Role of dense granule antigen 7 in vertical transmission of *Neospora caninum* in C57BL/6 mice infected during early pregnancy

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Abstract

Neosporosis is a parasitic disease affecting the health of dogs and cattle worldwide. It is caused by *Neospora caninum*, an obligate intracellular apicomplexan parasite. Dogs are its definitive host, it mostly infects livestock animals, especially cattle that acts as intermediate host. It is necessary to have well-established models of abortion and vertical transmission in experimental animals, in order to determine basic control measures for the *N. caninum* infection. We evaluated the role of *N. caninum* dense granule antigen 7 (NcGRA7) in the vertical transmission of *N. caninum* using the C57BL/6 pregnant mouse model. We inoculated mice on day 3.5 of pregnancy with parental Nc-1 or NcGRA7-deficient parasites (NcGRA7KO). Post-mortem analyses were performed on day 30 after birth and the surviving pups were kept until day 30 postpartum. The number of parasites in the brain tissues of offspring from NcGRA7KO-infected dams was significantly lower than that of the Nc-1-infected dams under two infection doses (**1×10^6** and 1×10^5 tachyzoites/mouse). The vertical transmission rates in the NcGRA7KO-infected group were significantly lower than those of the Nc1-infected group. To understand the mechanism by which the lack of NcGRA7 decreases the vertical transmission, pregnant mice were sacrificed on day 13.5 of pregnancy (10 days after infection), although parasite DNA was detected in the placentas, no significant difference was found between the two parasite lines. Histopathological analysis revealed a greater inflammatory response in the placentas from NcGRA7KO-infected dams than in those from the parental strain. This finding correlates with upregulated chemokine mRNA expression for CCL2, CCL8, and CXCL9 in the placentas from the NcGRA7KO-infected mice. In conclusion, these results suggest that loss of NcGRA7 triggers an inflammatory response in the placenta, resulting in decreased vertical transmission of *N. caninum*.

Keywords

Neosporosis; NcGRA7; placenta; brain; chemokines; inflammatory response

1. INTRODUCTION

Neospora caninum, an obligate intracellular apicomplexan parasite, causes abortion in infected livestock. The parasite can be horizontally transmitted through ingestion of oocysts shed in the faeces of definitive hosts, but vertical transmission is considered its main infection route in cattle [1]. *Neospora caninum* causes encephalomyelitis and myelitis in dogs [2], and abortion in both dairy and beef livestock at 5–6 months of pregnancy [3]. Furthermore, bovine fetuses can die *in utero* and then be resorbed, mummified, autolyzed or stillborn, or born alive with or without clinical signs and possibly persistently infected [3]. Transplacental transmission of *N. caninum* can occur over several generations, which explains the global persistence of this disease [4]. In the USA, South America, Switzerland, New Zealand, and the European Union, neosporosis is considered the main cause of bovine abortions [1,5-7]. Although antibodies to *N. caninum* have been detected in humans [1,8], including immunocompromised patients infected with the immunodeficiency virus [9], and two umbilical cord blood samples were positive for *N. caninum* infection in pregnant women [10], unlike *Toxoplasma gondii*, the zoonotic potential of *N. caninum* has not been confirmed so far. In Spain, a study was carried out where 600 DNA samples from humans with clinical signs compatible with toxoplasmosis but with negative PCR results for both *T. gondii* and *N. caninum* were analyzed [11].

Dense granule organelles are present in all apicomplexan parasites, and these granules play a role in the host-parasite relationship. After parasite invasion, the dense granule proteins secreted from these organelles are released into the parasite parasitophorous vacuole (PV) and its membrane (PVM) where they perform roles in nutrient uptake and waste excretion in infected host cells [12]. Among the 18 tested *N. caninum* dense granule antigens (NcGRAs), we previously found that NcGRA7 plays a role in regulating *N. caninum* pathogenesis and host immune response modulation in mice [13].

NcGRA7 has been reported to be a marker of primo-infection, recrudescence, and reinfection in serum samples from herds associated with abortion and/or vertical transmission [14]. In addition, the production of anti-NcGRA7 antibodies was correlated with the virulence in pregnant and non-pregnant mice. High levels of the antibodies were developed in the mice inoculated with high virulence isolates compared to those inoculated with low-to-moderate virulence *Neospora* isolates [15]. NcGRA7, a highly immunogenic antigen required during the initial development of the intracellular parasite, also performs an important role during the initial invasion of the parasite into host cells [16]. NcGRA7 antigen has also been used in combination with a plasmid-containing adjuvant for establishing immune resistance in mice infected with *N. caninum* [17]. Vaccination with recombinant NcGRA7 encapsulated alone or combined with recombinant NcSAG4 resulted in slight protection against challenge infection with Nc1 isolate in non-pregnant mice, but it elicits a strong humoral and cellular immune response [18]. Additionally, immunization before pregnancy with NcGRA7 entrapped in oligomannose-coated liposomes resulted in increased offspring survival and decreased infection rates in the brains of dam mice [19]. However, little is known about the role of NcGRA7 during pregnancy. Therefore, our main goal was to evaluate the role of NcGRA7 in the vertical transmission of *N. caninum* using a pregnant mouse model.

2. MATERIALS AND METHODS

2.1 Ethics statement

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine, Obihiro,

Japan (permit numbers 20-27, 21-36). To minimize animal suffering, all surgical operations, blood collection, and cervical dislocation were performed under isoflurane anesthesia.

2.2 Mice

C57BL/6 female and male mice, 8–10 weeks old, were obtained from Clea Japan (Tokyo, Japan). The animals were housed 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. These animals were treated and used according to the Guiding Principles for the Care and Use of Research Animals published by the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. The animals were kept under standard laboratory conditions and fed with commercial food and water *ad libitum* on a 12/12-h light/dark cycle at 21°C under 40% relative humidity.

2.3 Parasites and cell cultures

Neospora caninum Nc1 (parental strain), the NcGRA7-deficient parasite (NcGRA7KO) generated by the clustered regularly interspaced short palindromic repeats (CRISPR)-associated gene 9 (CRISPR/CAS9) system [13] were maintained in African green monkey kidney epithelial cells (Vero cells). Parasites were cultured in Eagle's minimum essential medium (Sigma, St. Louis, MO) supplemented with 8% heat-inactivated fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 100 U/mL penicillin, and 10 mg/mL streptomycin. At 72 hours after the infection, parasites and host cell debris were washed with cold phosphate-buffered saline (PBS) to exclude the extracellular parasites and the final pellet resuspended in cold PBS was passed through a 27-gauge needle and a 5.0-µm-pore size filter (Millipore, Bedford, MA). After washing the parasites with PBS by centrifugation at 1,500 ×

122 *g* for 10 min, the parasites were refiltered, and their numbers were hemocytometrically counted
123 for each experiment according to the previous study [13].
124
125 **2.4 Experimental design**
126 In our previous study using non-pregnant C57BL/6 mice [20,21], 10^6 *N. caninum*
127 tachyzoites of Nc1 strain were intraperitoneally injected into the mice for observation of mouse
128 survival rates, bodyweight, and clinical scores and analyses of immune response. In addition,
129 NcGRA7KO showed reduced virulence in non-pregnant C57BL/6 mice [13]. Therefore, we
130 used 10^6 *N. caninum* tachyzoites of Nc1 strain in addition to 10^5 tachyzoites for intraperitoneal
131 inoculation into pregnant C57BL/6 mice in this study (Fig. S1).
132 Eight mice were used for each group of each trial in the mating procedures. Estrus
133 synchronization was initiated in a group (8 mice /cage) by the Whitten effect [22-24]. Male
134 and female mice were mated for 14 h (one female with one male /cage) after 18:00 pm. Mating
135 procedures were done on 3 successive days and females were checked for vaginal plugs or
136 swelling the next morning at 8:00 am after each mating day. Mice with positive vaginal plugs
137 were considered at day 0.5 of pregnancy. Eight mice were mated for each group. The mice
138 without vaginal plugs were excluded from the experiment and their bodyweight was observed
139 to ensure that they were non-pregnant. Pregnancies were further confirmed by recording an
140 increased bodyweight percentage in each of the estimated pregnant dam mice until day 10 post-
141 mating. The number of pregnant mice used was shown in Fig. S1, Tables 2 and 3.
142 To compare parasite virulence in the mice based on survival rate and clinical score of
143 dams, number of offspring, and the daily survival of the offspring, pregnant C57BL/6 mice
144 were intraperitoneally inoculated with different doses of *N. caninum* tachyzoites (trial 1: 10^6
145 tachyzoites/mouse, Nc1, and NcGRA7KO, trial 2: 10^5 tachyzoites/mouse, Nc1, NcGRA7KO)
146 or RPMI-1640 medium (negative control) on day 3.5 of pregnancy. The daily bodyweight

measurement of each dam mouse including RPMI-1640 medium-injected mice (negative control), as recorded from −2 to 13 days post-infection (dpi) with Nc1 or NcGRA7KO, was compared with the weight of the same mouse on the first day of measurement before infection. Clinical scores were assigned based on hunching, piloerection, warm-seeking behavior, ptosis, sunken eyes, ataxia, latency of movement, flaccidity, touch reflexes, the skin and eye reflexes, and lying on belly. The scores varied from 0 (no signs) to 10 (all signs) [25]. Clinical scores of dam mice were estimated by recording the clinical signs manifested in each mouse from −2 to 13 dpi. To exclude the pregnancy parturition effects and avoid the disturbances of mice, only recording of the number of dams and offspring was performed from 14 dpi (correspond to day 17.5 of pregnancy) to the day 30 post-partum.

