



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

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1 **Title:**

2 ***Neospora* GRA6 possesses immune-stimulating activity and confers efficient protection**
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21 **ABSTRACT**

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

40

41 **Keywords**

42 *Neospora caninum*; Vaccine; Neosporosis; NcGRA6; Vaccination; Immunity

43

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

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55 1. Introduction

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

64 Humoral and cellular immunity are involved in the response to neosporosis, and both
65 types of immunity play essential roles in reducing its pathogenic effects. However, effective
66 protective immunity to neosporosis is triggered primarily through cell-mediated immunity,
67 especially that involving Th1 cell activation and interleukin (IL)-12 and gamma-interferon
68 (IFN- γ) cytokine production (Innes et al., 2000; Nishikawa, 2017). Macrophages, dendritic
69 cells, CD4⁺, and CD8⁺ T cells appear to be crucial for resistance to and protective immunity
70 from neosporosis (Nishikawa et al., 2001a; Dion et al., 2011; Abe et al., 2014). Nuclear
71 factor-kappa B (NF- κ B), a pivotal signaling pathway, can regulate the production of pro-
72 inflammatory cytokines, such as tumor necrosis factor alpha and IL-12 (Liu et al., 2017).
73 These cytokines are produced mainly by macrophages and are indispensable for controlling
74 neosporosis (Nishikawa, 2017). Antibody-mediated immunity is also beneficial for
75 combating extracellular *N. caninum* either in blood or body fluids by limiting their
76 dissemination through the complement system (Nishikawa et al., 2000).

77 Vaccines are recognized as the most successful control intervention in *N. caninum*
78 infections (Reichel et al., 2013). Considering their safety and ease of preparation, vaccine
79 development based on recombinant antigens offers many advantages over live-attenuated and

80 inactivated vaccines. Several dense granule (GRA) proteins in *N. caninum* have been
81 identified as highly antigenic molecules that could be used in diagnostics or as vaccine
82 candidates (e.g., GRA2, GRA6, GRA7 and NTPase) (Huang et al., 2007; Ramamoorthy et al.,
83 2007a; Ramamoorthy et al., 2007b; Nishikawa et al., 2009; Jin et al., 2015; Pastor- Fernández
84 et al., 2015). Similarly, the GRA proteins from *T. gondii*, a closely related parasite to *N.*
85 *caninum*, are abundantly secreted, and are considered candidate vaccines and/or diagnostic
86 tools in different animal species (Redlich and Müller, 1998; Hiszczyńska-Sawicka et al.,
87 2011; Sun et al., 2011). Numerous *T. gondii*-derived molecules have already been reported to
88 activate NF-κB signaling and relevant cytokines (Rosowski et al., 2011). Recently, we found
89 that the transfection of human embryonic kidneys cells with *Neospora* GRA6 (NcGRA6)
90 cDNA activated NF-κB signaling (Nishikawa et al., 2018). NcGRA6 is characterized as an
91 integral part of the parasitophorous vacuole (PV) of *N. caninum* because it was distributed in
92 the lumen and PV intravacuolar network of Madin–Darby bovine kidney cells as a host cell
93 model (Dong et al., 2017).

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

105 effects of rNcGRA6 in terms of its immune stimulating activity when used in combination
106 with OML in experimental lethal infections with *N. caninum* in mice.

107

108 **2. Materials and methods**

109 *2.1. Ethics statement*

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

119

120 *2.2. Animals*

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

125

126 *2.3. Parasites and cell cultures*

127 Maintenance of *N. caninum* (strain Nc-1) was performed in Vero cells (African
128 green monkey kidney epithelial cells) cultured in Eagle's minimum essential medium
129 (EMEM; Sigma, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine
130 serum (FBS; Nichirei Biosciences, Tokyo, Japan) and 1% streptomycin–penicillin (Sigma).

131 Regarding parasite purification, the host cell debris was removed by washing in cold
132 phosphate-buffered saline (PBS), the monolayer of infected cells was scraped off with a cell
133 scraper (BD Bioscience, San Jose, CA, USA), collected in medium, and centrifuged ($800 \times g$,
134 5 min, 20 °C). The harvested cell pellet was resuspended in RPMI 1640 medium (Sigma) and
135 passed through a 27-gauge needle and a 5.0 μm pore-sized filter (Millipore, Bedford, MA,
136 USA).

137

138 2.4. *NcGRA6* gene amplification and cloning

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

153

154 2.5. *Recombinant protein expression and purification*

155 Recombinant protein of NcGRA6 was expressed as glutathione S-transferase (GST)
156 fusion protein (NcGRA6+GST) in *Escherichia coli* BL21 (DE3) cells (New England
157 BioLabs Inc., Ipswich, MA, USA). Protein expression was performed at 37 °C for 6 h after
158 induction with 0.1 mM isopropyl β -D-1-thiogalactopyranoside (Wako Inc., Osaka, Japan).
159 The final *E. coli* pellet from a large-volume culture was resuspended in suspension buffer (50
160 mM Tris-HCl, pH 8; 50 mM NaCl, 1 mM EDTA and 1 mM DTT) then centrifuged (7,000
161 $\times g$, 10 mi, 4 °C). Lysozyme at 500 μ g/ml and 10% Triton in PBS (-) were added to the cell
162 homogenate followed by incubation on ice for 1 h before cell sonication. The supernatant
163 from sonicated cells was purified with Glutathione Sepharose 4B beads (GE Healthcare Life
164 Sciences), according to the manufacturer's instructions. The GST-fused protein was eluted in
165 elution buffer (100 mM Tris-HCl, pH 8.0; 100 mM NaCl, 5 mM EDTA, 25 mM reduced
166 glutathione powder; Wako Inc.). The amount and purity of each protein fragment were
167 examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
168 followed by staining of gel with Coomassie Brilliant Blue R250 (MP Biomedicals Inc.,
169 Illkirch-Graffenstaden, France). The concentrations of protein were measured using the
170 bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL,
171 USA). Proteins with the expected molecular weights were obtained in highly pure forms (Fig.
172 S1). Although several attempts were made to remove the GST-tag from NcGRA6 using
173 thrombin protease (GE Healthcare, Buckinghamshire, England) according to the
174 manufacturer's instructions, they were unsuccessful. Therefore, we used recombinant GST as
175 the control protein in all the *in vivo* and *in vitro* experiments to exclude its effect. This
176 approach has been validated in several vaccine studies from our group and others (Nishikawa
177 et al., 2009; Munkhjargal et al., 2016a, 2016b; Fereig and Nishikawa, 2016; Fereig et al.,
178 2017). For the mouse experiments and cell culture assays, the proteins were filtered through a
179 0.45 μ m low-protein binding Supor[®] membrane, and resident endotoxin was removed using

180 Acrodisc® Units with Mustang® E Membrane (Pall Life Sciences, Ann Arbor, MI, USA).
181 Additionally, the endotoxin level was estimated with Limulus Amebocyte Lysate reagents
182 (Seikagaku Inc., Tokyo, Japan), and no endotoxin was detected in the tested protein lots (Fig.
183 S2).

