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Research paper

Neospora GRA6 possesses immune-stimulating activity and confers efficient protection against *Neospora caninum* infection in mice

Ragab M. Fereig[a,b,c], Naomi Shimoda[a], Hanan H. Abdelbaky[a], Yasuhiro Kuroda[d], Yoshifumi Nishikawa[a,*]

[a] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[b] Research Center for Global Agromedicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
[c] Department of Animal Medicine, Faculty of Veterinary Medicine, South Valley University, Qena City, Qena 83523, Egypt
[d] Department of Applied Biochemistry, Tokai University, Kita-kaname, Hiratsuka, Kanagawa 259-1292, Japan

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ABSTRACT

Vaccination has the potential to be the most cost-effective control measure for reducing the economic burden of neosporosis in cattle. In this study, the immune-stimulatory effect of recombinant *Neospora caninum* dense granule protein 6 (NcGRA6) was confirmed via its triggering of IL-12p40 production in murine macrophages. BALB/c mice were immunized with recombinant NcGRA6 fused with glutathione *S*-transferase (GST) protein with or without oligomannose-coated-liposomes (OMLs) as the potential adjuvant. Specific IgG1 antibody production was observed from 21 and 35 days after the first immunization in NcGRA6 + GST- and NcGRA6 + GST-OML-immunized mice, respectively. However, specific IgG2a was detected 1 week after the infection, and IgG2a levels of the NcGRA6 + GST- group were higher than those of the NcGRA6 + GST-OML-group. Moreover, spleen cell proliferation with concomitant interferon-gamma production was detected in mice immunized with NcGRA6 + GST, indicating that a significant cellular immune response was induced. Mouse survival rates against *N. caninum* challenge infection were 91.7% for NcGRA6 + GST and 83.3% for NcGRA6 + GST-OML, which were significantly higher than those of control groups (GST-OML: 25%, phosphate-buffered saline: 16.7%). This indicates that naked NcGRA6 + GST induced protective immunity. Thus, our findings highlight the immune-stimulating potential of NcGRA6 and the ability to induce protective immunity against *N. caninum* infection in mice.

1. Introduction

Neospora caninum, an intracellular protozoan parasite causing neosporosis, is closely related to *Toxoplasma gondii*. This parasite infects dogs as the definitive host and a wide range of warm-blooded animals as intermediate hosts (Dubey and Schares, 2011). Neosporosis is transmitted by ingestion of oocysts or tissue cysts, or by transplacental transmission from an infected dam to her fetus. As a common cause of abortion in cattle worldwide, neosporosis induces high economic losses in farming (Dubey, 2003). With no effective drugs or vaccines available to control neosporosis (Dubey and Schares, 2011), developing a potent vaccine against it is vital.

Humoral and cellular immunity are involved in the response to neosporosis, and both types of immunity play essential roles in reducing its pathogenic effects. However, effective protective immunity to neosporosis is triggered primarily through cell-mediated immunity, especially that involving Th1 cell activation and interleukin (IL)-12 and gamma-interferon (IFN-γ) cytokine production (Innes et al., 2000; Nishikawa, 2017). Macrophages, dendritic cells, $CD4^+$, and $CD8^+$ T cells appear to be crucial for resistance to and protective immunity from neosporosis (Nishikawa et al., 2001a; Dion et al., 2011; Abe et al., 2014). Nuclear factor-kappa B (NF-κB), a pivotal signaling pathway, can regulate the production of pro-inflammatory cytokines, such as tumor necrosis factor alpha and IL-12 (Liu et al., 2017). These cytokines are produced mainly by macrophages and are indispensable for controlling neosporosis (Nishikawa, 2017). Antibody-mediated immunity

Abbreviations: BCA, bicinochoninic acid assay; CCK-8, cell counting kit-8; ConA, concanavalin A; DMEM, Dulbecco's Modified Eagle Medium; DPI, days-post infection; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HRP, horse radish peroxidase; IFN-γ, interferon γ; IgG, immunoglobulin G; IL-4, interleukin 4; LPS, lipopolysaccharide; NcGRA6, *N. caninum* dense granule protein 6; PB, polymixin B; PBS, phosphate buffered saline; Nc-1, a strain of *Neospora caninum*; NF-κB, nuclear factor of kappa B pathway; OML, oligomannaose-coated-liposomes; RPMI-1640 medium, Roswell Park Memorial Institute 1640 medium

* Corresponding author.
E-mail address: nisikawa@obihiro.ac.jp (Y. Nishikawa).

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is also beneficial for combating extracellular *N. caninum* either in blood or body fluids by limiting their dissemination through the complement system (Nishikawa et al., 2000).

Vaccines are recognized as the most successful control intervention in *N. caninum* infections (Reichel et al., 2013). Considering their safety and ease of preparation, vaccine development based on recombinant antigens offers many advantages over live-attenuated and inactivated vaccines. Several dense granule (GRA) proteins in *N. caninum* have been identified as highly antigenic molecules that could be used in diagnostics or as vaccine candidates (e.g., GRA2, GRA6, GRA7 and NTPase) (Huang et al., 2007; Ramamoorthy et al., 2007a, b; Nishikawa et al., 2009; Jin et al., 2015; Pastor- Fernández et al., 2015). Similarly, the GRA proteins from *T. gondii*, a closely related parasite to *N. caninum*, are abundantly secreted, and are considered candidate vaccines and/or diagnostic tools in different animal species (Redlich and Müller, 1998; Hiszczyńska-Sawicka et al., 2011; Sun et al., 2011). Numerous *T. gondii*-derived molecules have already been reported to activate NF-kB signaling and relevant cytokines (Rosowski et al., 2011). Recently, we found that the transfection of human embryonic kidneys cells with *Neospora* GRA6 (NcGRA6) cDNA activated NF-kB signaling (Nishikawa et al., 2018). NcGRA6 is characterized as an integral part of the parasitophorous vacuole (PV) of *N. caninum* because it was distributed in the lumen and PV intravacuolar network of Madin–Darby bovine kidney cells as a host cell model (Dong et al., 2017).