For pathological and mRNA expression analyses, experiment trial 3 was performed. Pregnant C57BL/6 mice were intraperitoneally inoculated with 10^6 *N. caninum* tachyzoites on day 3.5 of pregnancy and then postmortem examination was performed at day 13.5 of pregnancy (10 dpi). The fertility rates were as follows, negative control (5/6, 83.3%), Nc1-infected (3/5, 60.0%), NcGRA7KO-infected (4/5, 80.0%). To analyze mRNA expression, spleen (right half) and pooled placenta samples were collected for each dam. For the pathological analysis, placental tissues from each pregnant dam were collected. For determination of the parasite burden, spleen (left half), placental tissue, and whole fetal bodies were collected from each pregnant dam. The number of dams for above experiments was as follows, negative control, n = 5; Nc1-infected, n = 3; NcGRA7KO-infected, n = 4.

2.5 DNA isolation and quantitative PCR (qPCR) to determine *N. caninum* distribution

Parasite burdens were quantified in the brain, placenta, spleen and whole fetus bodies. DNA was extracted from the brain of dams and offspring from trials 1, 2 and 3, and from dam

placenta and spleen, and whole fetus bodies from trial 3 as follows: each tissue or organ was thawed in 10 volumes of extraction buffer (0.1 M Tris-HCl [pH 9.0], 1% SDS, 0.1 M NaCl, 1 mM EDTA) and 20 μg/ml of proteinase K at 50°C. The DNA was purified by phenol-chloroform extraction and ethanol precipitation. Parasite DNA was then amplified with the following *N. caninum* Nc5 gene-specific primers: forward 5′-ACT GGA GGC ACG CTG AAC AC-3′ and reverse 5′-AAC AAT GCT TCG CAA GAG GAA-3′ [20]. Amplification, data acquisition, and data analysis were performed on the ABI Prism 7900HT sequence detection system (Applied Biosystems), and the cycle threshold (Ct) values were calculated as described previously [29]. Standard curves were constructed using 10-fold serial dilutions of *N. caninum* DNA extracted from 10^5 parasites; thus, each curve ranged from 0.01 to 10,000 parasites. Parasite numbers were calculated from the standard curve.

2.6 Real-time reverse transcriptase (RT)-PCR analysis of chemokine expression

Total RNA was extracted from cells or homogenized tissues using TRI Reagent (Sigma-Aldrich). RNA was reverse transcribed with a Prime Script II First Strand cDNA synthesis kit (TaKaRa Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. The cDNA recovered was amplified using RT-PCR with PowerUp SYBR green master mix (Thermo Fisher Scientific, Inc., Waltham, MA) and 500 nM of gene-specific primers in a 10 μl reaction volume according to the manufacturer's protocol. The target molecules were interleukin-10 (IL-10), interleukin-4 (IL-4), interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF-α), C-C motif chemokine ligand 2 (CCL2), C-C motif chemokine ligand 8 (CCL8), cysteine-X-cysteine motif chemokine ligand 9 (CXCL9) and cysteine-X-cysteine motif chemokine ligand 10 (CXCL10). Gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the internal control by Ref Finder [28] and gene expression was

normalized against this control. The primer sequences of the target genes are shown in Table 1. The relative mRNA levels were calculated using the fold change Ct method.

2.7 Histopathological analysis

Placental tissue was fixed in neutral-buffered formalin and processed using routine methods. Serial sections (4 μm each) made from paraffin-embedded tissues were stained with hematoxylin and eosin. Immunohistochemistry was performed using the following procedures [28]. Deparaffinized sections were incubated with polyclonal antibodies: rabbit anti-*N. caninum* dense granule antigen 6 (NcGRA6), rabbit anti-NcSAG1, rabbit anti-CD3 (Abcam, Cambridge, UK) for T cell marker, and rabbit anti- ionized calcium binding adapter protein 1 **(**Iba1) (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) for macrophage marker at 4˚C for overnight. Antigen retrieval was performed at 98˚C for 45 min with Immunosaver (Nisshin EM Co., Ltd, Tokyo, Japan). Non-specific reactions and endogenous peroxidases were blocked by blocking solution (10 mM PBS pH7.4 with 8% skim milk and 3% tween 20 (Santa Cruz Biotechnology, inc., Dallas, TX)) and 10% H_2O_2 methanol. Signals were visualized on the Envision system (Agilent, Santa Clara, CA, USA) using 3, 3'-diaminobendidin. To characterize the inflammatory cells and evaluate the inflammatory response, we manually scan the slides and select an inflammatory region. CD3 and Iba1-positive cells were counted on three aleatory fields (×400; total 0.711mm^2 in each) in three placental zones (decidua, junctional zone, and labyrinth).

2.8 Statistical analysis

GraphPad Prism 6.0 software and its updated version 8.3.4 (GraphPad Software Inc., La Jolla, CA, USA) was used. Data represent the mean ± SD. Statistical analyses were performed using Student's t-test, Mann–Whitney U test, and one-way or two-way analysis of

variance (ANOVA) followed by the Tukey-Kramer hoc test for group comparisons. Survival rates, vertical transmission rates, and statistical comparisons were assessed using a χ^2 test. The levels of statistical significance are shown as asterisks and defined in each figure legend together with the name of the statistical test that was used. *P* value of < 0.05 was considered statistically significant.

3. Results

3.1 Parasite virulence in pregnant mouse model under a higher infection dose

During the infections with 1×10^6 tachyzoites of either parasite, we observed that the bodyweight gains in the infected groups did not show significant differences with respect to the uninfected group (negative control) (Fig. S2). However, the clinical scores of the infected dams were significantly higher than those of the uninfected animals (negative control). The clinical manifestations observed were hunching, piloerection, warm-seeking behavior, and sunken eyes (Fig. S2). All dams survived during the pregnancy until 15-16 dpi (Fig. 1A). The survival rate of the NcGRA7KO-infected dams (60%, 3/5) was lower than that of the Nc-1-infected dams (100%, 4/4) and negative control (100%, 5/5), but no significant difference was found (Fig. 1A). The fertility rates were as follows, negative control (5/6, 83.3%), Nc1-infected (4/5, 80.0%), NcGRA7KO-infected (5/5, 100.0%) (Table 2). The number of parasites in the brain also showed no significant difference between the Nc1- and NcGRA7KO-infected dams (Fig. 1B). There was no significant difference either in the mean number of offspring per litter among the experimental groups (Table 2). Although infection with *N. caninum* decreased survival in the offspring compared with the negative control mice, the survival rates of the offspring from the NcGRA7KO-infected dams (28.0%) were significantly higher than those from the Nc1-infected dams (7.7%) (Fig. 1C, Table 2). The number of parasites in the brains

of the offspring of the NcGRA7KO-infected dams was significantly lower than in the brains of the offspring of the Nc-1-infected dams (Fig. 1D). The proportion of vertical transmission was calculated retrospectively in both infected groups (Table 2). Parasite DNA was detected in 20/21 (95.2%) in the brains of offspring from Nc1-infected dams. However, a significantly lower percentage of positivity was seen in the brains of offspring from NcGRA7KO-infected dams (14/21, 66.7%) (Table 2). Additionally, parasite DNA was detected during acute death of offspring from Nc1-infected dams compared with those from NcGRA7KO-infected dams (Fig. 1E).