184

185 *2.6. Collection and stimulation of murine peritoneal macrophages*

186 Macrophage preparation and stimulation assay were performed as previously
187 described (Fereig et al., 2017), with slight modifications. In brief, four days after
188 intraperitoneal injection of BALB/c mice with 2 mL of 4.05% BBL™ Brewer modified
189 thioglycolate medium (Becton Dickinson, Sparks, MD, USA), the macrophages were
190 collected from mouse peritoneal lavages with cold PBS. After removal of red blood cells and
191 washing steps, the macrophage suspension was seeded to a 96-well microplate (3×10^5
192 cells/well) and incubated in a 5% CO₂ incubator at 37 °C for 4 h to allow the cell adherence
193 to the bottom. Before adding stimulants, the floating cells were removed, and the
194 macrophages were incubated again for 20 h with the indicated stimulants, including positive
195 and negative controls with and without polymixin B (PB) (Sigma) to exclude the effect of
196 any residual endotoxin in the protein samples. PB (10 µg/mL) was added to the samples
197 followed by incubation in a 37 °C water bath for 2 hr.

198

199 *2.7. OML preparation*

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

203

204 *2.8. Scheme used for mouse immunization and infection*

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

221

222 *2.9. Indirect ELISA to detect NcGRA6- specific antibodies*

223 Purified NcGRA6+GST and GST were used as the coating antigens. They were
224 diluted in coating buffer to a final concentration of 0.1 μ M after which the indirect ELISAs
225 proceeded as reported previously (Nishikawa et al., 2009), with slight modifications. The
226 plates were incubated with 50 μ L aliquots of serum samples from the immunized or control

227 mice (diluted 1:100), added to the wells in duplicate, followed by incubation with horseradish
228 peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (1:4,000).

229

230 2.10. *Neospora lysate antigen (NLA) preparation*

231 NLA was prepared from Nc-1 strain tachyzoites as stated previously (Ribeiro et al.,
232 2009). The harvested crude extract was filtered through a 0.45 µm low-protein binding
233 Supor® membrane, and the concentration was measured using a BCA protein assay kit.

234

235 2.11. *In vitro spleen cell stimulation*

236 Two weeks after the third immunization, the spleens from the vaccinated and PBS-
237 inoculated mice ($n = 4/\text{group}$) were aseptically dissected and single cell suspensions were
238 prepared as described previously (Nishikawa et al., 2009), with slight modifications. Briefly,
239 the splenocytes were placed into 96-well plates ($3 \times 10^5/100 \mu\text{l}/\text{well}$). The cells were
240 stimulated with 100 µL of NcGRA6+GST or GST recombinant proteins, or with NLA (at 10
241 and 50 µg/ml) or concanavalin A (ConA; Sigma-Aldrich, St Louis, MO) (at 0.5 and 5 µg/ml)
242 as the positive controls, or with stimulant-free medium as the negative control. The plates
243 were incubated for 48 h at 37 °C in 5% CO₂. Culture supernatants (100 µl aliquots) were
244 collected and assayed for cytokines (IL-4, IL-10 and IFN-γ). Simultaneously, 10 µl of Cell
245 Counting Kit-8 reagent (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was added to the
246 previously stimulated cell wells to estimate the extent of splenocyte proliferation. After 2 h of
247 incubation at 37 °C in 5% CO₂, the optical density of each well was measured using a plate
248 reader at 450 nm.

249

250 2.12. *Sandwich ELISA for measuring cytokine levels*

251 Cytokine levels IL-4, IL-10, and IFN- γ in the splenocyte culture supernatant, and IL-
252 12p40 in macrophage culture supernatant were determined via commercial sandwich ELISAs
253 (Pierce Biotechnology Inc., Rockford, IL, USA), according to the manufacturer's instructions.
254 The standard cytokine curves constructed from the samples run on the same plate was used
255 for the calculation of cytokine concentrations.

256

257 2.13. *Clinical scores and body weights*

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

264

265 2.14. *Statistical analyses*

266 Statistical analyses were performed using a one-way analysis of variance (ANOVA)
267 followed by the Tukey-Kramer test for group comparisons of parasite burden, with the data
268 presented as the mean values \pm the standard deviations. We used a two-way ANOVA
269 followed by the Tukey-Kramer test or Bonferroni test to estimate differences in cytokine
270 production, antibody levels, splenocyte response, body weight and clinical scores, with the
271 data for each presented as mean values \pm standard deviations. The significance of differences

272 in mouse survival was analyzed by χ^2 test. All statistical analyses were performed with
273 GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA).

274

275 **3. Results**

276 *3.1. IL-12p40 production of macrophages by NcGRA6*

277 The immune-stimulating potential of NcGRA6 was investigated by treatment of
278 peritoneal macrophages isolated from mouse with different doses of recombinant proteins. At
279 doses of 2,500 nM and 5,000 nM, NcGRA6+GST treatment promoted IL-12p40 production
280 in the murine peritoneal macrophages (Fig. 1). Treatment with *Neospora* lysates (20 μ g/ml
281 and 50 μ g/ml), also enhanced the production of IL-12p40. These findings were unchanged in
282 the presence of PB. In contrast, the GST and mock treatments failed to trigger any significant
283 responses. Treatment of the cells with lipopolysaccharide (LPS) also triggered IL-12p40
284 production, but PB treatment significantly reduced the production level. These results
285 indicate that NcGRA6 has immune stimulating activity.

286

287 *3.2. Specific anti-NcGRA6 antibody production in immunized mice*

288 Mice immunized with NcGRA6+GST alone or formulated with OML were positive
289 for specific IgG1 antibodies after the second and third immunizations, respectively. In
290 contrast, specific IgG2a antibodies were only detected in the above-mentioned groups at 7
291 dpi (49 days after the first immunization). No or low detectable antigen-specific antibodies
292 were observed in the control group receiving PBS or GST-OML, respectively (Fig. 2A, B).
293 These results suggest that immunization with rNcGRA6 primarily induced Th2 immune
294 responses against *N. caninum* in the mice. However, the production of specific IgG2a was

295 only initiated as a result of the boosting effect of immunization with NcGRA6 with or
296 without OML at 7 dpi. The proficiency of naked NcGRA6 in generating specific antibodies
297 against itself was higher than the entrapped OML version, as evidenced by the earlier IgG1
298 production and higher IgG2a production compared with the NcGRA6+GST-OML group (Fig.
299 2A, B). The reactivity of sera against recombinant GST as a coating antigen was seen in
300 GST-OML, NcGRA6+GST-OML ,and NcGRA6+GST groups after the third immunization
301 and 7 dpi (Fig. 2C, D). However, the antibody levels against GST were lower than those of
302 the NcGRA6-specific antibody. These results suggest that immunization with rNcGRA6
303 primarily induced Th2 immune responses against *N. caninum* in the mice. However, the Th1
304 immune response, evidenced by specific IgG2a production, was only initiated as a result of
305 the boosting effect of immunization with NcGRA6 with or without OML at 7 dpi.