Previous studies have shown that expressing NcGRA6 in *Brucella abortus* (strain RB51) led to the induction of protective immunity against lethal intraperitoneal infection and vertical transmission of *N. caninum* in a mouse infection model (Ramamoorthy et al., 2007a, b). However, safety concerns were raised because *B. abortus* RB51 is infectious to humans and is potentially resistant to rifampicin, the most commonly used drug for human brucellosis (Dorneles et al., 2015). In the present study, a non-pregnant BALB/c mouse model was used to comprehensively estimate the immunoprophylactic potential of rNcGRA6 as a naked and oligomannose-coated-liposome (OML)-entrapped antigen. Our previous studies in mice and cattle indicate that the adjuvant properties of OML (plus antigen) can enhance protective immunity against infection with *N. caninum* (Nishikawa et al., 2009; Nishimura et al., 2013; Nishikawa, 2017). Thus, we evaluated the effects of rNcGRA6 in terms of its immune stimulating activity when used in combination with OML in experimental lethal infections with *N. caninum* in mice.

2. Materials and methods

2.1. Ethics statement

This study followed all relevant guidelines and procedures. Mouse work, such as the collection of heart blood, injection with parasites, recombinant proteins, or thioglycolate medium, and surgery for the collection of brains and spleen were implemented under general anesthesia induced with isoflurane. Mice were euthanized by cervical dislocation when they became unconscious with no reaction against external stimuli. We followed the guidelines and recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The procedures were approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine (permission numbers 29–58 and 28–49).

2.2. Animals

BALB/c female mice of 6–7 weeks old were purchased from Clea Japan (Tokyo, Japan). The housing of mice was applied under specific-pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan.

2.3. Parasites and cell cultures

Maintenance of *N. caninum* (strain Nc-1) was performed in Vero cells (African green monkey kidney epithelial cells) cultured in Eagle's minimum essential medium (EMEM; Sigma, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan) and 1% streptomycin–penicillin (Sigma). Regarding parasite purification, the host cell debris was removed by washing in cold phosphate-buffered saline (PBS), the monolayer of infected cells was scraped off with a cell scraper (BD Bioscience, San Jose, CA, USA), collected in medium, and centrifuged (800 × *g*, 5 min, 20 °C). The harvested cell pellet was resuspended in RPMI 1640 medium (Sigma) and passed through a 27-gauge needle and a 5.0 µm pore-sized filter (Millipore, Bedford, MA, USA).

2.4. NcGRA6 gene amplification and cloning

The total RNA from the *N. caninum* (Nc-1) strain using TRI reagent (Sigma) was reverse transcribed using the SuperScript first strand synthesis system for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, CA) and then used as a template to amplify the target genes. The cDNA of the target gene (gene ID: NCLIV_052880) corresponding to amino acid positions 43 to 154 and lacking amino acids 1–43 (signal peptide) and 155–172 (transmembrane domain) was PCR amplified using oligonucleotide primers. The primers included an *Eco*RI site (underlined) in the forward primer (5′-AT **GAA TTC** ATG GAT CCG GTT GAA TCC GTG GAG-3′) and an *Xho*I site (underlined) in the reverse primer (5′-AT **CTC GAG** CTA TCT GTG ACG TGC CTG CTG CCG-3′). The PCR products of target genes digested with above-mentioned restriction enzymes were inserted into a pGEX-4T1 plasmid vector digested with the same enzymes (Amersham Pharmacia Biotech, Madison, CA, USA). To confirm the successful insertion, sequencing of the inserted PCR product was conducted using the Big Dye Terminator Cycle Sequencing Kit (AB Applied Biosystems, Carlsbad, CA, USA), and the ABI PRISM 3100 genetic analyzer (AB Applied Biosystems).

2.5. Recombinant protein expression and purification

Recombinant protein of NcGRA6 was expressed as glutathione S-transferase (GST) fusion protein (NcGRA6 + GST) in *Escherichia coli* BL21 (DE3) cells (New England BioLabs Inc., Ipswich, MA, USA). Protein expression was performed at 37 °C for 6 h after induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (Wako Inc., Osaka, Japan). The final *E. coli* pellet from a large-volume culture was resuspended in suspension buffer (50 mM Tris-HCl, pH 8; 50 mM NaCl, 1 mM EDTA and 1 mM DTT) then centrifuged (7000 × *g*, 10 mi, 4 °C). Lysozyme at 500 µg/ml and 10% Triton in PBS (-) were added to the cell homogenate followed by incubation on ice for 1 h before cell sonication. The supernatant from sonicated cells was purified with Glutathione Sepharose 4B beads (GE Healthcare Life Sciences), according to the manufacturer's instructions. The GST-fused protein was eluted in elution buffer (100 mM Tris-HCl, pH 8.0; 100 mM NaCl, 5 mM EDTA, 25 mM reduced glutathione powder; Wako Inc.). The amount and purity of each protein fragment were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining of gel with Coomassie Brilliant Blue R250 (MP Biomedicals Inc., Illkirch-Graffenstaden, France). The concentrations of protein were measured using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Proteins with the expected molecular weights were obtained in highly pure forms (Fig. S1). Although several attempts were made to remove the GST-tag from NcGRA6 using thrombin protease (GE Healthcare, Buckinghamshire, England) according to the manufacturer's instructions, they were unsuccessful. Therefore, we used recombinant GST as the control protein in all the *in vivo* and *in vitro* experiments to exclude its effect. This approach has been validated in several vaccine studies from our group