3.2 Parasite virulence in pregnant mouse model under a lower infection dose

To confirm the virulence of NcGRA7KO, trial 2 experiment under a lower infection dose was conducted. The bodyweight gains in the infected groups did not show significant differences with respect to the uninfected group (negative control) (Fig. S3). However, the clinical scores of both infected dams were significantly higher than those of the uninfected animals (negative control). The clinical manifestations observed were hunching, piloerection, warm-seeking behavior, and sunken eyes (Fig. S3). Survival of all dams was seen during the pregnancy until 15-16 dpi (Fig. 2A). The survival rate of the NcGRA7KO-infected dams (80%, 4/5) was lower than that of the Nc-1-infected dams (100%, 5/5) and negative control (100%, 4/4), but no significant difference was found (Fig. 2A). The fertility rates were as follows, negative control (4/6, 66.6%), Nc1-infected (5/6, 83.3%), NcGRA7KO-infected (5/7, 71.4%) (Table 3). The number of parasites in the brain also showed no significant difference between the Nc1- and NcGRA7KO-infected dams (Fig. 2B). Furthermore, there was no significant difference either in the mean number of offsprings per litter among the experimental groups (Table 3). In contrast, the offspring survival rates for the NcGRA7KO-infected dams (50.0%) were significantly higher than those for the Nc1-infected dams (19.4%) (Fig. 2C, Table 3).

Parasite numbers in the brain tissues of the offspring from the NcGRA7KO infected dams were significantly lower than those from the Nc-1-infected dams (Fig. 2D). The proportion of vertical transmission was monitored in both infected groups (Table 3). The percentage of the parasite DNA positive in the brain of offspring from NcGRA7KO-infected dams (14/20, 70.0%) was significantly lower than that from Nc1-infected mice (36/36, 100.0%). As shown in Fig. 2E, parasite DNA was detected in the offspring from the Nc-1-infected dams. Together with the results shown in Fig. 1, NcGRA7KO parasite numbers in the brains of the offspring were significantly lower than those for Nc1, indicating lower vertical transmission of NcGRA7KO.

3.3 Histopathological and qPCR analyses of parasite numbers in placentas and fetuses

To estimate the tissue damage caused by *N. caninum* infection, histopathological analysis of the placentas on day 13.5 of pregnancy (10 dpi with 1×10^6 tachyzoites) was performed as trial 3. As we observed in trial 1, the bodyweight gains in the infected groups did not show significant differences with respect to the uninfected group (negative control) (Fig. S4). On the other hand, the clinical scores of the infected dams were significantly higher than those of the uninfected animals (negative control) (Fig. S4). The fertility rates were as follows, negative control (5/6, 83.3%), Nc1-infected (3/5, 60.0%), NcGRA7KO-infected (4/5, 80.0%). In Nc1- and NcGRA7KO-infected dams, mild edema, and mononuclear cell infiltration were observed in the decidua and junctional zone. In decidua, T lymphocyte-rich inflammation was more severe in NcGRA7KO- than Nc1-infected groups (Fig. 3B). Hemorrhage and necrosis were not observed in the placenta. There was only one case where a NcGRA7KO-infected mouse had observable *N. caninum* tachyzoites in the trophoblast with focal vasculitis and neutrophil infiltration (Fig. S5; Table S1). T lymphocyte and macrophage numbers were higher in the

decidua and junctional zones of the Nc1- and NcGRA7KO-infected groups than in the negative control mice (Figs. 3B, C, and 4), unlike in the labyrinths (Figs. 4 and S6). Furthermore, the number of T lymphocytes in the NcGRA7KO-infected dams was significantly higher in the deciduas and junctional zones than that in the Nc1-infected animals (Fig. 4, Table S1).

To determine whether parasites were present in the placentas or fetuses, parasite loads were estimated by qPCR. Although parasite DNA was detected in the placentas of both experimental groups, no significant difference in the parasite numbers was found between Nc1 and NcGRA7KO parasites (Fig. 5A). The parasite DNA was not detected in the spleen of the dams (data not shown). Moreover, parasite DNA was not detectable in the whole bodies of fetuses from most of the infected dams (Fig. 5B).

3.4 Proinflammatory marker expression in the spleen and placenta

To examine the inflammatory responses during the pregnancy, we analyzed mRNA expression, which we have previously found to be regulated by *N. caninum* infection [13], in both placenta and spleen tissues on day 13.5 of pregnancy (10 dpi) (Fig. 6). In the spleen, TNF-α expression was lowered by *N. caninum* infection, and CCL8 expression was raised in the NcGRA7KO-infected mice, as compared with negative control mice (Fig. 6A). The expression levels of CCL2, CCL8 and CXCL9 were higher in the placentas from the NcGRA7KO-infected dams than in the placentas from Nc1-infected animals (Fig. 6B). Under our experimental conditions, changes in the expression of IL-4, IL-10, IFN-γ and CXCL-10 were not seen (Fig. S7).

4. DISCUSSION

Neospora caninum has one of the highest known vertical transmission rates of parasites and its transplacental transmission results in the persistent transfer of parasites from infected dams to their fetuses during gestation [29]. Vertical transmission has been previously evaluated by many studies in dairy cows [30,31], dogs [32], and mice such as Swiss-Webster mice [33], Qs out-bred mice [34], BALB/c mice [35,36] and C57BL/6 mice [37]. In this study, the route infection (intraperitoneal), and the use of animal strain (C57BL/6) of our experimental model was based on a previous study [37], while the doses of inoculation, time of vertical transmission monitored and the parasite lines used were different [37]. The reported study [38] evaluated the vertical transmission in the fetuses at days 18-20 of gestation using one parasite strain (Nc1), while in this study in trials 1 and 2 the vertical transmission of the offspring was determined until day 30 post-partum, using two parasite lines, Nc1 and NcGRA7KO. These studies prove that the vertical transmission varies according to the use of the mouse strain and the parasite strain.

In our study, 95.2%-100.0% PCR positive offspring were observed after the intraperitoneal infection with Nc1 strain at the early period of pregnancy (day 3.5), resulting in the successful vertical transmission in C57BL/6 mice in both high and low doses of the infection. However, the vertical transmission rate of NcGRA7KO in C57BL/6 mice was 66.7%-70.0%. Moreover, the fertility rates of uninfected dams (negative control), dams infected with Nc1, dams infected with NcGRA7KO were 66.6%-83.3%, 80.0%-83.3%, 71.4%-100.0%, and the mean numbers of offspring obtained were 5.0-6.4, 6.5-7.2, and 4.4-5.0, respectively. Previous study using C57BL/6 mice [37] showed the vertical transmission rate in the mice infected with 5×10^6 tachyzoites of Nc1 strain at day 12-14 of gestation were 100%. Moreover, the fertility rates and mean number of fetuses of the uninfected controls and mice infected during pregnancy were 71.4% and 8.2, 57.1% and 8.0, respectively, while there was no significant difference between the uninfected and infected mice. Together, the infection of

C57BL/6 mice with Nc1 strain induced vertical transmission, but did not affect the fertility rate and litter size. Therefore, these results indicated C57BL/6 mice can be used for the study of the vertical transmission of *N. caninum* and vertical transmission of NcGRA7KO was reduced compared with Nc1.

On the basis of the mouse model of vertical transmission, vertical transmission prevention has been reported in BALB/c mice [35] and C57BL/6 mice [39]. Type 1 and type 2 immune responses are involved in both vertical transmission and abortion after *N. caninum* infection [40]. A study illustrated that modulation of a Th2 cytokine can reduce the frequency of transplacental transmission of *N. caninum*. The reduction in the transplacental transmission was associated with lower levels of maternal mRNA expression of IL-4 and elevated levels of IFN-γ production [41]. The type 1 immune response is associated with protection against intracellular *N. caninum* infection, and its downregulation during pregnancy may trigger transplacental transmission [42]. However, the mechanism of abortion and vertical transmission caused by *N. caninum* infection is poorly understood. Studies on the relationship between the infection and the inflammatory response occurring during pregnancy are vital if we are able to understand the local immune response at the site of the infection. IFN-γ and TNF-α are reportedly associated with the fetal loss caused by *N. caninum* infection-related placental damage [42-44]. Another hypothesis is that induction of the T helper type 1 (Th1) immune response at the maternal level and modulation to a T helper type 2 (Th2) immune response can prevent vertical transmission of *N. caninum* in mice [45]. In fact, the immune response to *N. caninum* is typically associated with IFN-γ and $CD4^+$ T cells [42,46-48]. It has been reported that the placental pro-inflammatory response against *N. caninum* infection causes placental damage and fetal death via the destruction of fetal trophoblast cells and the effects of cytotoxic T cells and pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-2 [29,42,46]. Elevated Th1 and Th2 cytokine expression have also been reported to occur after *N. caninum*

infection [44, 49-51]. In the present study, the *N. caninum* Nc1 strain and NcGRA7KO enhanced mRNA expression of IFN-γ in the placenta on day 13.5 of pregnancy (10 dpi) while there was no significant difference among the experimental groups including negative control mice. Therefore, *N. caninum* infection might trigger the placental pro-inflammatory response.

