PLK: Δgra9 Live Attenuated Strain Induces Protective Immunity Against Acute and Chronic Toxoplasmosis

Jixu Li1,2,3, Eloiza May Galon3, Huanping Guo3, Mingming Liu3, Yongchang Li3, Shengwei Ji3, Iqra Zafar3, Yang Gao3, Weiqing Zheng3,4, Paul Franck Adjou Moumouni3, Mohamed Abdo Rizk3,5, Maria Agnes Tumwebaze3, Byamukama Benedicto3, Aaron Edmond Ringo3, Tatsunori Masatani6 and Xuenan Xuan3*

1 State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xining, China, 2 College of Agriculture and Animal Husbandry, Qinghai University, Xining, China, 3 National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan, 4 The Collaboration Unit for Field Epidemiology of State Key Laboratory for Infectious Disease Prevention and Control, Jiangxi Provincial key Laboratory of Animal-origin and Vector-borne Diseases, Nanchang Center for Disease Control and Prevention, Nanchang, China, 5 Department of Internal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt, 6 Transboundary Animal Diseases Research Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan

Toxoplasmosis is a zoonotic parasitic disease caused by the obligate intracellular protozoan *Toxoplasma gondii*, which threatens a range of warm-blooded mammals including humans. To date, it remains a challenge to find safe and effective drug treatment or vaccine against toxoplasmosis. In this study, our results found that the development of a mutant strain based on gene disruption of dense granule protein 9 (gra9) in type II PLK strain decreased parasite replication in vivo, severely attenuated virulence in mice, and significantly reduced the formation of cysts in animals. Hence, we developed an immunization scheme to evaluate the protective immunity of the attenuated strain of Δgra9 in type II PLK parasite as a live attenuated vaccine against toxoplasmosis in the mouse model. Δgra9 vaccination-induced full immune responses characterized by significantly high levels of pro-inflammatory cytokine interferon gamma (IFN-γ) and interleukin-12 (IL-12), maintained the high *T. gondii*-specific immunoglobulin G (IgG) level, and mixed high IgG1/IgG2a levels. Their levels provided the complete protective immunity which is a combination of cellular and humoral immunity in mouse models against further infections of lethal doses of type I RH, type II PLK wild-type tachyzoites, or type II PLK cysts. Results showed that Δgra9 vaccination proved its immunogenicity and potency conferring 100% protection against acute and chronic *T. gondii* challenges. Together, Δgra9 vaccination provided safe and efficient immune protection against challenging parasites, suggesting that PLK: Δgra9 is a potentially promising live attenuated vaccine candidate.

**Keywords:** Toxoplasma gondii, toxoplasmosis, live attenuated vaccine, PLK: Δgra9, protective immunity

INTRODUCTION

Toxoplasmosis is a zoonotic parasitic disease caused by the obligate intracellular protozoan *Toxoplasma gondii* (Montoya and Liesenfeld, 2004; Saadatnia and Golkar, 2012). *T. gondii* has the ability to infect all nucleated cells, and thus, has a broad host range of warm-blooded mammals, including humans (Loh et al., 2019). One-third of the global population is estimated...
to have *T. gondii* infection, most of which are asymptomatic in immunocompromised people, but causes severe complications in immunocompromised individuals and pregnant women (Tenter et al., 2000; Innes, 2010; Robert-Gangneux and Darde, 2012; Le Roux et al., 2020). Additionally, reproductive problems, i.e., abortion and stillbirth, induced by *T. gondii* infection in livestock presents a grave challenge to the animal industry (Robert-Gangneux and Darde, 2012; Wang et al., 2019). The ensuing public health problems and agricultural economic losses necessitate the search for and development of safe and effective drug treatments and vaccines against toxoplasmosis in humans and animals.

Despite unsparing research efforts in recent decades, treatment and vaccine options against toxoplasmosis are still limited, owing to the unique characteristics of *T. gondii*. For instance, *Toxoplasma* infection has multiple routes of transmission in humans or animals. One route is ingestion of raw or undercooked meat containing tissue cysts with bradyzoites which transmits the parasite to humans. Although a combination of pyrimethamine and sulfadiazine or other compounds has been used to treat active toxoplasmosis in animals or humans (Alday and Doggett, 2017; Dunay et al., 2018), there is no significant therapeutic efficiency on the bradyzoite residing within tissue cysts. Furthermore, *T. gondii* develops complex population structures, in which North America and Europe strains are classified into three major clonal lineages, type I, II, and III (Howe and Sibley, 1995; Loh et al., 2019). The composition of these complex strains will inevitably bring new challenges to the control of toxoplasmosis because of the different proliferative ability and degree of pathogenicity in mouse models. Therefore, due to the current unsatisfactory status in drug treatment of toxoplasmosis, such as the inability of eliminating tissue cysts, the development of a vaccine to control *Toxoplasma* infections caused by different strains and contracted through multiple routes has been a priority.