306

307 3.3. *Th1 and Th2 cytokines and spleen cell proliferation*

308 Spleen cell proliferation was equally enhanced in the NcGRA6+GST and
309 NcGRA6+GST-OML-immunized mice after stimulation with rNcGRA6 antigen at 10 and 50
310 $\mu\text{g/ml}$ (Fig. 3A). Moreover, the rNcGRA6-treated cells revealed higher levels of IFN- γ
311 production from the mice immunized with NcGRA6+GST alone or with OML than the cells
312 from mice injected with PBS or GST-OML (Fig. 3B). IFN- γ production was detected in
313 spleen cells from the NcGRA6+GST-immunized mice during stimulation with NLA,
314 although there were no significant differences among the groups (Fig. 3B). On the contrary,
315 IL-4 and IL-10 production in the spleen cells from all mouse groups was not significantly
316 detectable, whereas high production levels were noted when ConA was used at 5 $\mu\text{g/ml}$ (Fig.
317 3C, D). Collectively, these results indicate that immunization with naked rNcGRA6 triggered
318 the antigen-specific cell-mediated immune responses in the mice.

319

320 3.4. Protection against lethal experimental infections of *N. caninum* in mice

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

339

340

341 4. Discussion

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

357 GRA proteins from *N. caninum* and *T. gondii* are pivotal weapons used by these
358 apicomplexan parasites for establishing infections in their hosts. This competence requires
359 the successful interaction with host molecules. Numerous *T. gondii*-derived GRAs have been
360 reported to modulate the host's cell signaling pathways, with TgGRA6 reported to enhance
361 the immune response via the NFAT4-dependent pathway (Ma et al., 2014). TgGRA15
362 activation of the NF-kB pathway leads to proinflammatory-biased immunity (Rosowski et al.,
363 2011). TgGRA16 and TgGRA24 have also been shown to enhance the host's p53 tumor
364 suppressor signaling pathway (Bougdour et al., 2013), and p38 MAP kinase activation (Braun
365 et al., 2013), respectively. Unlike the situation for *Toxoplasma*, little information about the

366 interaction between *Neospora* and host cell-transduction pathways has been published. In a
367 recent study by our group, NcGRA6 activated the NF- κ B pathway in human embryonic
368 kidneys cells (Nishikawa et al., 2018). In the present study, we found that NcGRA6 promoted
369 IL-12p40 production from murine macrophages. Because IL-12 is an essential cytokine for
370 triggering cell-mediated immunity and resistance against *N. caninum* infection, it indicates a
371 potential immune-stimulatory effect (Khan et al., 1997; Baszler et al., 1999).

372 Because NcGRA6 was able to enhance murine macrophage activation and specific
373 antibody production, as well as triggering antigen-specific IFN- γ production in the spleen
374 cells of the immunized mice, we expect that this recombinant antigen should have protective
375 efficacy as a vaccine candidate. In this study, the boosting effect of the rNcGRA6 antigen in
376 terms of specific antibody production was confirmed. Additionally, the mitogenic effect and
377 IFN- γ production of spleen cells indicated the high induction of cell-mediated immunity
378 against NcGRA6. Consequently, immunization with NcGRA6+GST alone conferred 91.7%
379 protection against infection with *N. caninum* in the mouse model. Nevertheless, the role of
380 IFN- γ in NcGRA6-induced protection should be further investigated using IFN- $\gamma^{-/-}$ mice or
381 specific neutralization antibodies. Although our previous studies revealed the utility of OML
382 as a potential adjuvant for vaccine antigens against neosporosis (Nishikawa et al., 2009;
383 Zhang et al., 2010; Nishimura et al., 2013), here, we found that OML is not required to
384 achieve a substantial protective effect from rNcGRA6 immunization. The delay in the
385 development of IgG1 responses of the NcGRA6+GST-OML-immunized group compared
386 with the NcGRA6+GST-immunized group suggests that the liposomes are retaining the
387 recombinant protein and retarding its exposure to immune cells. They could also be
388 modifying local immune responses and antigen presentation, which could explain why
389 splenocytes from mice vaccinated with rNcGRA6-GST-OML secreted lower levels of IFN- γ
390 when stimulated with NLA. Our results also suggest that naked NcGRA6 possesses adjuvant

391 activity, as evidenced by its triggering of IL-12 production and NF- κ B activation in
392 macrophages. This effect may explain the robust immune response and protective potential of
393 NcGRA6 without requiring OML adjuvant activity. A better combination of OML and
394 antigen will be required for induction of the ideal immune response. Antigen with immune-
395 stimulating activity may not be suitable for entrapping OML, and further investigation will be
396 needed to clarify this uncertainty. We have previously confirmed that *Toxoplasma*
397 peroxiredoxin 1 and 3 possess immune-stimulating activities and protect mice against murine
398 toxoplasmosis when used as naked vaccine antigens (Fereig and Nishikawa, 2016; Fereig et
399 al., 2017). Thus, the strategy of using an antigen or antigens that possess immune-stimulating
400 activity in a vaccine holds merit.

401 Elimination of intracellular protozoan parasites like *N. caninum* depends critically on
402 the action of cellular immunity, whereby cross-talk between numerous effector cells and
403 molecules is achieved and various immune cells cooperate actively to combat the infection.
404 Cumulative evidence from previous studies has indicated that the IL-12/IFN- γ axis is critical
405 for resistance against intracellular parasites such as *N. caninum* and the closely related
406 parasite, *T. gondii* (Khan et al., 1997; Baszler et al., 1999; Innes et al., 2000). IFN- γ is the key
407 molecule for combating such parasites via the following pathways: 1) macrophage priming,
408 which exerts an anti-parasitic effect by enhancing macrophage functioning (Nishikawa et al.,
409 2001a) and nitric oxide production (Green et al., 1999); 2) MHC class I expressional boosting
410 on antigen presenting cells, which in turn enhances CD8⁺ T cell functioning (Ely et al.,
411 1999); 3) PV disruption via immunity-related GTPases and p65 guanylate-binding proteins
412 (Haldar et al., 2013); 4) tryptophan depletion through incrementally increased indoleamine
413 2,3-dioxygenase levels (Taylor and Feng, 1991); and 5) oxygen radical production
414 enhancement (Aline et al., 2002). Antibody-mediated immunity plays an essential role in
415 protection against *N. caninum* and *T. gondii* in another pathway also, although it has a partial

416 effect. Mice lacking B cells are markedly more susceptible to infection than their wild-type
417 counterparts, mostly because of impaired antibody production. This effect is attributable to
418 the blocking of tachyzoite invasion of the host cells (Sayles et al., 2000). Additionally, mice
419 passively immunized with antisera or purified antibodies showed improved protection against
420 infection with *N. caninum* or *T. gondii* (Fereig et al., 2017; Wang et al., 2017; Ferreira et
421 al., 2018). Antibody-mediated immunity is mostly related to the enhancement of the
422 complement system or by paralyzing parasites via an agglutinating effect (Nishikawa et al.,
423 2000; Nishikawa et al., 2001b; Ferreira et al., 2014). However, another study also
424 reported a role for B cells in cytokine production in mouse spleen cells, especially IFN- γ and
425 IL-10. Higher levels of both cytokines in wild-type but not B cell-deficient mice were
426 produced from spleen cells re-stimulated with *Neospora* antigens. The mechanism for this
427 process is not clearly understood, but it may be related to the suppression of
428 lymphoproliferation in the B cell-lacking mice (Eperon et al., 1999).