The Th2 immune response is characterized by IL-4 production. IL-4 has been reported to be related to susceptibility to *N. caninum* infection [52,53]. IL-4 may aid *N. caninum* replication at the maternal-fetal junction [51,54] and lead to high levels of vertical transmission [55-57]. Moreover, increased IL-4 expression might reduce the harmful effects of the pro-inflammatory immune response to maintain pregnancy [57]. In the present study, no statistically significant difference in placental IL-4 expression levels was found between the infected and uninfected dams on day 13.5 of pregnancy (10 dpi), suggesting that the Th2 immune response in the host may not contribute to vertical transmission under the present experimental conditions.

Several *N. caninum* dense granule proteins such as NcGRA7 play a role in the expression and production of cytokines, chemokines, and chemokine receptors through the activation of NF-$_{K}$B (nuclear factor kappa-light-chain-enhancer of activated B cells), NFAT (nuclear factor of activated T-cells), and CAMP/PKA (cyclic adenosine monophosphate dependent protein kinase) signals, and therefore NcGRA7KO parasites appear to reduce parasite virulence in immunocompetent and immunocompromised non-pregnant mice, as evidenced by lower parasite burdens and mild brain lesions [13]. Unexpectedly, in the present study, the expression levels of CCL2, CCL8, and CXCL9 were higher in the placentas from the NcGRA7KO-infected dams than in the placentas from Nc1-infected animals. This suggests that NcGRA7 has a different activity in the host under non-pregnant and pregnant conditions. An unknown factor produced by the placenta might interact with NcGRA7, resulting in inhibition of chemokine expression. Alternatively, a gene downregulated by NcGRA7 may predominantly

affect pathogenesis in the placenta. In fact, several genes that are involved in the cell cycle, RAS signaling, apoptosis, cell differentiation, and metabolism are downregulated by NcGRA7 in macrophages [13]. Therefore, loss of NcGRA7 may enhance chemokine expression in the placenta, resulting in the immune reaction for prevention of vertical transmission of NcGRA7KO parasites. But, further studies are needed for confirmation.

Neospora caninum secretes molecules that initiate monocyte migration to the site of infection to enhance parasite invasion and multiplication [58]. Moreover, *N. caninum* cyclophilin (NcCyp) causes CCR5 (cysteine–cysteine chemokine receptor 5)-dependent migration of murine and bovine cells and is consistently detected in tachyzoites distributed within or around brain lesions [59]. Because mouse placenta reaches to full maturity at 12.5 day of pregnancy [60], the mice were sacrificed at 13.5 day of pregnancy. Both Nc1 and NcGRA7KO parasites were not detected in whole fetus bodies at 13.5 day of pregnancy (10 dpi), suggesting that vertical transmission may occur after this period. Moreover, both Nc1 and NcGRA7KO parasites were detected in the placenta at similar levels on day 13.5 of pregnancy (10 dpi). However, the histopathology analysis reported here reveals that more inflammatory cell infiltration was seen in the placentas from the NcGRA7KO-infected dams than in those from Nc1-infected dams. These results are consistent with the upregulated mRNA chemokine expression levels that we observed in the placenta. This immune reaction might affect parasite viability in the placenta, resulting in decreased vertical transmission of NcGRA7KO parasites.

The placenta of mice and humans is hemochorial type [61]. Among the placenta of mammals, the layered structure of mice and humans that exists between the blood on the maternal side and the blood on the fetal side is simple. The infection into fetus is established when the pathogen crossed the three layers of trophoblastic cells and two layers of fetal vascular endothelial cells from the maternal side. On the other hand, the placenta of cattle and dogs is epitheliochorial and endotheliochorial type, respectively [61]. To establish the vertical

transmission, pathogens must pass three layers consisting of maternal vascular endothelial cells, trophoblastic cells, and fetal vascular endothelial cells in dogs, and four layers consisting of maternal vascular endothelial cells, endometrial epithelium, trophoblastic cells, and fetal vascular endothelial cells in cattle. Compared with the placenta structure of dogs and cattle, it might be easily infected in mice. However, the molecular mechanism for crossing the placental barrier by pathogens including *N. caninum* should be elucidated in future studies.

In conclusion, to our knowledge, our study is the first to evaluate the role of an *N. caninum* dense granule antigen in pregnant mice. Our findings show that NcGRA7 might play a role in the vertical transmission of *N. caninum*. Further studies are vital for understanding the role played by NcGRA7 in the host immune response related to the regulation of vertical transmission of *N. caninum* in mice.

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Declaration of competing interests

The authors declare that there is no competing of interest.