Several studies on *T. gondii* vaccines have been done and reported in animal models. Although compared with nucleic acid vaccines (Li et al., 2015; Zhang et al., 2015; Lu et al., 2017) and recombinant protein vaccines (El Bissati et al., 2014; Tanaka et al., 2014; Wang et al., 2014a,b), the higher protection against acute or chronic *T. gondii* infection provided by live attenuated vaccines (Fox and Bzik, 2015; Lagal et al., 2015; Abdelbaset et al., 2017; Wang et al., 2017, 2018; Xia et al., 2018b; Yang et al., 2019; Liang et al., 2020) was highlighted both in short- and long-term vaccination using different strains in the mouse model. However, the saveness of these mutants still needs to be tested in animal models. With the advent of the genomic era, the widespread application of the CRISPR/Cas9 system has permitted precise and efficient genetic manipulations in *T. gondii*, such as gene editing and gene deletion resulting in attenuated strains which can be functionally selected (Shen et al., 2017). Advantages of this system facilitate the development of a live attenuated vaccine with reduced virulence but retaining its ability for limited replication in order to induce an immune response, which is considered as the ideal vaccine for resisting toxoplasmosis (Wang et al., 2019).

Dense granule proteins (GRAs) play major functions within the structural formation of the parasitophorous vacuole (PV) and the cyst wall of *T. gondii* (Guevara et al., 2019). One of the GRAs, the dense granule protein 9 (GRA9), has been characterized in *T. gondii*. Recent studies reported that GRA9 was investigated as one of the intravacuolar-network-associated GRAs during cyst development *in vitro*, and loss of gra9 in type II Prugniaud (Pru) strain induced severe defects in the development of chronic-stage cysts *in vivo* (Fox et al., 2019; Guevara et al., 2019). More so, our previous study revealed that disruption of gra9 gene in *T. gondii* type II PLK strain significantly reduced the growth of tachyzoites *in vitro* (Guo et al., 2019). In the current study, we confirmed that the development of a mutant strain based on gene disruption of gra9 in type II PLK strain decreased parasite replication *in vivo*, severely attenuated virulence in mice, and significantly reduced the formation of cysts in animals. These suggest that Δgra9 could be considered a vaccine candidate. Hence, we developed an immunization scheme to evaluate the protective immunity of the attenuated strain of Δgra9 in type II PLK parasite as a live attenuated vaccine against toxoplasmosis in the mouse model. Results showed that Δgra9 vaccination proved its immunogenicity and potency with 100% protection against acute and chronic *T. gondii* challenge infections.

### MATERIALS AND METHODS

#### Animals and Parasite Strains

The recommendations in the Guide for the Care and Use of Laboratory Animals of Obihiro University of Agriculture and Veterinary Medicine, Japan were strictly followed. The protocol of this study was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine, Japan (permission numbers: 190246). Six-week-old female BALB/c mice were purchased from Clea Japan. All animals were housed in the animal facility of the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, with adequate temperature (25 ± 2°C) and luminosity (12-h light and 12-h dark) under specific pathogen-free conditions, and free access to food and water. All animals were used at least one week after habituation.

All procedures of pathogen experiments were carried out according to the guidelines of Obihiro University of Agriculture and Veterinary Medicine (permission number: 2018728). In this study, *T. gondii* type I (RH strain with hypoxanthine-xanthine-guanine phosphoribosyl transferase deficiency), and type II (PLK strain, which is a clone of ME49 strain) strain (Kirkman et al., 2001) were used. Mutant PLK:Δgra9 attenuated strain with green fluorescent protein (GFP) was generated and cultured in our laboratory, which has been passed for more than 250 generations (Guo et al., 2019). All parasites were cultured in human foreskin fibroblast (HFF) cells maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma), as previously described (Li et al., 2020).