429 Previous studies have revealed high variability in the involvement of different *N.*
430 *caninum* dense granule proteins as vaccine antigens against infection with *N. caninum*. In a
431 previous study, *B. abortus* RB51 strain expressing NcGRA6 conferred protection in non-
432 pregnant and pregnant mouse models, although this did not differ significantly compared
433 with the control group receiving RB51 vector alone (Ramamoorthy et al., 2007a;
434 Ramamoorthy et al., 2007b). In regard to NcGRA7, the extensively evaluated
435 immunodominant antigen in vaccine studies of *N. caninum*, mucosal immunization of mice
436 with *Neospora* extract containing NcGRA7 as a predominant antigen induced long-term
437 protective effects when enriched with CpG adjuvant (Ferreira et al., 2016). A formulation
438 of NcGRA7 with OML elicited robust immune responses and protected mouse dams and
439 pups against such parasites (Nishikawa et al., 2009). Similarly, the inclusion of CpG with
440 NcGRA7-encoding DNA plasmids protected against congenital neosporosis in mice (Jenkins

441 et al., 2004). Low or no protective efficacy of NcGRA7 formulated in potential adjuvants was
442 reported in non-pregnant and pregnant mouse models (Aguado-Martínez et al., 2009;
443 Jiménez-Ruiz et al., 2012; Pastor- Fernández et al., 2015). Similarly, NcNTPase, a putative
444 dense granule antigen, could not confer any protective efficacy when formulated in Quil-A
445 adjuvant either alone or in combination with NcGRA7 (Pastor- Fernández et al., 2015). In
446 cattle, NcGRA7 encapsulated in OML triggered protective immunity in immunized calves,
447 accompanied by a significantly lower parasite burden in the brains of immunized animals
448 (Nishimura et al., 2013). Conversely, immune stimulating complexes formulated with
449 cocktail antigens including NcGRA7 could not protect heifers against *N. caninum* infection at
450 the materno–fetal interface (Hecker et al., 2015). Accordingly, subunit vaccines based on
451 dense granule proteins appear to be a promising tool to develop vaccines against *N. caninum*
452 infection in cattle in the field.

453 During the last three decades, numerous vaccination studies using native or
454 recombinant antigens against neosporosis have been performed. As a preliminary crucial step,
455 evaluation of any vaccine antigen using the immunocompetent non-pregnant mouse model is
456 considered the gold standard for defining the appropriate effector molecules for protective
457 immunity against *N. caninum* (Aguado-Martínez et al., 2017; Marugan-Hernandez, 2017;
458 Nishikawa, 2017). The main challenge resides in finding a vaccine that protects against
459 vertical transmission and abortion, but previous vaccination studies have shown variable
460 degrees of success in blocking vertical transmission of *N. caninum* in different animal models.
461 In the mouse model, although great numbers of vaccine antigens have been found to induce
462 potential immune responses, far fewer were protective against vertical transmission. The
463 various vaccine antigen types with protective effects that have been evaluated in mice include
464 live, attenuated or killed vaccines, recombinant DNA or antigens, and vector-based vaccines
465 (Aguado-Martínez et al., 2017; Nishikawa, 2017). Although numerous vaccines have elicited

466 remarkable immune responses in cattle and sheep, few of them have conferred protection
467 against abortion, particularly when live or killed tachyzoites have been used (Horcajo et al.,
468 2016; Marugan-Hernandez, 2017). Consequently, with regard to the optimal immune
469 response and protective efficacy of naked NcGRA6 in non-pregnant mice, further evaluation
470 of this antigen should be considered to determine its potential protective effect against
471 vertical transmission of *N. caninum* in the mouse infection model.

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

477

478 **5. Conclusions**

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

488

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496

497 **Author Contributions**

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

501

502 **Competing financial interests**

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

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506 **References**

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701

702 **Figure legends**

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

712

713 **Figure 2. Time course of specific antibody generation against NcGRA6 in the**
714 **immunized and control mice.** Sera were collected from all the mouse groups at day -2, 7,
715 21, 35 and 49 after first immunization. The antibody responses for each experimental group
716 were tested against recombinant NcGRA6+GST for IgG1 (A) and IgG2a (B), and against
717 recombinant GST for IgG1 (C) and IgG2a (D). The mean optical density (OD) was
718 determined at a wavelength of 415 nm. Each bar represents the mean \pm standard deviation for
719 the mice used per group ($n = 6$), and the results represent two independent experiments with
720 similar results. The different letters above the bars in the graphs indicate statistically
721 significant differences among the same immunization group, $P < 0.05$. *, the statistically
722 significant differences observed for the same immunized group were based on the
723 comparisons against the day 0 values, $P < 0.05$.