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Graphical Abstract

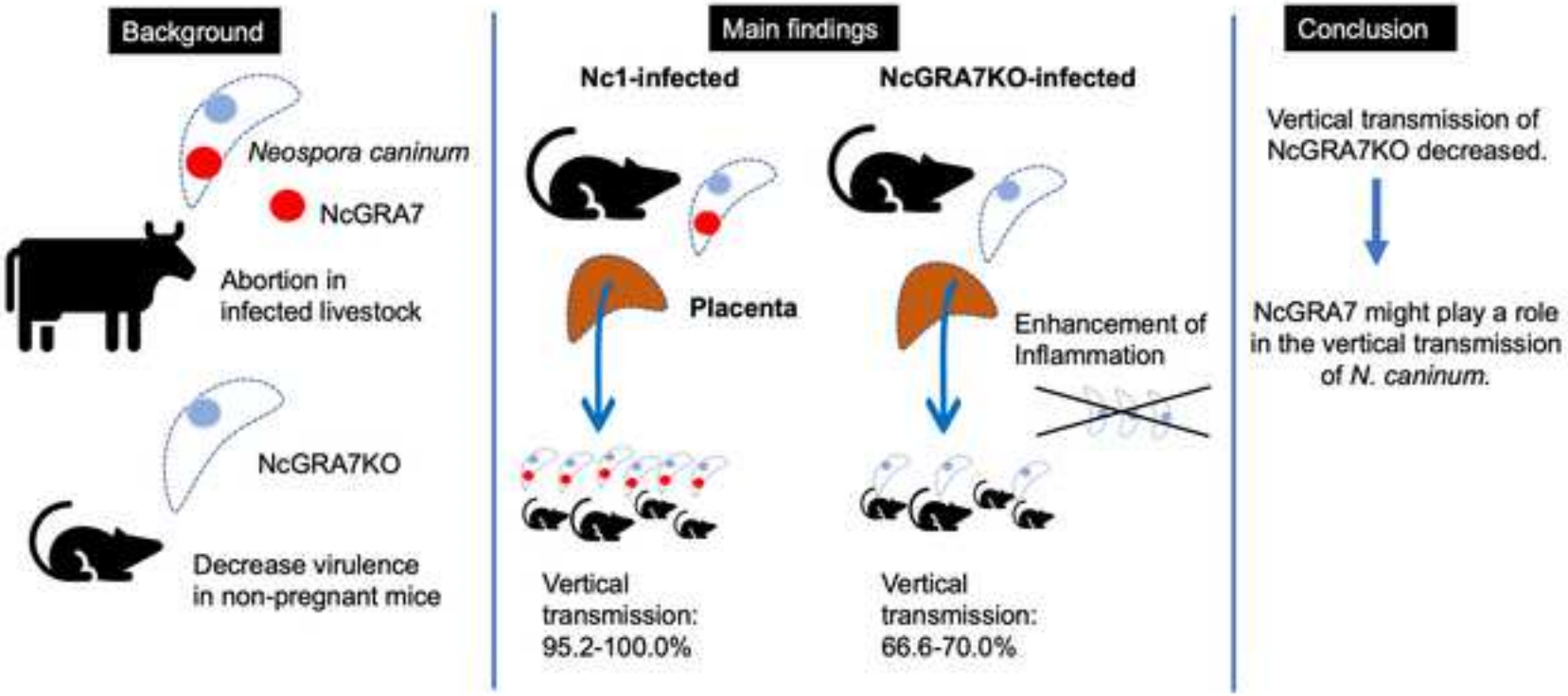


Figure 1

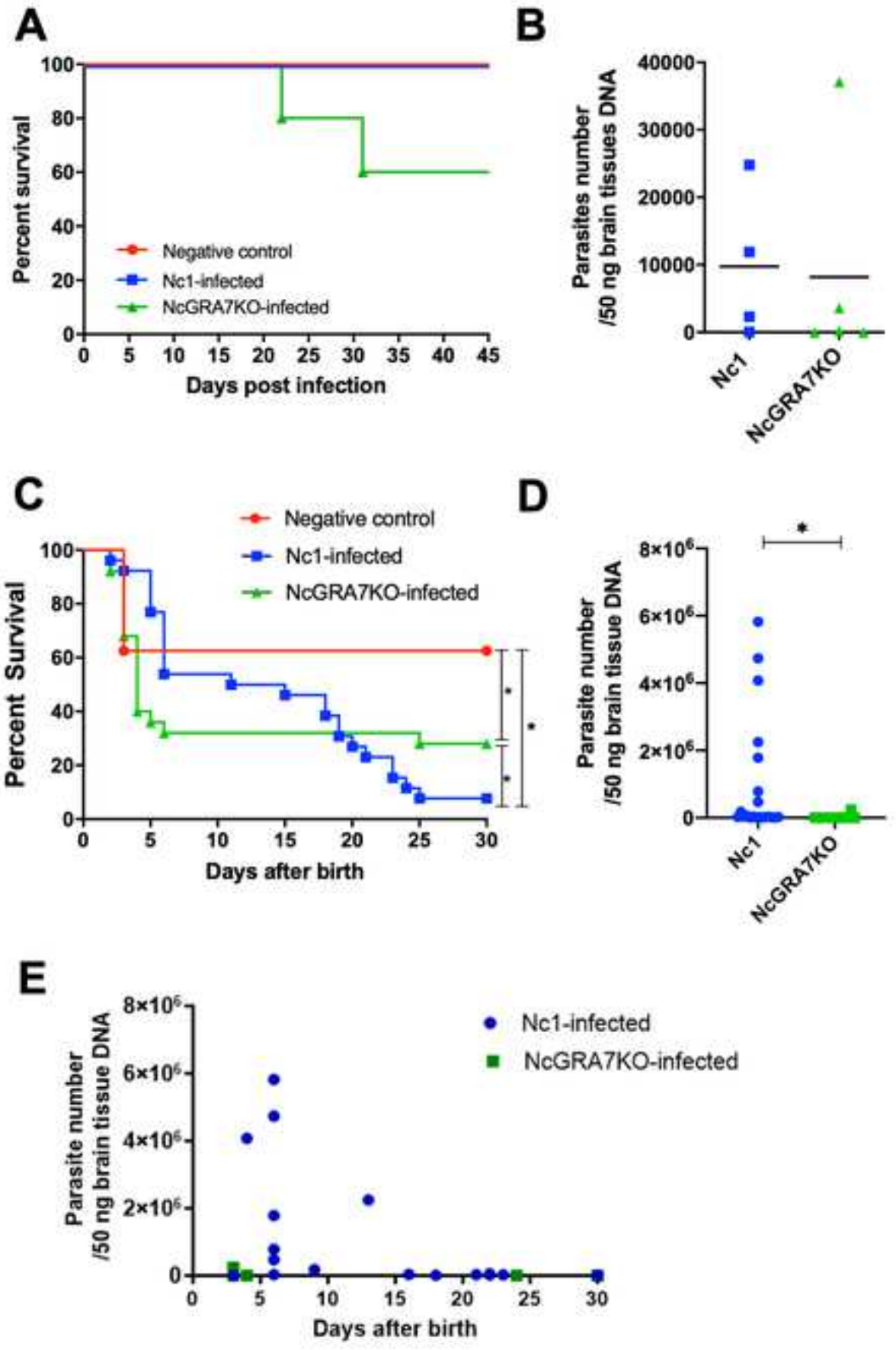


Figure 2

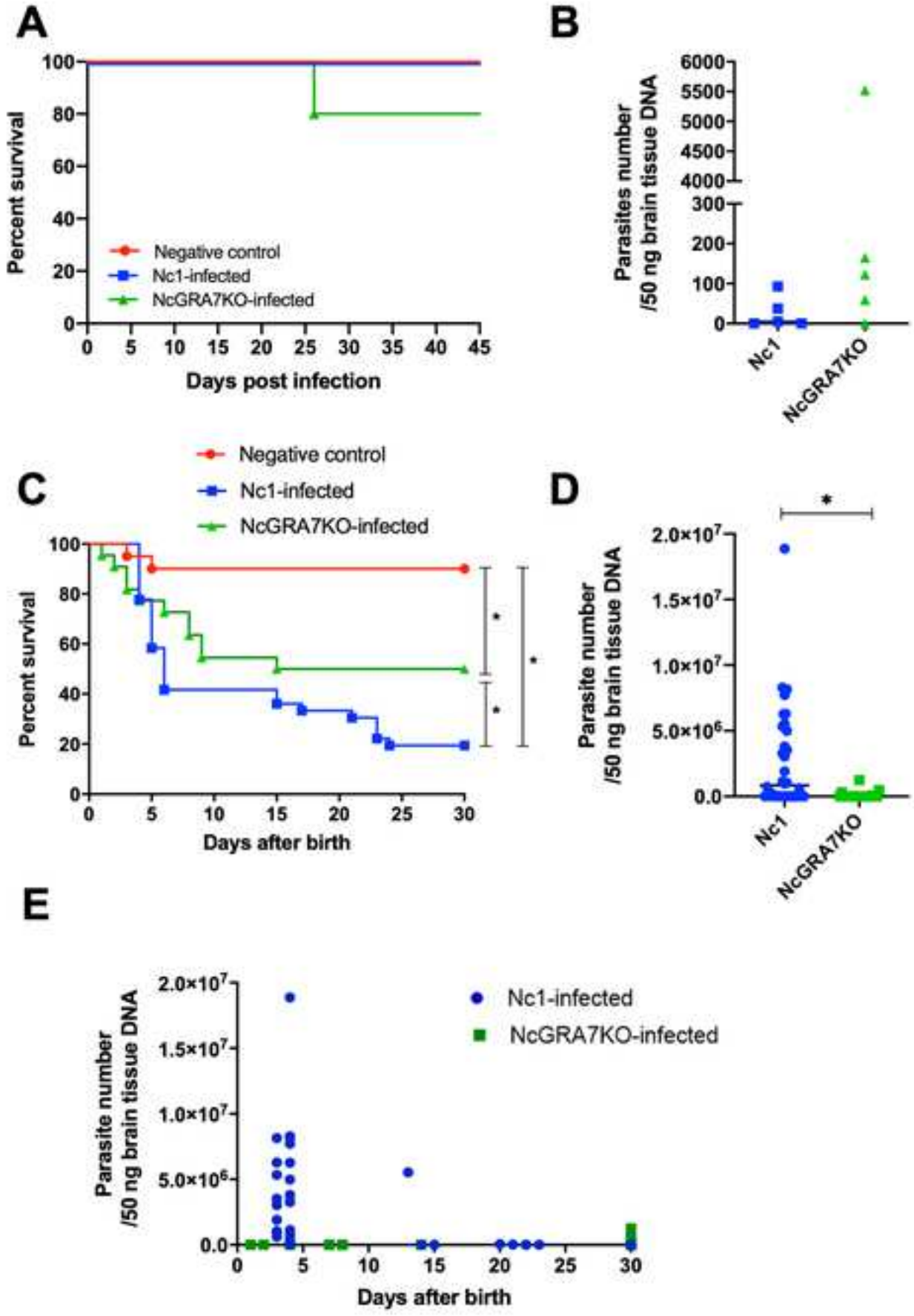


Figure 3

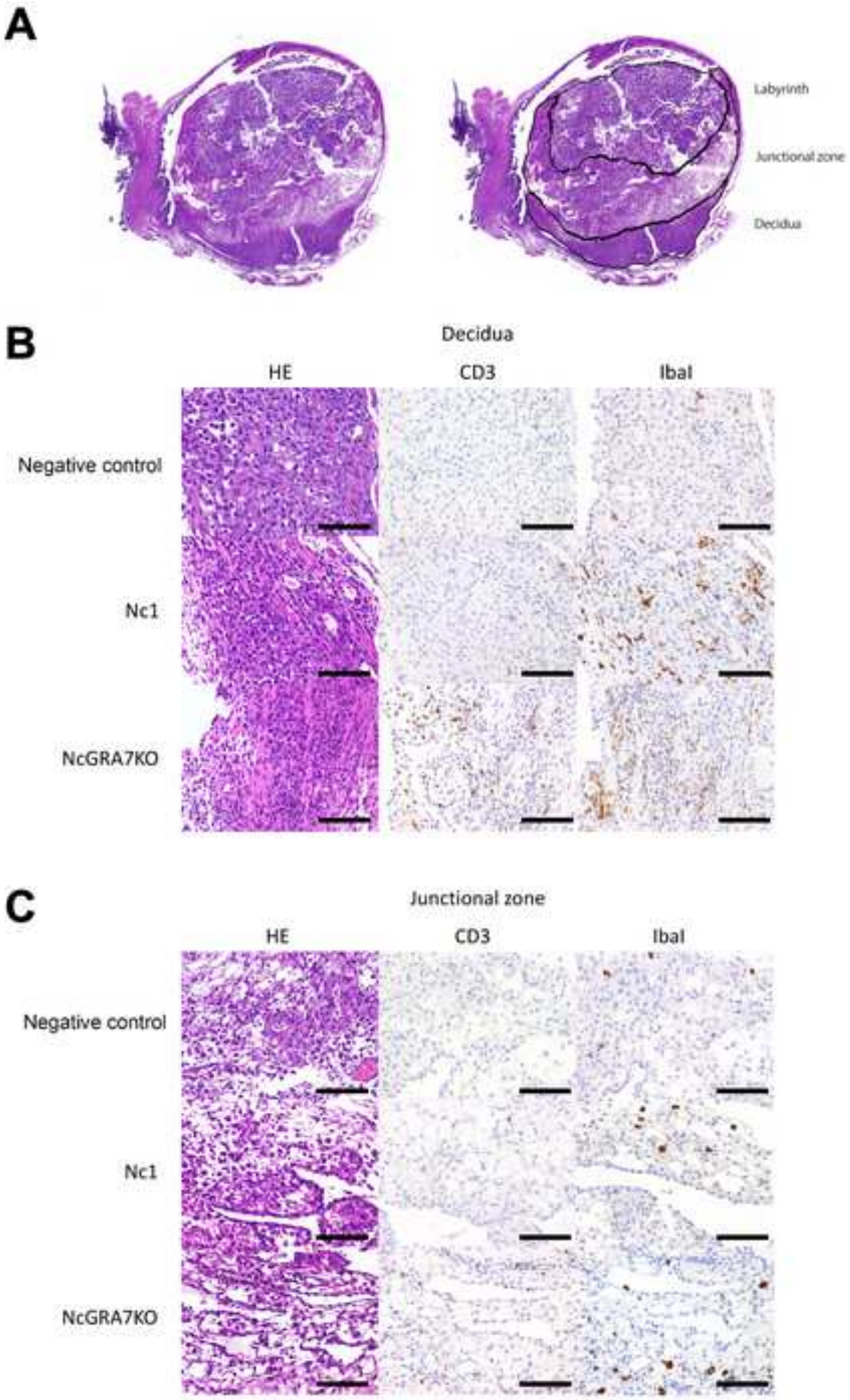


Figure 4

Click here to access/download;Figure;Fig. 4R.tif

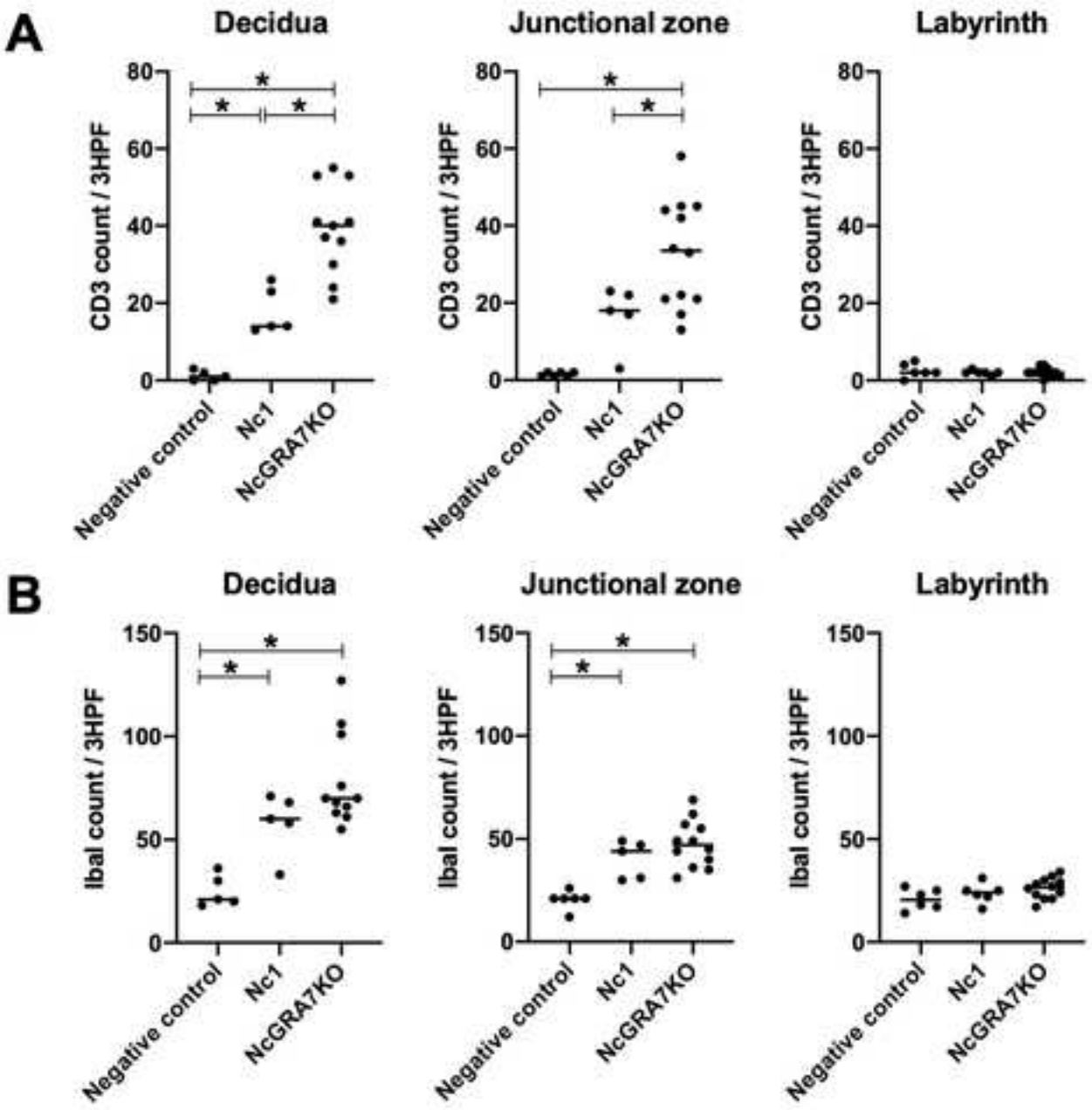


Figure 5

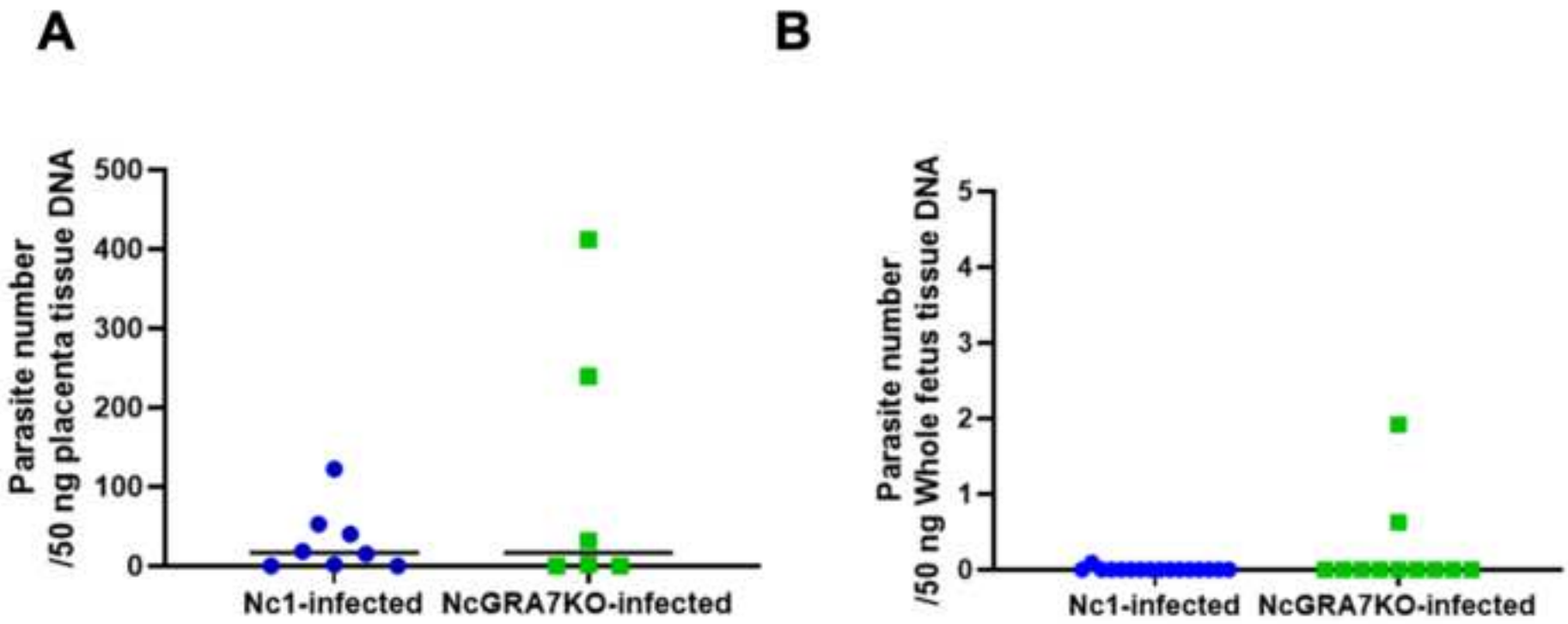
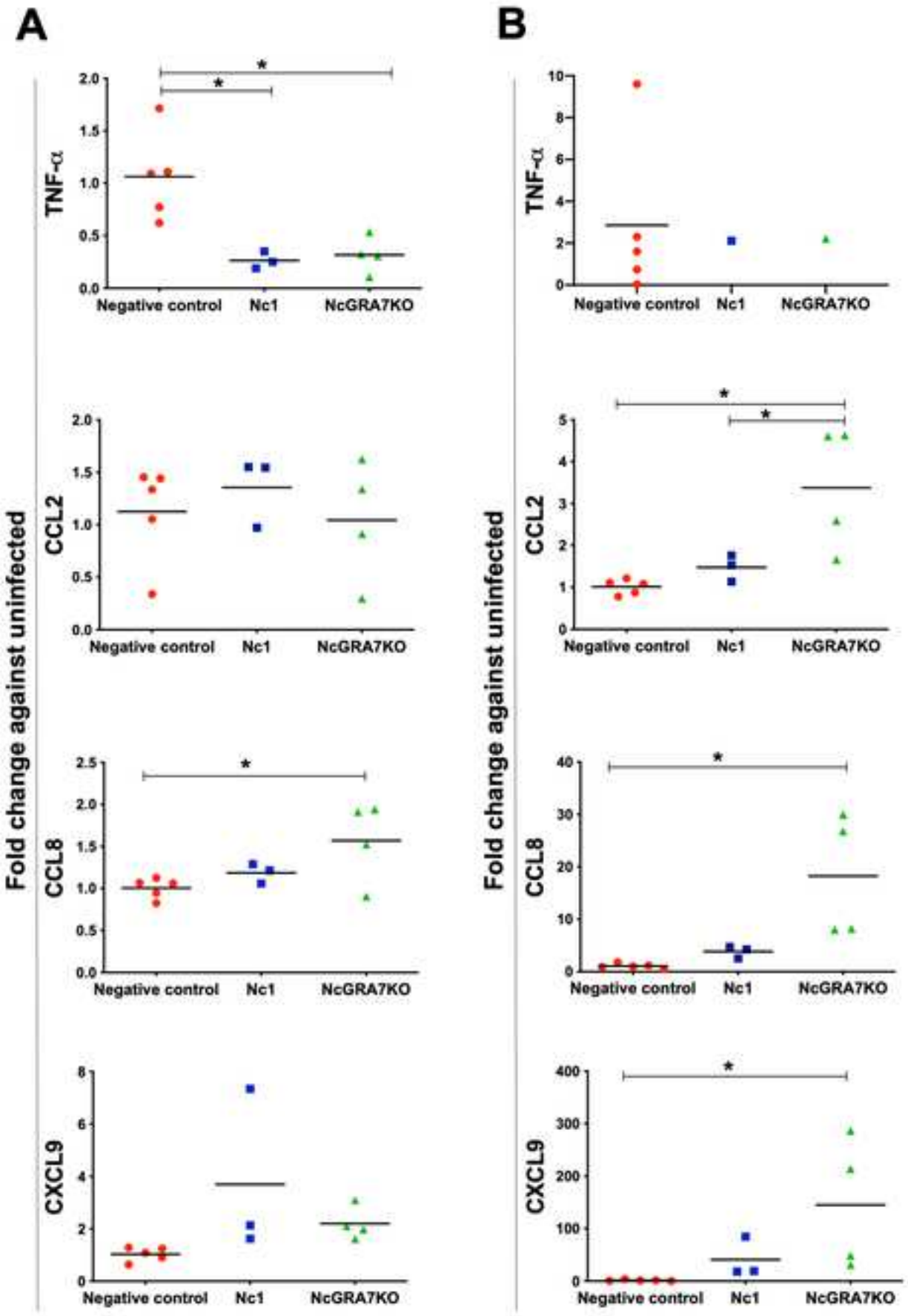


Figure 6

Click here to access/download;Figure;Fig. 6R.tif



704 **Table 1** Primers used in this study

Primer	**Sequence (5′-3′)**	**Use**
Nc5 gene-specific	5′-ACT GGA GGC ACG CTG AAC AC-3′ 5′-AAC AAT GCT TCG CAA GAG GAA-3′	Quantitative PCR for measuring the parasite numbers according to the detection of *N. caninum* control DNA
Mouse GAPDH	5′-TGT GTC CGT CGT GGA TCT GA-3′ 5′-CCT GCT TCA CCA CCT TCT TGA T-3′	Internal control gene (housekeeping gene) for real-time RT-PCR analysis
Mouse IL-10	5′-CCT GGT AGA AGT GAT GCC CC-3′ 5′- TCC TTG ATT TCT GGG CCA TG-3′	Real-time PCR of mouse IL-10 mRNA expression
Mouse IFN-γ	5′-GCC ATC AGC AAC AAC ATA AGC GTC-3′ 5′-CCA CTC GGA TGA GCT CAT TGA ATG-3′	Real-time PCR of mouse IFN-γ mRNA expression