#### Bradyzoite Differentiation *in vitro* of Δgra9 Strain

*Toxoplasma gondii* PLK wild-type (WT) or PLK:Δgra9 parasites were cultured in RPMI 1640 medium supplemented with 50 mM HEPES and 1% fetal bovine serum, pH 8.2, ambient...
CO₂ for 4 days, as previously described (Xia et al., 2018a). The parasites (2 × 10⁵ per well) forced to egress were allowed to invade cell monolayer (1 × 10⁵ per well) for 3 h, then washed, and each culture was allowed to grow under bradyzoite-inducing conditions for 24, 48, 72, and 96 h, as described above. Subsequently, samples were fixed by 4% paraformaldehyde. After permeabilizing with 0.3% Triton X-100/phosphate-buffered saline (PBS), samples were incubated with rabbit anti-SAG1 polyclonal antibody diluted to 1:500. The Alexa Fluor 594-conjugated goat anti-rabbit IgG (Life Technologies, Inc., United States) and Dolichos biflorus Agglutinin, FITC Conjugate (DBA-FITC) (Vector Laboratories, United States) were used to detect primary antibody and cyst wall. Samples were examined using the All-in-one Fluorescence Microscope (BZ-900, Keyence, Japan). Conversion rates were counted in at least 100 vacuoles, wherein DBA-FITC (green) was used for cyst wall staining and anti-SAG1 (red) antibody was used for tachyzoite marker. All assays were conducted in triplicate and repeated at least three times.

**Mutant Δgra9 Parasite Infection Tests in Mice**

To determine the virulence of PLK:Δgra9 in animals, six BALB/c mice were infected with a lethal dose (freshly egressed tachyzoites, 1 × 10⁵ per mouse) by intraperitoneal injection (i.p.). Daily observations of body weight and clinical signs were noted. Clinical scores ranged from 0 to 10, denoting presence of no signs or all signs, respectively. Evaluated clinical signs included hunching, piloerection, worm-seeking, behavior, ptosis, sunken eyes, ataxia, the latency of movement, deficient evacuation and touch reflexes, and lying on belly (Lesembun et al., 2016). Surviving mice were monitored for 30 days and blood was drawn at day 30 to confirm infection by an ELISA. Tissues were collected to determine parasite burdens through an examination of cyst wall. Samples were examined using the All-in-one Fluorescence Microscope (BZ-900, Keyence, Japan). Conversion rates were counted in at least 100 vacuoles, wherein DBA-FITC (green) was used for cyst wall staining and anti-SAG1 (red) antibody was used for tachyzoite marker. All assays were conducted in triplicate and repeated at least three times.

Vaccination of Mice and Immunogenicity Measurements

Mice were either vaccinated once with 10³ freshly harvested PLK:Δgra9 tachyzoites or mock-vaccinated in a total of 200 µl PBS i.p. 30 and 70 days post-infection (dpi), then, sera were collected to test total T. gondii-specific IgG and IgG subclasses (IgG1 and IgG2a) levels. Briefly, the 96-well ELISA plates were coated with 0.5 µg/ml soluble tachyzoite antigens of PLK parasites diluted in coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) and incubated at 4°C overnight. The ELISA plates were washed by PBS-T (0.05% Tween-20) three times, and then blocked with 3% BSA, then washed once. Collected sera were diluted by 1:50 and incubated for 1 h at 37°C. The plates were washed with PBS-T six times, the HRP conjugated goat antimouse IgG, IgG1 and IgG2a secondary antibodies were added and incubated for another 1 h at 37°C. After washing six times, ABTS (2, 2’-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate was used to develop the reaction and measure the results at OD 415 nm. All samples were analyzed three times. Meanwhile, cytokine productions IL-12, IL-10, and IFN-γ were determined using ELISA kits according to the manufacturer’s recommendations, as above.

**Cytokine Productions of Splenocytes by T. gondii Antigens Stimulation After Vaccination**

Vaccinated mouse spleens were isolated to test stimulating cytokine productions of splenocytes at 70 days post-vaccination (dpv), and unvaccinated mouse spleens were also collected to use as controls. In brief, the splenocytes were washed three times with RPMI 1640 (Sigma, United States), and hemolyzed in a lysing buffer (0.83% NH₄Cl and 0.01 M Tris-HCl, pH 7.2) for 5 min, then washed with RPMI 1640. The viability of the splenocytes was determined by trypan blue staining. A total of 3 × 10⁵ viable splenocytes each well of 96-well cell culture plates were plated and cultured in RPMI 1640 supplemented with 20% FBS maintained 24 h. The final concentration of 50 µg/ml of T. gondii soluble antigens (TSA) of PLK parasites were used to stimulate cytokine productions for 3 days. Then supernatants from each well were harvested for cytokine level measurements, as above. For negative and positive controls, the same number of splenocytes was also plated into 96-well cell culture plates at the same time and stimulated with RPMI 1640 with 20% FBS only or 5 µg/ml concanavalin A (Sigma, United States) for 3 days, respectively. Each spleen-harvested splenocytes were plated in at least three wells for TSA, negative and positive control, and each supernatant sample was tested three times.