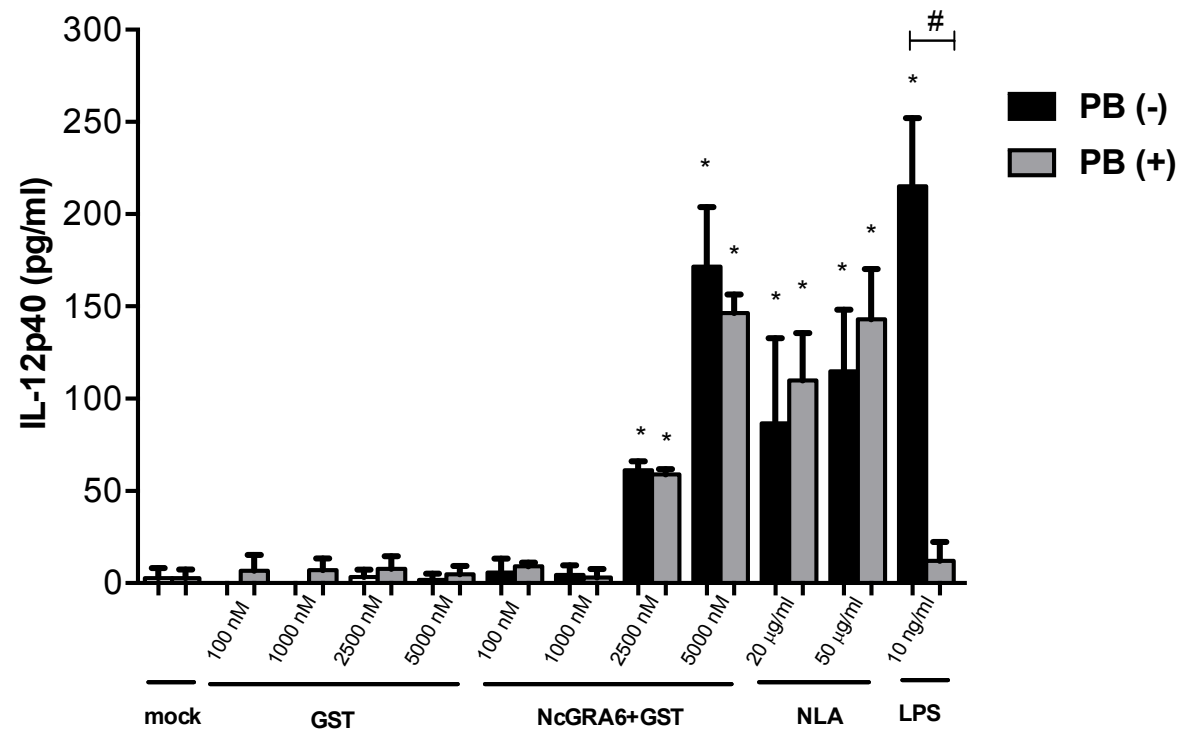
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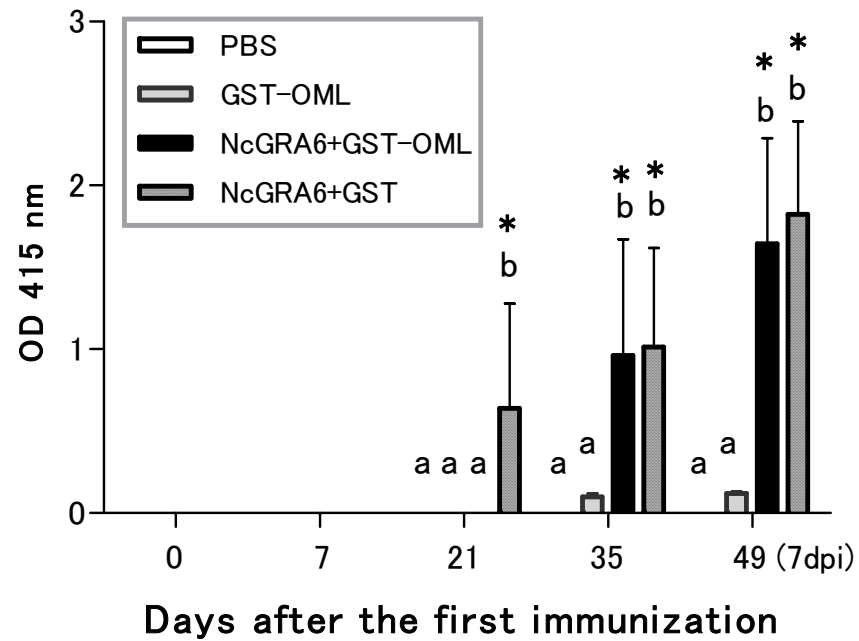
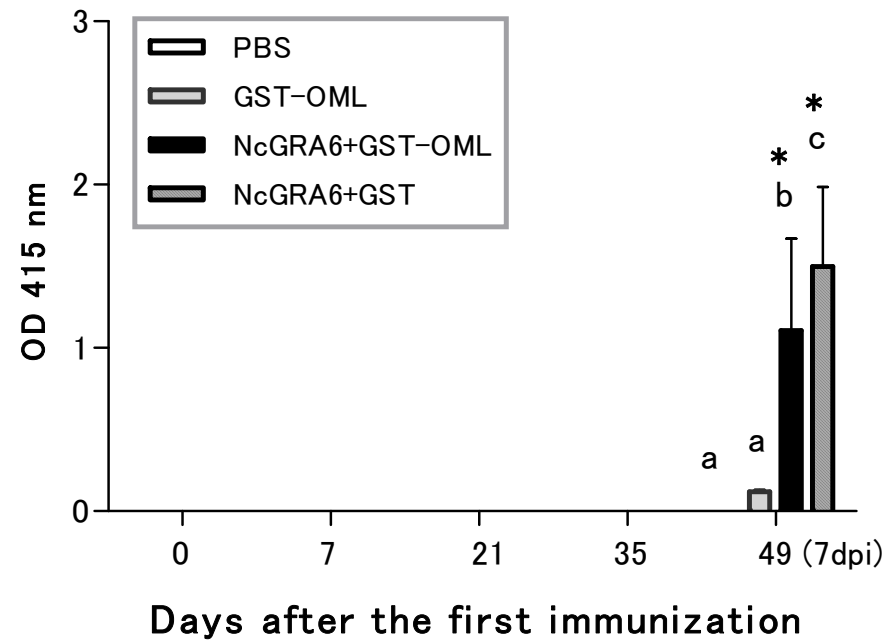
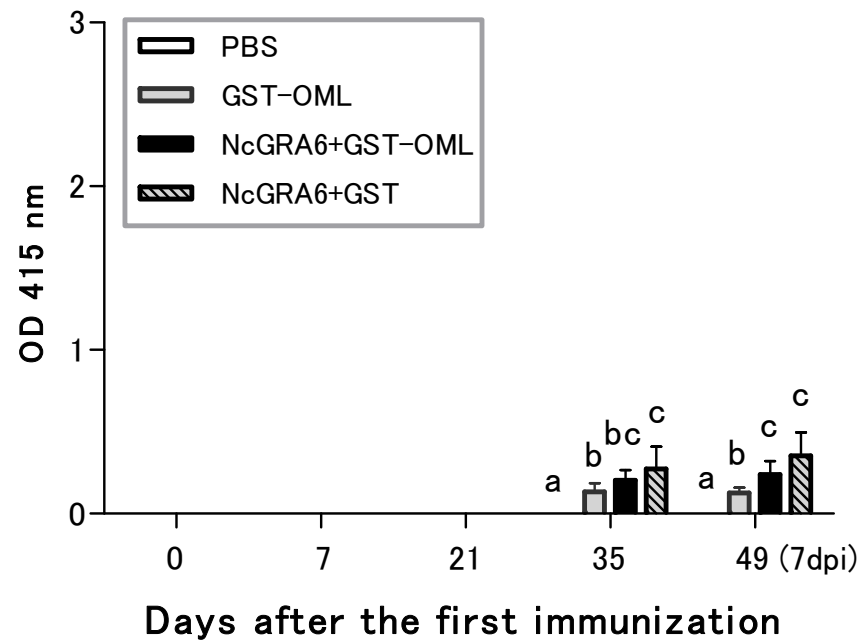
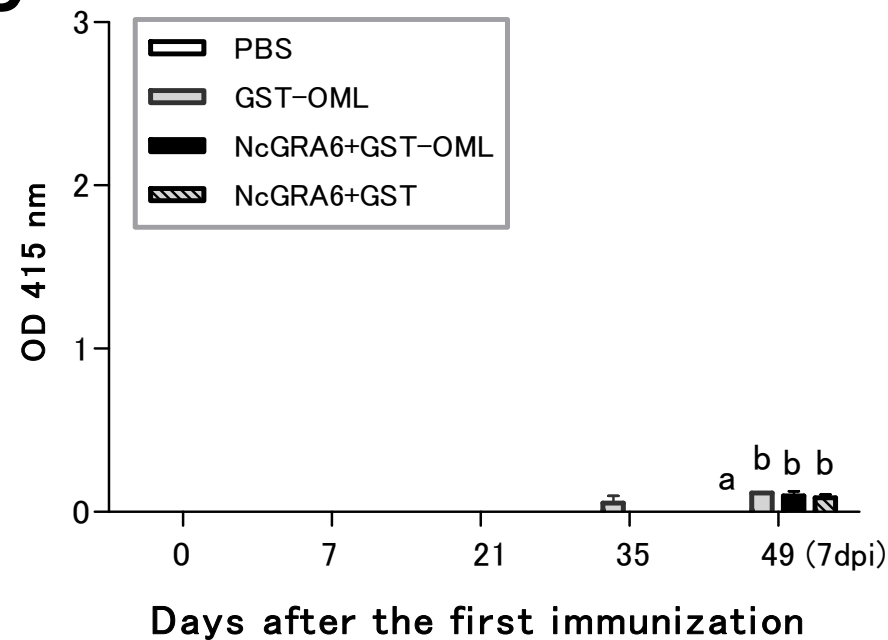
725 **Figure 3. Spleen cell assessment and cellular immunity.** The cell suspensions prepared
726 from the individual mouse spleens, and then cultured without any stimulator (culture
727 medium) or in the presence of NcGRA6, GST, *N. caninum* lysate antigen (NLA), or
728 concanavalin A (Con A), each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