and others (Nishikawa et al., 2009; Munkhjargal et al., 2016a, 2016b; Fereig and Nishikawa, 2016; Fereig et al., 2017). For the mouse experiments and cell culture assays, the proteins were filtered through a 0.45 μm low-protein binding Supor® membrane, and resident endotoxin was removed using Acrodisc® Units with Mustang® E Membrane (Pall Life Sciences, Ann Arbor, MI, USA). Additionally, the endotoxin level was estimated with Limulus Amebocyte Lysate reagents (Seikagaku Inc., Tokyo, Japan), and no endotoxin was detected in the tested protein lots (Fig. S2).

2.6. Collection and stimulation of murine peritoneal macrophages

Macrophage preparation and stimulation assay were performed as previously described (Fereig et al., 2017), with slight modifications. In brief, four days after intraperitoneal injection of BALB/c mice with 2 mL of 4.05% BBL™ Brewer modified thioglycolate medium (Becton Dickinson, Sparks, MD, USA), the macrophages were collected from mouse peritoneal lavages with cold PBS. After removal of red blood cells and washing steps, the macrophage suspension was seeded to a 96-well microplate (3×10^5 cells/well) and incubated in a 5% CO_2 incubator at 37 °C for 4 h to allow the cell adherence to the bottom. Before adding stimulants, the floating cells were removed, and the macrophages were incubated again for 20 h with the indicated stimulants, including positive and negative controls with and without polymixin B (PB) (Sigma) to exclude the effect of any residual endotoxin in the protein samples. PB (10 μg/mL) was added to the samples followed by incubation in a 37 °C water bath for 2 h.

2.7. OML preparation

Recombinant NcGRA6 + GST and GST were formulated and entrapped in OML as reported previously (Nishikawa et al., 2009). The protein concentrations were assayed with the BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

2.8. Scheme used for mouse immunization and infection

The female mice were inoculated subcutaneously in the neck region with 25 pmol of recombinant NcGRA6 + GST, NcGRA6 + GST-OML, or GST-OML in PBS, or with PBS alone (each 100 μL). The antigens were administered three times at 14-day intervals (Total number = 12 mice per group from two independent trials). Two weeks after the third immunization, each mouse was challenged intraperitoneally with a lethal dose of *N. caninum* Nc-1 tachyzoites (1×10^6). Such applied route of infection and infective dose was already established a model for lethal infection in BALB/c mice (Ybañez et al., 2016; Nishikawa et al., 2018). Survival and clinical observations in the mice were recorded for 32 dpi. We monitored the health of the animals twice a day. We sometimes observed unexpected deaths of infected mice because of acute symptoms. Serum (20 μL) was collected from the mice via their tail veins at 7, 21, and 35 days after the first immunization, and at 7 dpi to investigate the dynamicity of the specific antibodies generated against NcGRA6, using indirect enzyme-linked immunosorbent assays (ELISAs). To confirm the lack of an antibody response in an unvaccinated or uninfected mouse, control sera were collected from all the animals two days before their immunizations. At 32 dpi, serum and brain samples were collected from all the surviving mice after they were euthanized.

2.9. Indirect ELISA to detect NcGRA6- specific antibodies

Purified NcGRA6 + GST and GST were used as the coating antigens. They were diluted in coating buffer to a final concentration of 0.1 μM after which the indirect ELISAs proceeded as reported previously (Nishikawa et al., 2009), with slight modifications. The plates were incubated with 50 μL aliquots of serum samples from the immunized or control mice (diluted 1:100), added to the wells in duplicate, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (1:4000).

2.10. Neospora lysate antigen (NLA) preparation

NLA was prepared from Nc-1 strain tachyzoites as stated previously (Ribeiro et al., 2009). The harvested crude extract was filtered through a 0.45 μm low-protein binding Supor® membrane, and the concentration was measured using a BCA protein assay kit.

2.11. In vitro spleen cell stimulation

Two weeks after the third immunization, the spleens from the vaccinated and PBS-inoculated mice ($n = 4$/group) were aseptically dissected and single cell suspensions were prepared as described previously (Nishikawa et al., 2009), with slight modifications. Briefly, the splenocytes were placed into 96-well plates (3×10^5/100 μl/well). The cells were stimulated with 100 μL of NcGRA6 + GST or GST recombinant proteins, or with NLA (at 10 and 50 μg/ml) or concanavalin A (ConA; Sigma-Aldrich, St Louis, MO) (at 0.5 and 5 μg/ml) as the positive controls, or with stimulant-free medium as the negative control. The plates were incubated for 48 h at 37 °C in 5% CO_2. Culture supernatants (100 μl aliquots) were collected and assayed for cytokines (IL-4, IL-10 and IFN-γ). Simultaneously, 10 μl of Cell Counting Kit-8 reagent (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was added to the previously stimulated cell wells to estimate the extent of splenocyte proliferation. After 2 h of incubation at 37 °C in 5% CO_2, the optical density of each well was measured using a plate reader at 450 nm.

2.12. Sandwich ELISA for measuring cytokine levels

Cytokine levels IL-4, IL-10, and IFN-γ in the splenocyte culture supernatant, and IL-12p40 in macrophage culture supernatant were determined via commercial sandwich ELISAs (Pierce Biotechnology Inc., Rockford, IL, USA), according to the manufacturer's instructions. The standard cytokine curves constructed from the samples run on the same plate was used for the calculation of cytokine concentrations.

