4 in culture supernatants of spleen cells.

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Three mice per group were immunized subcutaneously with PBS, M3-GST,

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NcAMA1, or M3-NcAMA1 three times at intervals of one week and then

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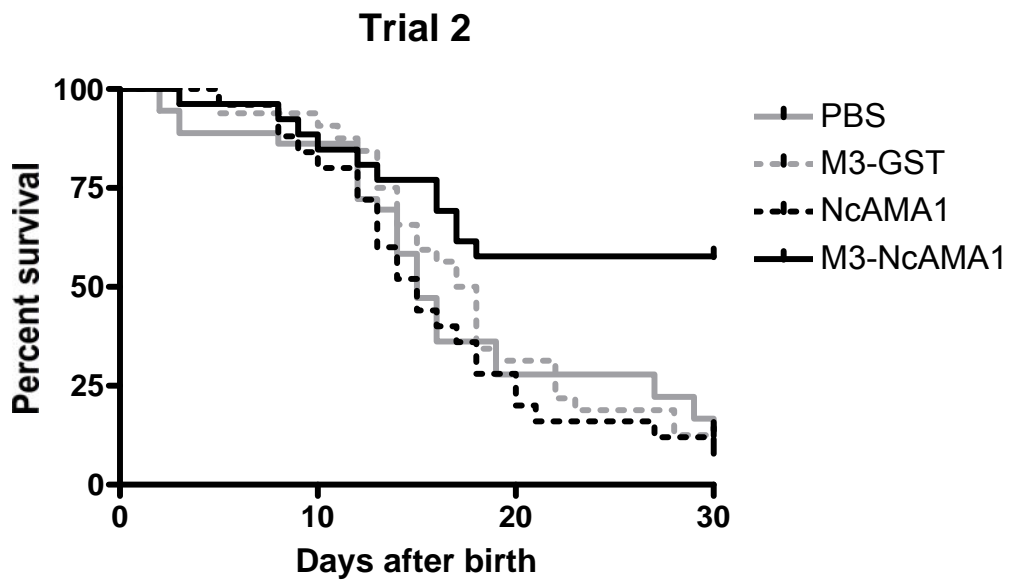
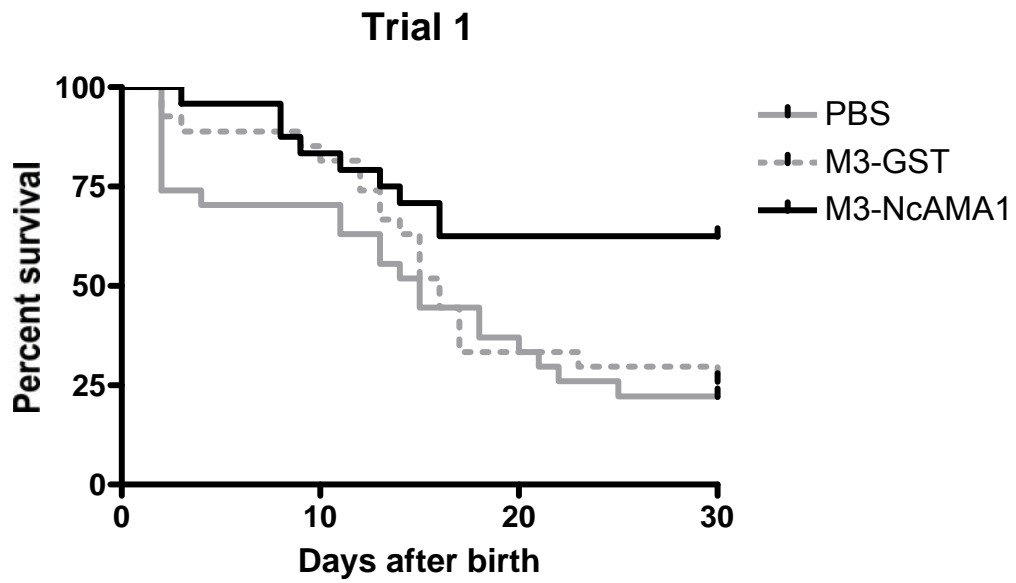
sacrificed one week after the last immunization. Single cell suspensions were

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prepared from the spleens of individual mice in the groups and cultured in

1 triplicate for 48 h in the presence of 2 µg/ml ConA, 50 µg/ml NLA, 10 µg/ml
2 NcGRA7, 10 µg/ml NcAMA1, or 40 µg/ml NcAMA1 or without any stimulator.
3 The IFN-γ and IL-4 secreted in culture supernatants were assayed by ELISA.
4 Each bar represents the mean ± SD of three mice per group. Results are
5 representative of two independent experiments. Statistically significant
6 differences were observed between groups of NcAMA1 and M3-NcAMA1
7 (* P <0.01).
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5 **Figure 3.** Survival rates of offspring in trials 1 and 2. Female mice were
6 immunized subcutaneously with PBS, M3-GST, NcAMA1, or M3-NcAMA1 three

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

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