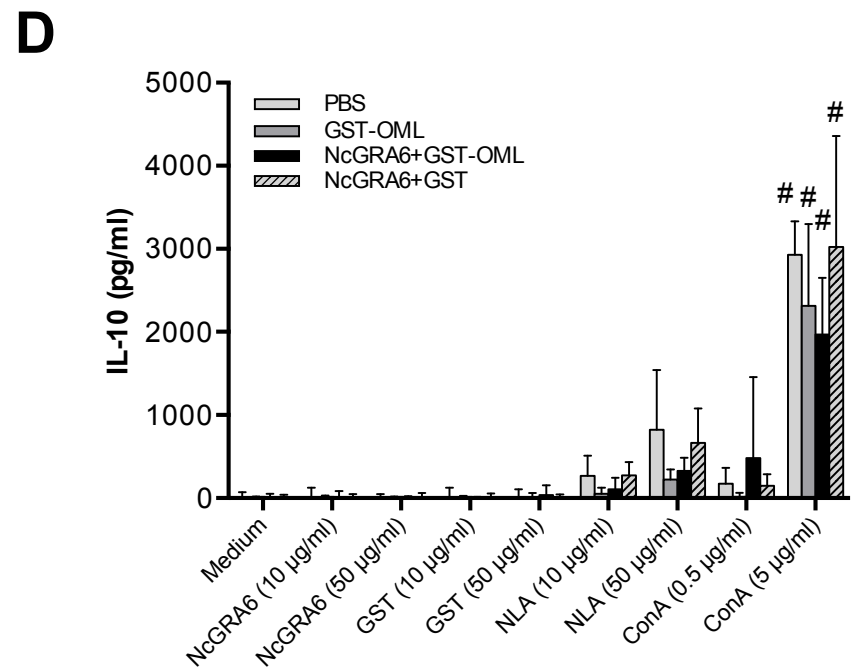
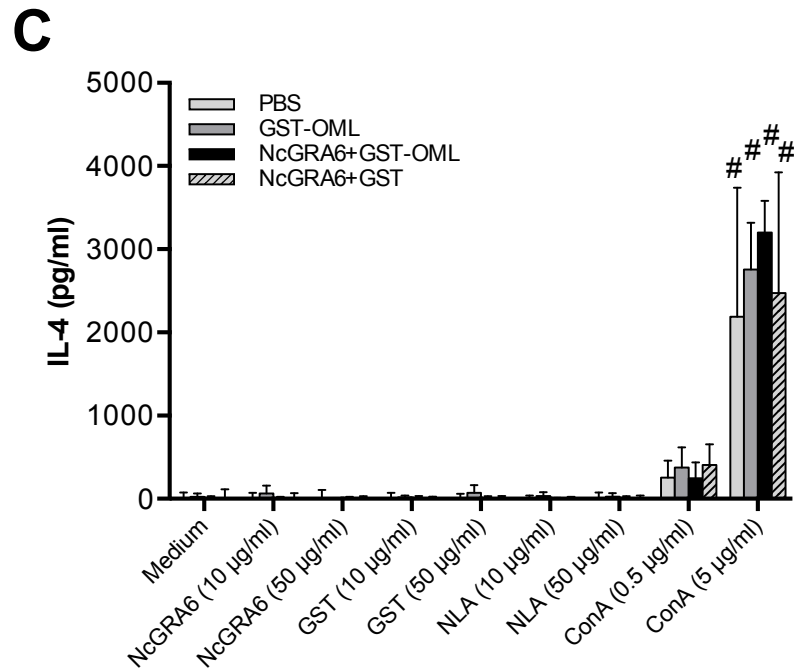
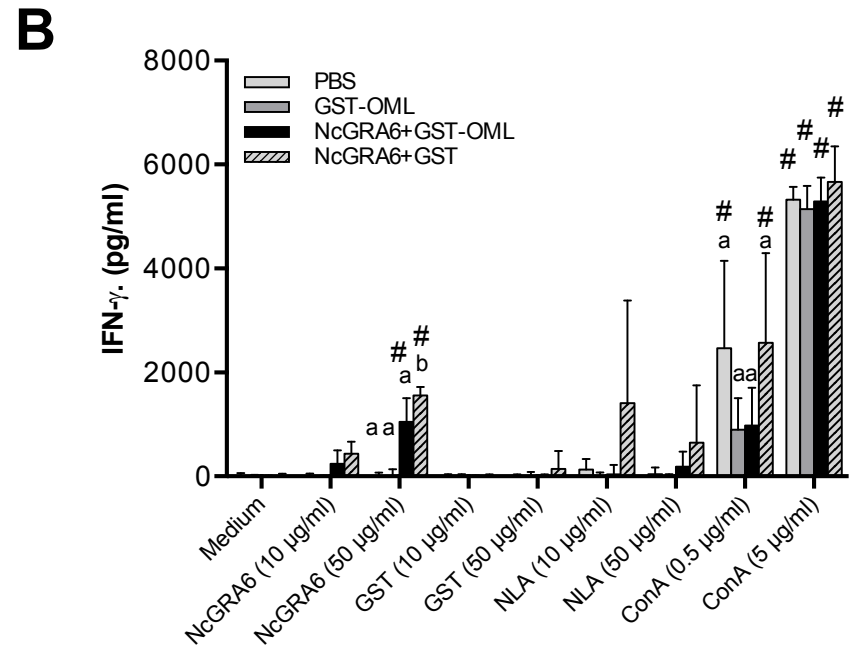
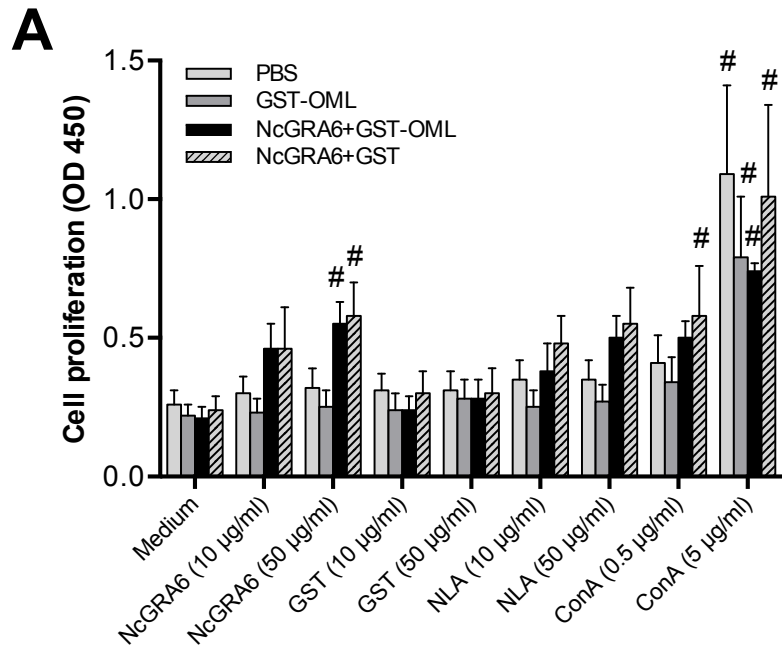
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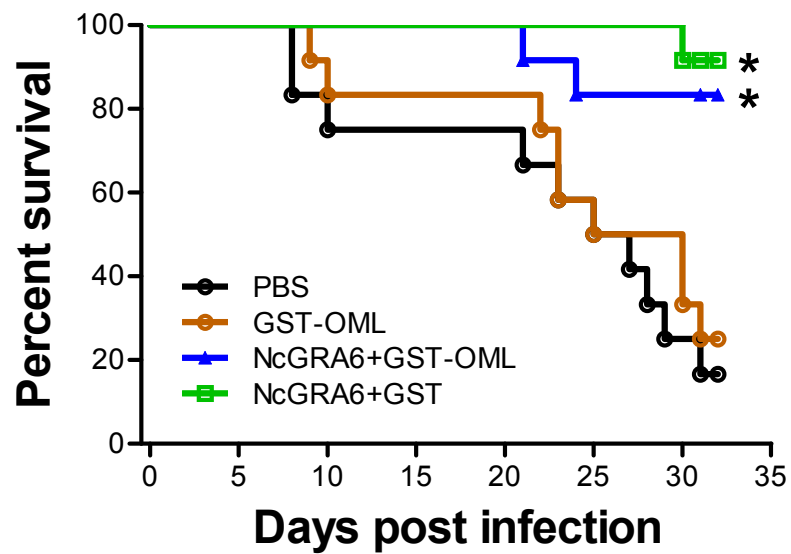
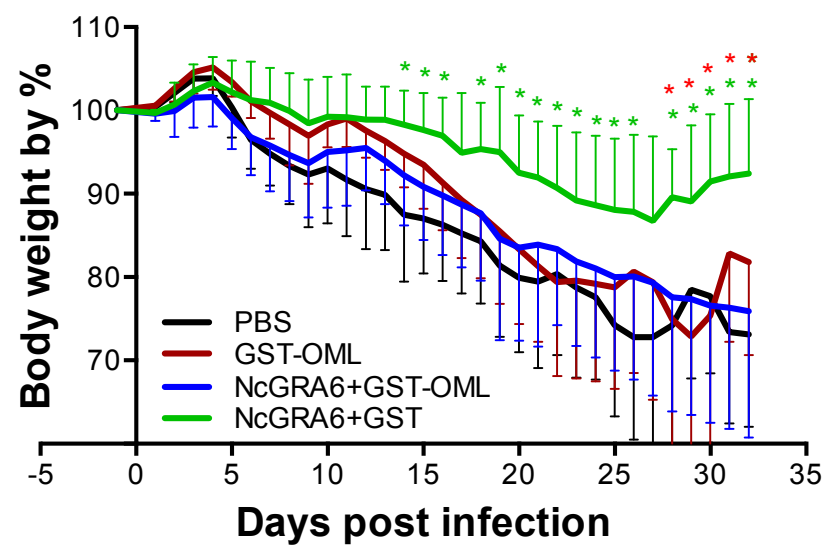
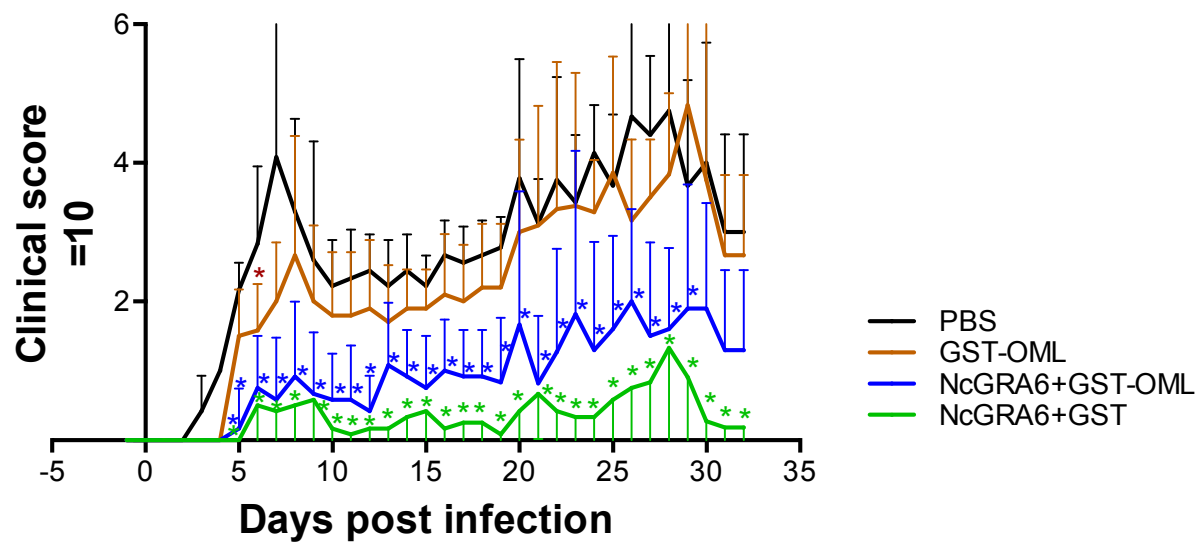
735 **Figure 4. Survival rates and clinical observations of mice.** (A) The survival rates
736 (surviving mice/total mice) are calculated from two pooled independent experiments: PBS:
737 2/12 (16.7%), GST-OML: 3/12 (25%), NcGRA6+GST: 11/12 (91.7%) and NcGRA6+GST-
738 OML: 10/12 (83.3%). *, The significance of differences in mouse survival was analyzed by
739 the χ^2 test. The differences were significant between PBS- and GST-OML-injected groups,
740 and NcGRA6+GST- and NcGRA6+GST-OML-immunized groups ($P < 0.05$). Body weight
741 alterations (B) and clinical scores (C) were calculated as means \pm SD of the body weights and
742 clinical score values for all the mice in a group from -2 until 32 days post-infection (dpi). *,
743 the differences were significant between the PBS- and test groups, ($P < 0.05$). **, the
744 differences were significant between the NcGRA6+GST and NcGRA6+GST-OML-injected
745 groups, ($P < 0.05$).