2.13. Clinical scores and body weights

Alterations in the body weights of the individual mice, which were recorded daily from −2 to 32 dpi with *N. caninum*, were compared with the weights of the individual mice on the first day of measurement. The clinical score was adjusted by recording the clinical signs manifested in each mouse and the mouse group overall starting from −2 to 32 dpi with *N. caninum* as described in our previous study (Abe et al., 2015). Briefly, each recorded clinical sign was represented by a score ranging from 0 (no signs) to 10 (all signs) (Table S1).

2.14. Statistical analyses

Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for group comparisons of parasite burden, with the data presented as the mean values ± the standard deviations. We used a two-way ANOVA followed by the Tukey–Kramer test or Bonferroni test to estimate differences in cytokine production, antibody levels, splenocyte response, body weight and clinical scores, with the data for each presented as mean values ± standard deviations. The significance of differences in mouse survival was analyzed by χ^2 test. All statistical analyses were performed with GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA).

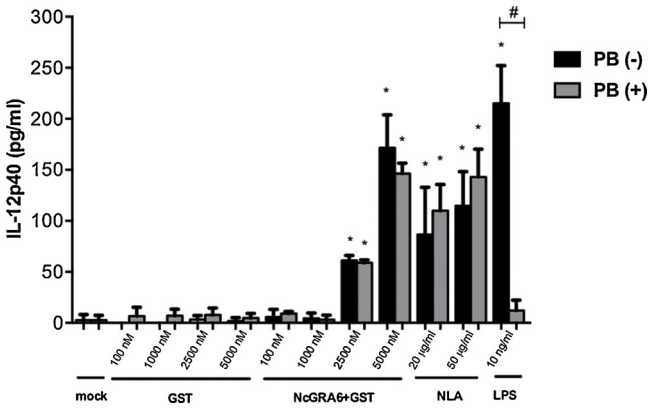

Fig. 1. Immune-stimulatory effect of NcGRA6. IL-12p40 production in murine peritoneal macrophages. Macrophages were treated with culture medium alone (mock), GST, recombinant NcGRA6 + GST, *N. caninum* lysate antigen (NLA), or lipopolysaccharide (LPS, 10 ng/mL) in the presence or absence of polymixin B (PB). The IL-12p40 value represents the mean ± standard deviation of quadruplicate samples. The results represent two repeat experiments with similar results. #, statistically significant differences for the same stimulant in the presence and absence of polymixin B were observed, $P < 0.05$. *, statistically significant differences for the mock control were observed in the presence or the absence of polymixin B, $P < 0.05$.

3. Results

3.1. IL-12p40 production of macrophages by NcGRA6

The immune-stimulating potential of NcGRA6 was investigated by treatment of peritoneal macrophages isolated from mouse with different doses of recombinant proteins. At doses of 2500 nM and 5000 nM, NcGRA6 + GST treatment promoted IL-12p40 production in the murine peritoneal macrophages (Fig. 1). Treatment with *Neospora* lysates (20 μg/ml and 50 μg/ml), also enhanced the production of IL-12p40. These findings were unchanged in the presence of PB. In contrast, the GST and mock treatments failed to trigger any significant responses. Treatment of the cells with lipopolysaccharide (LPS) also triggered IL-12p40 production, but PB treatment significantly reduced the production level. These results indicate that NcGRA6 has immune stimulating activity.

3.2. Specific anti-NcGRA6 antibody production in immunized mice

Mice immunized with NcGRA6 + GST alone or formulated with OML were positive for specific IgG1 antibodies after the second and third immunizations, respectively. In contrast, specific IgG2a antibodies were only detected in the above-mentioned groups at 7 dpi (49 days after the first immunization). No or low detectable antigen-specific antibodies were observed in the control group receiving PBS or GST-OML, respectively (Fig. 2A, B). These results suggest that immunization with rNcGRA6 primarily induced Th2 immune responses against *N. caninum* in the mice. However, the production of specific IgG2a was only initiated as a result of the boosting effect of immunization with NcGRA6 with or without OML at 7 dpi. The proficiency of naked NcGRA6 in generating specific antibodies against itself was higher than the entrapped OML version, as evidenced by the earlier IgG1 production and higher IgG2a production compared with the NcGRA6 + GST-OML group (Fig. 2A, B). The reactivity of sera against recombinant GST as a coating antigen was seen in GST-OML, NcGRA6 + GST-OML, and NcGRA6 + GST groups after the third immunization and 7 dpi (Fig. 2C, D). However, the antibody levels against GST were lower than those of the NcGRA6-specific antibody. These results suggest that immunization with rNcGRA6 primarily induced Th2 immune responses against *N. caninum* in the mice. However, the Th1 immune response, evidenced by specific IgG2a production, was only initiated as a result of the boosting effect of immunization with NcGRA6 with or without OML at 7 dpi.

3.3. Th1 and Th2 cytokines and spleen cell proliferation

Spleen cell proliferation was equally enhanced in the NcGRA6 + GST and NcGRA6 + GST-OML-immunized mice after stimulation with rNcGRA6 antigen at 10 and 50 μg/ml (Fig. 3A). Moreover, the rNcGRA6-treated cells revealed higher levels of IFN-γ production from the mice immunized with NcGRA6 + GST alone or with OML than the cells from mice injected with PBS or GST-OML (Fig. 3B). IFN-γ production was detected in spleen cells from the NcGRA6 + GST-immunized mice during stimulation with NLA, although there were no significant differences among the groups (Fig. 3B). On the contrary, IL-4 and IL-10 production in the spleen cells from all mouse groups was not significantly detectable, whereas high production levels were noted when ConA was used at 5 μg/ml (Fig. 3C, D). Collectively, these results indicate that immunization with naked rNcGRA6 triggered the antigen-specific cell-mediated immune responses in the mice.