Mouse IL-4	5′-CAC GGA TGC GAC AAA AAT CA-3′ 5′ -CTC GTT CAA AAT GCC GAT GA-3′	Real-time PCR of mouse IL-4 mRNA expression
Mouse TNF-α	5′-GGC AGG TCT ACT TTG GAG TCA TTG C-3′ 5′-ACA TTC GAG GCT CCA GTG AA-3′	Real-time PCR of mouse TNF-α mRNA expression
Mouse CCL2	5′-GGC TCA GCC AGA TGC AGT TAA-3′ 5′-CCT ACT CAT TGG GAT CAT CTT GCT-3′	Real-time PCR of mouse CCL2 mRNA expression

Mouse CCL8	5′-ACC TCA AAC AGT TTG CCC CA-3′	Real-time PCR of mouse CCL8 mRNA expression
	5′-TTC ACA TTT GCC GAG TCC G-3′	
Mouse CXCL9	5′-ACC TCA AAC AGT TTG CCC CA-3′	Real-time PCR of mouse CXCL9 mRNA expression
	5′-TTC ACA TTT GCC GAG TCC G-3′	
Mouse CXCL10	5′-TGC CGT CAT TTT CTG CCT CA-3′	Real-time PCR of mouse CXCL10 mRNA expression
	5′-TCA CTG GCC CGT CAT CGA TAT-3′	

705

706 **Table 2.** Number of offspring per litter and offspring survival rates (30 days postpartum) at high dose of infection (10^6

707 tachyzoites/dam).

Groups	Number of litters (no. of used mice) , fertility rate	Mean number of offspring/litter (SD)	No. of surviving offspring /no. of offspring in each litter (Number of PCR positive offspring in each litter / number of analyzed samples in each litter)	Total no. of surviving offspring /total no. of offspring (%) (Total number of PCR positive offspring / total number of analyzed samples, %)