746



A**B****C****D**



A**B****C**

1 **Supplemental information**

2 **Materials and methods**

3 *1. Real time-PCR for measuring parasite burdens*

4 The parasite DNA from each mouse brain was extracted, purified, and quantified as
5 reported formerly (Ihara et al., 2016). Tissues homogenates were obtained after lysis with
6 extraction buffer (0.1 M Tris-HCl, pH 9.9; 1% SDS, 0.1 M NaCl, 1 mM EDTA, 1 mg/mL
7 proteinase K) at 55 °C. Phenol–chloroform extraction and ethanol precipitation were used to
8 purify the DNA. Parasite DNA was counted and analyzed by real-time PCR using specific
9 primers for the Nc5 gene, which is specific for *N. caninum* quantification (Kaufmann et al.,
10 1996; Liddell et al., 1999) (forward primer: nucleotides 248–257, 5'-ACT GGA GGC ACG
11 CTG AAC AC-3'; reverse primer: nucleotides 303–323, 5'-AAC AAT GCT TCG CAA GAG
12 GAA-3'). The PCR was performed on genomic DNA and guided by the SYBR green detection
13 method (Applied Biosystems). Amplifications, data processing and analysis were performed
14 with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Establishment of
15 standard curve was generated from *N. caninum* DNA extracted from 1×10^5 parasites using 1 μ L
16 of a serial dilution ranging from 10,000 to 0.01 parasites. The data were analyzed using
17 Dissociation Curves version 1.0 F (AB Applied Biosystems).