3.4. Protection against lethal experimental infections of N. Caninum in mice

Substantial protection occurred in both immunized groups (NcGRA6 + GST and NcGRA6 + GST-OML) unlike the control groups (PBS and GST-OML). The survival rates for the immunized groups were 91.7% for NcGRA6 + GST and 83.3% for NcGRA6 + GST-OML, the values of which were statistically significant unlike those for the control groups (GST-OML: 25%, PBS: 16.7%) (Fig. 4A). Furthermore, minimal body weight decrease and lower clinical score occurred in the NcGRA6 + GST-immunized mice compared with the other groups, particularly those that received PBS or GST-OML (Fig. 4B, C). A statistically significant difference in the clinical observations (body weight loss and clinical signs of infection) was recorded from the early stage of infection and continued until the end of the experiments in the NcGRA6 + GST-immunized mice (Fig. 4B, C). Although we did not include a non-vaccinated non-challenge group in this study, weight gain over 30 days was about 14% in naïve BALB/c mice in our experimental conditions. Because the body weights of NcGRA6 + GST-immunized mice increased from 28 dpi, the body conditions of these mice recovered during this period (Fig. 4B). Moreover, the number of parasites in the NcGRA6 + GST-immunized group was lower than those in other groups, although the difference was not statistically significant (Fig. S3). These results imply that rNcGRA6 showed effective protection in the mouse model following intraperitoneal infection with a lethal dose of *N. caninum* tachyzoites.

4. Discussion

The efficacy of antigens derived from *N. caninum* as potential vaccine candidates against murine or bovine neosporosis has been widely evaluated (Reichel and Ellis, 2009; Monney et al., 2011), and numerous vaccine antigens have been tested as plasmid DNA, recombinant protein, or vector-based vaccines. Although these types of vaccine offer a highly flexible vaccination technology capable of inducing a substantive immune response, safety issues and costly manufacturing processes are hampering the use of DNA or vector-based vaccines in large scale or field applications (Innes and Vermeulen, 2006). The first trials of vaccine development against *N. caninum* primarily focused on live or attenuated vaccines. Live vaccines could elicit both humoral and cellular immunity and confer a variable degree of protection. However, worries about safety, resuming virulence, and increasing numbers of carrier animals restrict their use in field applications. Attenuated, killed, or lysate antigen vaccines are safer than live vaccines, but an adjuvant is required for the induction of an effective immune responses (Andrianarivo et al., 1999; Innes et al., 2002). Thus, vaccination using a recombinant antigen triggering appropriate levels of protective

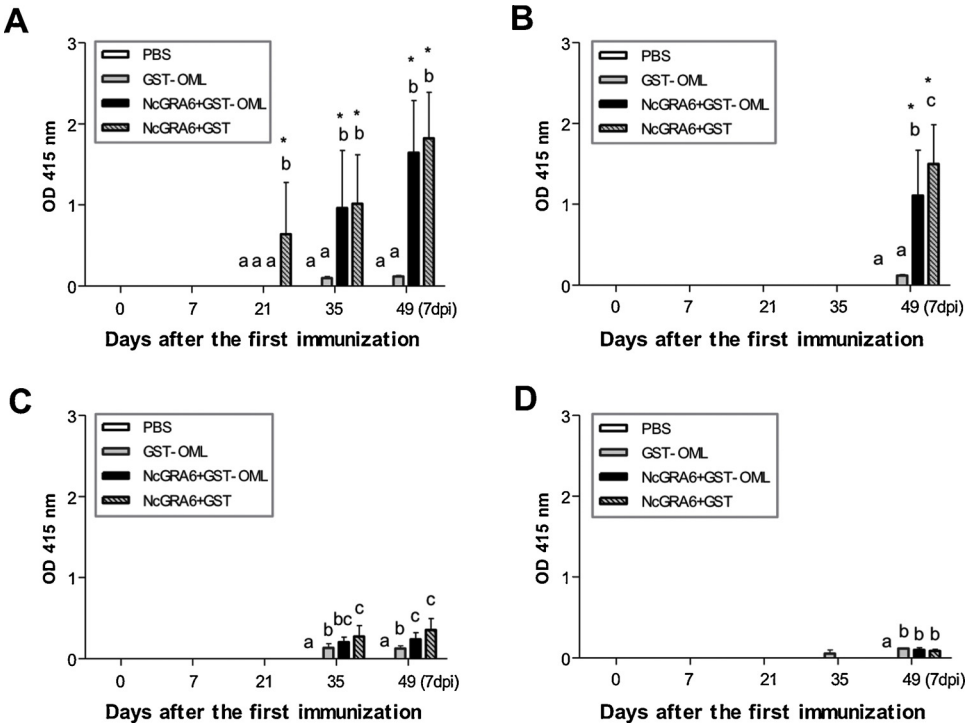

Fig. 2. Time course of specific antibody generation against NcGRA6 in the immunized and control mice. Sera were collected from all the mouse groups at day –2, 7, 21, 35 and 49 after first immunization. The antibody responses for each experimental group were tested against recombinant NcGRA6 + GST for IgG1 (A) and IgG2a (B), and against recombinant GST for IgG1 (C) and IgG2a (D). The mean optical density (OD) was determined at a wavelength of 415 nm. Each bar represents the mean ± standard deviation for the mice used per group ($n = 6$), and the results represent two independent experiments with similar results. The different letters above the bars in the graphs indicate statistically significant differences among the same immunization group, $P < 0.05$. *, the statistically significant differences observed for the same immunized group were based on the comparisons against the day 0 values, $P < 0.05$.