Negative control	5 (6) , 83.3%	6.4 (2.70)	0/2, 7/9, 0/8, 7/7, 6/6	20/32 (62.5%)
Nc1-infected	4 (5), 80%	6.5 (2.38)	2/8, 0/3, 0/7, 0/8 (3/4, 3/3, 7/7 ,7/7)	2/26 (7.7%) (20/21, 95.2%)
NcGRA7KO-infected	5 (5) , 100%	5.0 (3.67)	0/7, 0/8, 7/8, 0/1, 0/1 (5/5 ,3/6 ,4/8 ,1/1 ,1/1)	7/25 (28.0%) [#, *] (14/21, 66.7%) [#]

708

709 Mean number of offspring/litters in all experimental groups was analyzed with one-way ANOVA followed by the Tukey-Kramer post
710 hoc test, but no significant differences were observed. Mice were injected with RPMI-1640 medium as negative control of uninfected
711 dams. PCR was not performed to offspring from the negative control dams. Mortality rate of negative control was 37.5% (12/32). The
712 pregnant mice were used for analysis of fertility rate. The total number of PCR positive offsprings/ litters among infected groups were
713 analyzed with a χ^2 test, (# $P < 0.05$). Offspring survival rates at 30 days post-partum were analyzed with a χ^2 test, (* $P < 0.05$ against
714 uninfected mice, # $P < 0.05$ against Nc1-infected mice). SD, standard deviation.