18

19

20 2. An ethogram for measurement of clinical score

21 Clinical score was adjusted by recording the exhibited clinical signs manifested on each mouse
22 and the mice group started from -2 to 32 dpi in infected mice with *N. caninum* within and
23 outside their naïve cages. Each recorded sign was represent a certain score ranged from 0 (no
24 signs) to 10 (all signs).

25 **Table S1.** Different types of sign, behavior or activity observed in an animal and relevant score.

Score	Signs	Description
1	Kyphosis	The back of mouse is curved and hunched upwardly
2	Piloerection	The hair is stiff, dispersed and shaggy
3	Warm- seeking	The mouse is hiding below the bedding particularly in the cage corners
4	Sunken eye	As a result of dehydration due to inability to feed and drink the eyes become hollowed
5	Ptosis	The eyes become closed primarily due to dropping of upper eye lids
6	Move reluctance	The mouse refuses to move and latency may continue for more than 15 seconds
7	Ataxia	Under forced movements, the mouse walks in staggering gait and swaying from side to another
8	Lack of reflexes	The urine and faecal matter is voided involuntary, the skin and eye reflexes are latent

9 **Dizziness** The mouse is semi-comatosed and all its muscles are partially
flaccid

10 **Laying on the belly** Lateral recumbency accompanied with complete loss of
(LOB) reflexes and muscular tone

26

27

28

29 **References**

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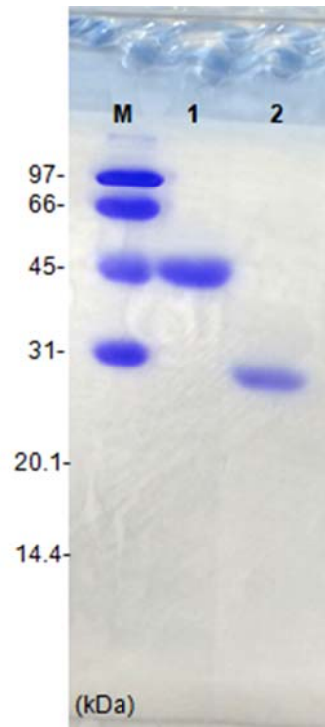
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39 **Supplemental figures**

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41

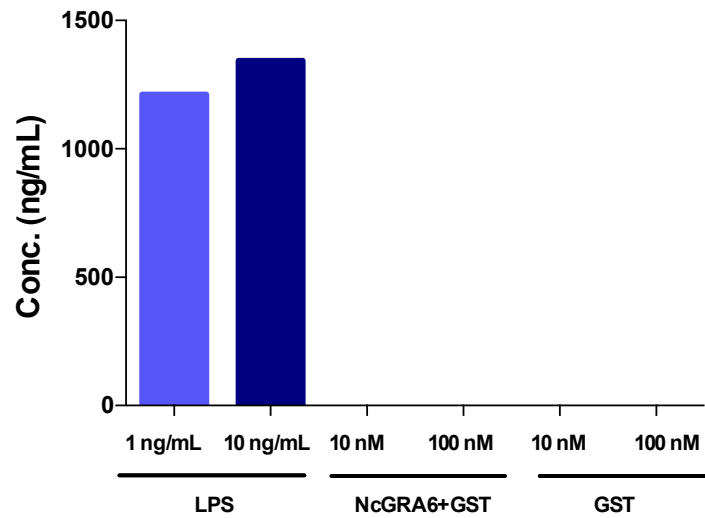
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43 **Fig. S1.** Expression and purification of recombinant proteins. Sodium dodecyl sulfate
44 polyacrylamide gel electrophoresis of recombinant proteins with Coomassie Blue staining.

45 Lanes: M, molecular mass marker; lane 1, NcGRA6+GST; lane 2, GST. The photo was taken
46 from the same gel.

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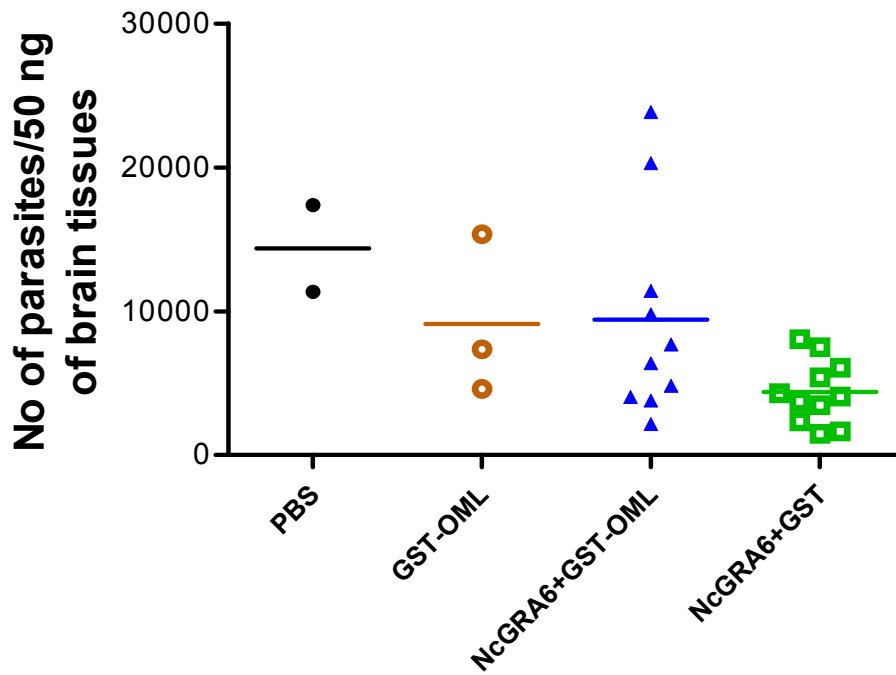


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51 **Fig. S2.** Endotoxin measurement levels of antigens in this study. Endotoxin levels in
52 NcGRA6+GST, glutathione S-transferase (GST) and lipopolysaccharide (LPS) were measured
53 by a Limulus Amebocyte Lysate assay. The test was standardized against different concentrations
54 of an *Escherichia coli*-derived standard endotoxin control supplied by the company and
55 validated against LPS which has been used in the experiments of the current study. No detectable
56 level of endotoxin was detected for NcGRA6+GST or GST. The values represent the mean
57 values of duplicate samples.

58



59

60

61 **Fig. S3.** Parasite numbers in the brains of the surviving mice. At 32 days post infection, the
 62 brains of surviving mice were aseptically collected. Quantification of the parasite was performed
 63 using real-time PCR and analyzed against standard curve prepared from genomic *N. caninum*
 64 DNA extracted from a well known number of parasite. The parasite burden was analyzed using
 65 one-way ANOVA plus Tukey–Kramer *post hoc* analysis, and no significant difference was
 66 observed between the different groups. Results are calculated from two pooled independent
 67 experiments (PBS: $n = 2$, GST-OML: $n = 3$, NcGRA6+GST: $n = 11$, NcGRA6+GST-OML: $n =$
 68 10).

69