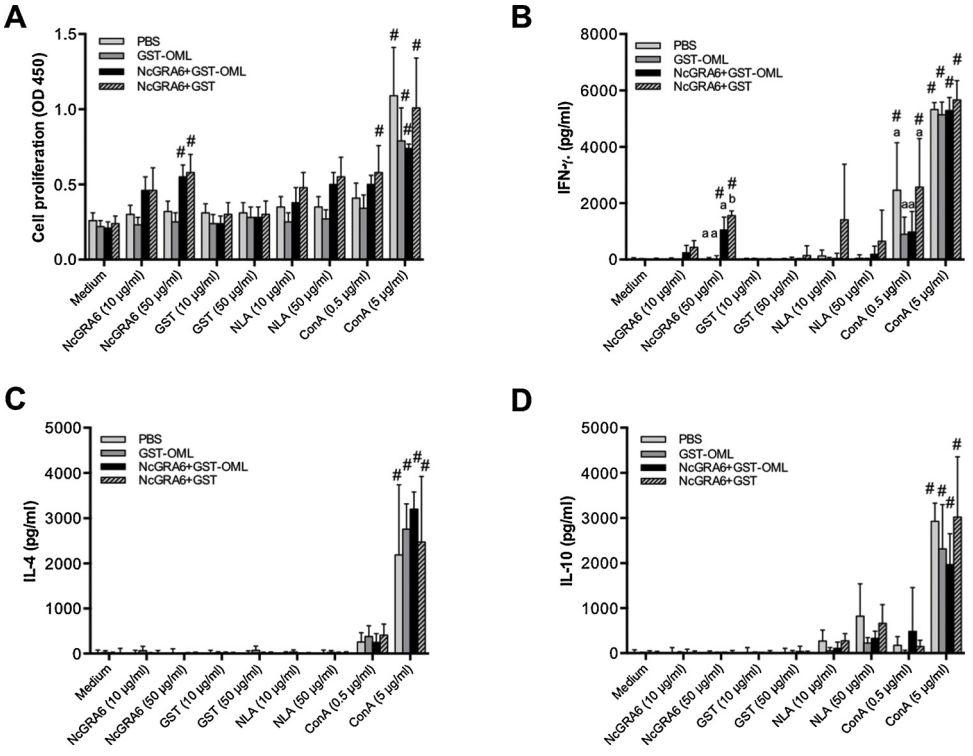

Fig. 3. Spleen cell assessment and cellular immunity. The cell suspensions prepared from the individual mouse spleens, and then cultured without any stimulator (culture medium) or in the presence of NcGRA6, GST, *N. caninum* lysate antigen (NLA), or concanavalin A (Con A), each at two different concentrations. (A) Cell proliferation was measured at 48 h. The culture supernatants were assayed for IFN-γ (B), IL-4 (C), and IL-10 (D) production with ELISAs. Each bar represents the mean ± standard deviation ($n = 4$ for all groups). The different letters above the bars indicate statistically significant differences among the groups for the same stimulator. #, statistically significant differences were observed for the culture medium wells for the same immunized group, $P < 0.05$.

immunity for effective protection could offer the most appropriate vaccination tool.

GRA proteins from *N. caninum* and *T. gondii* are pivotal weapons used by these apicomplexan parasites for establishing infections in their hosts. This competence requires the successful interaction with host molecules. Numerous *T. gondii*-derived GRAs have been reported to modulate the host's cell signaling pathways, with TgGRA6 reported to enhance the immune response via the NFAT4-dependent pathway (Ma et al., 2014). TgGRA15 activation of the NF-kB pathway leads to proinflammatory-biased immunity (Rosowski et al., 2011). TgGRA16 and TgGRA24 have also been shown to enhance the host's p53 tumor suppressor signaling pathway (Bougdour et al., 2013), and p38 MAP kinase activation (Braun et al., 2013), respectively. Unlike the situation for *Toxoplasma*, little information about the interaction between *Neospora* and host cell-transduction pathways has been published. In a recent study by our group, NcGRA6 activated the NF-kB pathway in human embryonic kidneys cells (Nishikawa et al., 2018). In the present study, we found that NcGRA6 promoted IL-12p40 production from murine macrophages. Because IL-12 is an essential cytokine for triggering cell-mediated immunity and resistance against *N. caninum*