715 **Table 3.** Number of offspring per litter and offspring survival rates (30 days postpartum) at the low dose of infection (10^5

716 tachyzoites/dam).

Groups	Number of litters (no. used mice), fertility rate	Mean number of offspring/litter (SD)	No. of surviving offspring/no. of offspring in each litter (Number of PCR positive offspring in each litter / number of analyzed samples in each litter)	Total no. of surviving offspring /total no. of offspring (Survival rate %) (Total Number of PCR positive offspring / total number of analyzed samples, %)
Negative control	4 (6) , 66.6%	5.0 (2.8)	5/5, 0/1, 7/7, 6/7	18/20 (90.0%)
Nc1-infected	5 (6) , 83.3%	7.2 (0.4)	2/7, 0/7, 5/8, 0/7, 0/7 (7/7, 7/7, 8/8, 7/7, 7/7)	7/36 (19.4%) # (36/36, 100.0%)
NcGRA7KO-infected	5 (7) , 71.4%	4.4 (2.3)	0/6, 5/5, 0/2, 0/2, 6/7 (5/5, 3/5, 2/2, 0/1, 4/7)	11/22 (50.0%)$^{*, \#}$. (14/20, 70.0%)$^{\#}$

717

718 Mean number of offspring/litters in all experimental groups was analyzed with one-way ANOVA followed by the Tukey-Kramer post
719 hoc test, but no significant differences were observed. Mice were injected with RPMI-1640 medium as negative control of uninfected
720 dams. PCR was not performed to offspring from the negative control dam. Mortality rate of negative control was 10 % (2/20). The
721 pregnant mice were used for analysis of fertility rate. The total number of PCR positive offsprings/ litters among infected groups were
722 analyzed with a χ^2 test, (# $P < 0.05$). Offspring survival rates at 30 days post-partum were analyzed with a χ^2 test (* $P < 0.05$ against
723 uninfected mice, # $P < 0.05$ against Nc1-infected mice). SD, standard deviation.

FIGURE LEGENDS

Fig. 1. Parasite virulence in mice under 1×10^6 parasite infection. Pregnant mice were infected with 1×10^6 *N. caninum* tachyzoites of the parental Nc1 strain, the NcGRA7-deficient parasite (NcGRA7KO), or they were injected with RPMI-1640 medium to represent the negative control on day 3.5 of pregnancy. **(A)** Survival rates in the dams were calculated until day 30 postpartum (45 days post infection) (negative control, 5/5, 100%; Nc1-infected, 4/4, 100%; NcGRA7KO-infected, 3/5, 60%). Statistically significant differences in the survival rates were analyzed with a χ^2 test but none were found. **(B)** Parasite burdens in brains of dams on day 30 postpartum. Values are the number of parasites in 50 ng of brain tissue DNA. Statistically significant differences between Nc1 and NcGRA7-infected groups were analyzed by Student's t-test, but none were found. **(C)** Kaplan Meier survival curve in the offspring was generated until day 30 postpartum (Survival rates: Negative control, 20/32, 62.5%; Nc1, 2/26, 7.7%; NcGRA7KO, 7/25, 28.0%). Statistically significant differences were analyzed with a χ^2 test (* $P < 0.05$). **(D)** Parasite burdens in the brains of offspring. Parasite numbers were measured in the brains of the dead and surviving offspring on day 30 postpartum. Values are the number of parasites in 50 ng of brain tissue DNA. Undetectable values were expressed as "0". Statistically significant differences were analyzed with a Mann–Whitney U test (* $P < 0.05$). (**E**) Parasite number in the brain of offspring corresponding to the day of death after birth from day 0 until 30 days post-partum. Parasite numbers in the brains of the dead and surviving offspring were measured. Values are the number of parasites in 50 ng of brain tissue DNA. Undetectable values were expressed as "0".

Fig. 2. Parasite virulence in mice under 1×10^5 parasite infection. Pregnant mice were infected with 1×10^5 *N. caninum* tachyzoites of the parental Nc1 strain or the NcGRA7-deficient parasite (NcGRA7KO), or they were injected with RPMI-1640 medium to represent the negative control on day 3.5 of pregnancy. **(A)** Survival rates in the dams were calculated until day 30 postpartum (45 days post infection) (negative control, 4/4, 100%; Nc1-infected, 5/5, 100%; NcGRA7KO-infected, 4/5, 80%). Statistically significant differences in the survival rates were analyzed with a χ^2 test but none were found. **(B)** Parasite burdens in the brains of the dams on day 30 postpartum. Values are the number of parasites in 50 ng of brain tissue DNA. Statistically significant differences between Nc1 and NcGRA7-infected groups were analyzed by a Mann–Whitney U test, but none were found. **(C)** Kaplan Meier survival curve in the offspring were calculated until

day 30 postpartum (Survival rates: Negative control, 18/20, 90.0%; Nc1, 7/36, 19.4%; NcGRA7KO, 11/22, 50.0%). Statistically significant differences were analyzed with a χ^2 test (* $P < 0.05$). **(D)** Parasite burdens in the brains of offspring. Parasite numbers were measured in the brains of the dead and surviving offspring on day 30 postpartum. Values are the number of parasites in 50 ng of brain tissue DNA. Undetectable values were expressed as "0". Statistically significant differences were analyzed with a Mann–Whitney U test (* $P < 0.05$). (**E**) Parasite number in the brain of offspring corresponding to the day of death after birth from day 0 until 30 days post-partum. Parasite numbers in the brains of the dead and surviving offspring were measured. Values are the number of parasites in 50 ng of brain tissue DNA. Undetectable values were expressed as "0".

Fig. 3. Histopathological analysis of *N. caninum* in fetoplacental tissue on day 13.5 of pregnancy (10 days post-infection). **(A)** The placenta was divided into three layers (decidua, junctional zone, and labyrinth). A representative image of hematoxylin and eosin staining (HE). Bar = 1 mm. HE staining and immunohistochemistry targeting of CD3 and Iba1 in the decidua **(B)** and junctional zone **(C)** of RPMI-1640 medium-injected (negative control), parental strain Nc1-infected, and NcGRA7-deficient (NcGRA7KO)-infected mice. **(B)** In decidua of Nc1-infected group, few T lymphocytes (arrow) and some macrophages (arrowhead) are infiltrated. In NcGRA7KO-infected group, T lymphocyte infiltration is more severe than Nc1-infected. Bar = 100 μm (C) In junctional zone, slight to mild inflammation were observed in both Nc1- and NcGRA7KO-infected groups. Bar = 100 μm, 3HPF: 3 high power fields.

Fig. 4. The number of T lymphocytes (A) and macrophages (B) in three high-power fields in three layers (decidua, junctional zone, and labyrinth) of placentas from RPMI-1640 medium-injected (negative control), parental strain Nc1-infected, and NcGRA7-deficient (NcGRA7KO)-infected mice. The immunohistochemistry analysis targeted CD3 (T lymphocytes) or Iba1 (macrophages) on day 13.5 of pregnancy (10 days post-infection). Negative control, n = 6; Nc1-infected, n = 6; NcGRA7KO-infected, n = 13. * Significant differences were analyzed by one-way ANOVA plus Tukey-Kramer hoc test ($P < 0.05$).

Fig. 5. Parasite burden in the placentas and fetuses of pregnant mice on day 13.5 of pregnancy and 10 days post-infection with 1×10^6 tachyzoites of Nc1 and NcGRA7-deficient (NcGRA7KO) parasites. **(A)** Values are the number of parasites in 50 ng of placental tissue DNA. (Nc1, n = 3; NcGRA7KO, n = 4). Statistically significant differences were analyzed by Student's t-test but none were found ($P < 0.05$). **(B)** Values are the number of parasites in 50 ng of tissue DNA from the body of each fetus (Nc1-infected, n = 16; NcGRA7KO-infected, n = 11). Statistically significant differences were analyzed by the Student's t-test, but none were found.

Fig. 6. Relative expression of TNF-α, CCL2, CCL8 and CXCL9 levels in the spleen (A) and placenta (B) of RPMI-1640 medium-injected pregnant mice (negative control) and pregnant mice on day 10 post-infection with 1×10^6 tachyzoites of Nc1 and NcGRA7-deficient (NcGRA7KO) parasites (day 13.5 pregnancy). Pooled placenta samples were used for each dam. Negative control, n = 5; Nc1-infected, n = 3; NcGRA7KO-infected, n = 4. Undetectable values are not shown. Statistically significant differences were analyzed by one-way ANOVA plus Tukey-Kramer post hoc test (* $P < 0.05$).