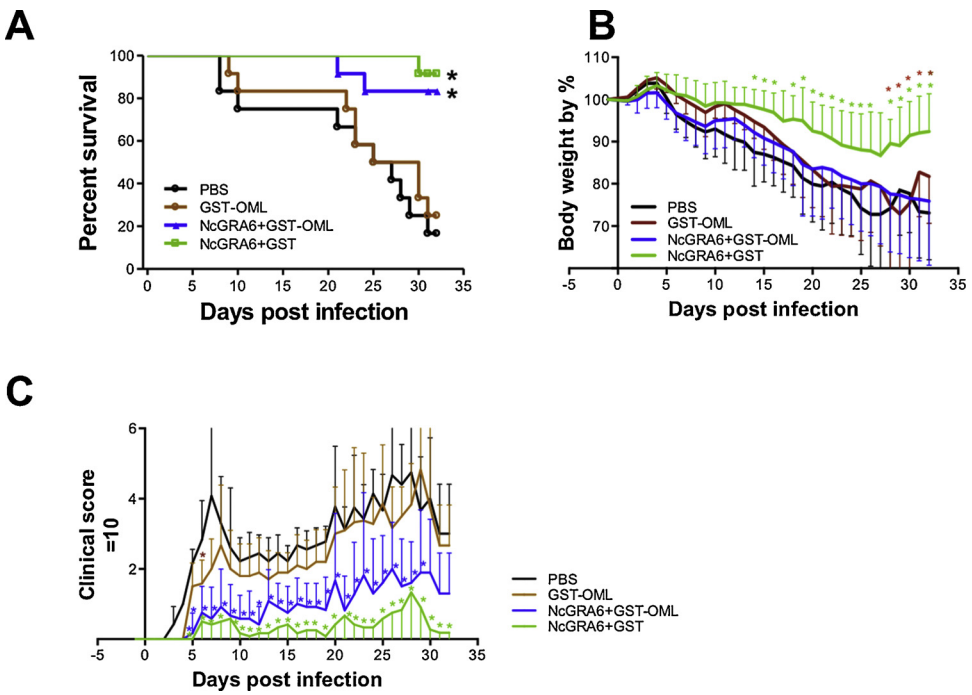

Fig. 4. Survival rates and clinical observations of mice. (A) The survival rates (surviving mice/total mice) are calculated from two pooled independent experiments: PBS: 2/12 (16.7%), GST-OML: 3/12 (25%), NcGRA6 + GST: 11/12 (91.7%) and NcGRA6 + GST-OML: 10/12 (83.3%). *, The significance of differences in mouse survival was analyzed by the χ^2 test. The differences were significant between PBS- and GST-OML-injected groups, and NcGRA6 + GST- and NcGRA6 + GST-OML-immunized groups ($P < 0.05$). Body weight alterations (B) and clinical scores (C) were calculated as means ± SD of the body weights and clinical score values for all the mice in a group from −2 until 32 days post-infection (dpi). *, the differences were significant between the PBS- and test groups, ($P < 0.05$). **, the differences were significant between the NcGRA6 + GST and NcGRA6 + GST-OML-injected groups, ($P < 0.05$).

infection, it indicates a potential immune-stimulatory effect (Khan et al., 1997; Baszler et al., 1999).

Because NcGRA6 was able to enhance murine macrophage activation and specific antibody production, as well as triggering antigen-specific IFN-γ production in the spleen cells of the immunized mice, we expect that this recombinant antigen should have protective efficacy as a vaccine candidate. In this study, the boosting effect of the rNcGRA6 antigen in terms of specific antibody production was confirmed. Additionally, the mitogenic effect and IFN-γ production of spleen cells indicated the high induction of cell-mediated immunity against NcGRA6. Consequently, immunization with NcGRA6 + GST alone conferred 91.7% protection against infection with *N. caninum* in the mouse model. Nevertheless, the role of IFN-γ in NcGRA6-induced protection should be further investigated using IFN-$\gamma^{-/-}$ mice or specific neutralization antibodies. Although our previous studies revealed the utility of OML as a potential adjuvant for vaccine antigens against neosporosis (Nishikawa et al., 2009; Zhang et al., 2010; Nishimura et al., 2013), here, we found that OML is not required to achieve a substantial protective effect from rNcGRA6 immunization. The delay in the development of IgG1 responses of the NcGRA6 + GST-OML-immunized group compared with the NcGRA6 + GST-immunized group suggests that the liposomes are retaining the recombinant protein and retarding its exposure to immune cells. They could also be modifying local immune responses and antigen presentation, which could explain why splenocytes from mice vaccinated with rNcGRA6-GST-OML secreted lower levels of IFN-γ when stimulated with NLA. Our results also suggest that naked NcGRA6 possesses adjuvant activity, as evidenced by its triggering of IL-12 production and NF-kB activation in macrophages. This effect may explain the robust immune response and protective potential of NcGRA6 without requiring OML adjuvant activity. A better combination of OML and antigen will be required for induction of the ideal immune response. Antigen with immune-stimulating activity may not be suitable for entrapping OML, and further investigation will be needed to clarify this uncertainty. We have previously confirmed that *Toxoplasma* peroxiredoxin 1 and 3 possess immune-stimulating activities and protect mice against murine toxoplasmosis when used as naked vaccine antigens (Fereig and Nishikawa, 2016; Fereig et al., 2017). Thus, the strategy of using an antigen or antigens that possess immune-stimulating activity in a vaccine holds merit.

Elimination of intracellular protozoan parasites like *N. caninum* depends critically on the action of cellular immunity, whereby cross-talk between numerous effector cells and molecules is achieved and various immune cells cooperate actively to combat the infection. Cumulative evidence from previous studies has indicated that the IL-12/IFN-γ axis is critical for resistance against intracellular parasites such as *N. caninum* and the closely related parasite, *T. gondii* (Khan et al., 1997; Baszler et al., 1999; Innes et al., 2000). IFN-γ is the key molecule for combating such parasites via the following pathways: 1) macrophage priming, which exerts an anti-parasitic effect by enhancing macrophage functioning (Nishikawa et al., 2001a) and nitric oxide production (Green et al., 1991); 2) MHC class I expressional boosting on antigen presenting cells, which in turn enhances $CD8^+$ T cell functioning (Ely et al., 1999); 3) PV disruption via immunity-related GTPases and p65 guanylate-binding proteins (Haldar et al., 2013); 4) tryptophan depletion through incrementally increased indoleamine 2,3-dioxygenase levels (Taylor and Feng, 1991); and 5) oxygen radical production enhancement (Aline et al., 2002). Antibody-mediated immunity plays an essential role in protection against *N. caninum* and *T. gondii* in another pathway also, although it has a partial effect. Mice lacking B cells are markedly more susceptible to infection than their wild-type counterparts, mostly because of impaired antibody production. This effect is attributable to the blocking of tachyzoite invasion of the host cells (Sayles et al., 2000). Additionally, mice passively immunized with antisera or purified antibodies showed improved protection against infection with *N. caninum* or *T. gondii* (Fereig et al., 2017; Wang et al., 2017; Ferreirinha et al., 2018). Antibody-mediated immunity is mostly related to the enhancement of the complement system or by paralyzing parasites via an agglutinating effect (Nishikawa et al., 2000, 2001b; Ferreirinha et al., 2014). However, another study also reported a role for B cells in cytokine production in mouse spleen cells, especially IFN-γ and IL-10. Higher levels of both cytokines in wild-type but not B cell-deficient mice were produced from spleen cells re-stimulated with *Neospora* antigens. The mechanism for this process is not clearly understood, but it may be related to the suppression of lymphoproliferation in the B cell-lacking mice (Eperon et al., 1999).

Previous studies have revealed high variability in the involvement of different *N. caninum* dense granule proteins as vaccine antigens against infection with *N. caninum*. In a previous study, *B. abortus* RB51

strain expressing NcGRA6 conferred protection in non-pregnant and pregnant mouse models, although this did not differ significantly compared with the control group receiving RB51 vector alone (Ramamoorthy et al., 2007a, b). In regard to NcGRA7, the extensively evaluated immunodominant antigen in vaccine studies of *N. caninum,* mucosal immunization of mice with *Neospora* extract containing NcGRA7 as a predominant antigen induced long-term protective effects when enriched with CpG adjuvant (Ferreirinha et al., 2016). A formulation of NcGRA7 with OML elicited robust immune responses and protected mouse dams and pups against such parasites (Nishikawa et al., 2009). Similarly, the inclusion of CpG with NcGRA7-encoding DNA plasmids protected against congenital neosporosis in mice (Jenkins et al., 2004). Low or no protective efficacy of NcGRA7 formulated in potential adjuvants was reported in non-pregnant and pregnant mouse models (Aguado-Martínez et al., 2009; Jiménez-Ruiz et al., 2012; Pastor- Fernández et al., 2015). Similarly, NcNTPase, a putative dense granule antigen, could not confer any protective efficacy when formulated in Quil-A adjuvant either alone or in combination with NcGRA7 (Pastor- Fernández et al., 2015). In cattle, NcGRA7 encapsulated in OML triggered protective immunity in immunized calves, accompanied by a significantly lower parasite burden in the brains of immunized animals (Nishimura et al., 2013). Conversely, immune stimulating complexes formulated with cocktail antigens including NcGRA7 could not protect heifers against *N. caninum* infection at the materno–fetal interface (Hecker et al., 2015). Accordingly, subunit vaccines based on dense granule proteins appear to be a promising tool to develop vaccines against *N. caninum* infection in cattle in the field.

During the last three decades, numerous vaccination studies using native or recombinant antigens against neosporosis have been performed. As a preliminary crucial step, evaluation of any vaccine antigen using the immunocompetent non-pregnant mouse model is considered the gold standard for defining the appropriate effector molecules for protective immunity against *N. caninum* (Aguado-Martínez et al., 2017; Marugan-Hernandez, 2017; Nishikawa, 2017). The main challenge resides in finding a vaccine that protects against vertical transmission and abortion, but previous vaccination studies have shown variable degrees of success in blocking vertical transmission of *N. caninum* in different animal models. In the mouse model, although great numbers of vaccine antigens have been found to induce potential immune responses, far fewer were protective against vertical transmission. The various vaccine antigen types with protective effects that have been evaluated in mice include live, attenuated or killed vaccines, recombinant DNA or antigens, and vector-based vaccines (Aguado-Martínez et al., 2017; Nishikawa, 2017). Although numerous vaccines have elicited remarkable immune responses in cattle and sheep, few of them have conferred protection against abortion, particularly when live or killed tachyzoites have been used (Horcajo et al., 2016; Marugan-Hernandez, 2017). Consequently, with regard to the optimal immune response and protective efficacy of naked NcGRA6 in non-pregnant mice, further evaluation of this antigen should be considered to determine its potential protective effect against vertical transmission of *N. caninum* in the mouse infection model.

Overall, previous studies have shown that vaccination can lead to the development of exacerbated symptoms and higher parasite burdens, and highlighted the varying outcomes of infection when animal models are vaccinated with the same antigen and different adjuvants. Thus, the animal model, physiological status, type of adjuvant, and vaccination scheme must be considered when evaluating antigens as vaccine candidates.

5. Conclusions

This study indicated the immunogenicity and potency of NcGRA6 as a vaccine candidate against lethal infection with *N. caninum* in a murine model. We first identified that the production of IL-12 from macrophages was initiated via NcGRA6 + GST recombinant protein, supporting the hypothesis of the induction of cell-mediated immunity via NcGRA6. Indeed, the immunization of mice with NcGRA6 + GST enhanced the proliferation and IFN-γ production of spleen cells treated with NcGRA6. Because effective protection was confirmed in NcGRA6 + GST-immunized mice, our strategy of vaccine development using antigen(s) that possess immune-stimulating activity will be an advantage for the control of *N. caninum* infection.

Author contributions

R.M.F and Y.N conceived and designed the experiments and analyzed the data. R.M.F., N.S., H.H.A., Y.K. and Y.N performed the experiments and contributed the reagents. R.M.F. and Y.N. wrote the manuscript. All authors have read and approved the final manuscript.

Competing financial interests

The authors declare that they have no financial or competing interests concerning this study.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.02.003.

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