**Protective Immunity Against T. gondii Challenges**

BALB/c mice were vaccinated with 10³ tachyzoites of PLK:Δgra9 through i.p. At 70 dpv, mice were challenged with 10² type I
RH or $10^5$ type II PLK tachyzoites by i.p., or 50 PLK cysts by oral administration (six mice for each group). Unvaccinated mice with same ages and numbers were used as control and infected with the same doses and routes. All challenged mice were monitored for another 30 days for tachyzoite-challenge or 35 days for cyst-challenge infections to record daily body weights, clinical signs, and survival rates in detail. Meanwhile, at 7 days tachyzoite post-challenges or 14 days cyst post-challenges, peritoneal fluid and serum samples of all experimental mice were collected to test cytokine productions. Parasite burdens were examined in peritoneal fluids during acute challenges at day 7 post-challenges. Furthermore, for chronic infection, the number of cysts in survival mouse brains was detected as above at 35 days cyst post-challenges.

### Passive Immunization Test of Δgra9-Vaccinated Sera

BALB/c mice were infected with $10^5$ type II PLK tachyzoites through i.p. At the day 0 and 3 post-infection, the 100 µl sera from naïve mice or the day 70 after PLK:Δgra9-vaccinated per mouse were administered into WT parasite infected mice by intraperitoneal injection, which includes four mice for naïve sera as negative control, and five mice for PLK:Δgra9-vaccinated sera as test group. Survival rates were recorded. Parasite burdens were examined in peritoneal fluids at day 5 or 7 post-infection by qPCR as follows to evaluate parasite growth under passive immunization.

### DNA Isolation and Quantitative PCR (qPCR) Detection of Parasite Burdens in Infected Mice

DNA was extracted from the tissues or peritoneal fluid of parasite-challenged mice by DNeasy Blood & Tissue Kit (Qiagen, Germany), according to the manufacturer’s instructions. The 50 ng DNA was then amplified with primers specific to the T. gondii B1 gene (forward primer 5′-AAC GGG CGA GTA GCA CCT GAG GAG-3′ and reverse primer 5′-TGG GTC TAC GTC GAT GGC ATG ACA AC-3′) by qPCR, as previously described (Li et al., 2020). A standard curve was constructed using 10-fold serial dilutions of T. gondii DNA extracted from $10^5$ parasites; thus, the curve ranged from 0.01 to 10,000 parasites. The parasite number was calculated from the standard curve.

### Statistical Analysis

To graph and analyze the data, GraphPad Prism 7 software (GraphPad Software Inc., United States) was used. In this study, statistical analyses were performed using unpaired Student’s t-test, Tukey’s Multiple Comparison Test, and one-way ANOVA plus Tukey-Kramer post hoc analysis. Data represent the mean ± Standard Error of Mean. Survival curves were generated using the Kaplan–Meier method and statistical comparisons were made by the log-rank method. A P value < 0.05 was considered statistically significant.

### RESULTS

#### Δgra9 Generation in Type II PLK Strain Markedly Reduces Cyst Formation in vitro and in vivo

Our previous study revealed that (Guo et al., 2019). To see whether a single mutant of Δgra9 affects bradyzoite differentiation from tachyzoite, WT or Δgra9 (with GFP) tachyzoites were cultured under bradyzoite-inducing conditions to count the conversion rates. Both strains formed cysts, as cysts were stained by DBA-FITC (green) (Figure 1A). However, compared with the cyst differentiation rate of WT parasites, Δgra9 reduced by 74.19, 94.04, 63.07, and 41.52% at 24, 48, 72, and 96 h post-induction, respectively (Figure 1B). In vivo, with the dose of $10^5$ tachyzoites infections, no mortalities were recorded until day 35 in mice infected with parasites either strains (data not shown), while as shown in Figure 1C, the cyst number in the brain of mice with Δgra9 infection significantly reduced by 73.68% compared with that of the PLK. Collectively, the loss of gra9 in type II PLK strain, did not abolish, but markedly reduced cyst formation in vitro and in vivo.

#### Δgra9 Generation in Type II PLK Strain Severely Attenuates Virulence in Mice

To evaluate the virulence of Δgra9, a lethal dose of $10^5$ parasites was injected into mice by i.p., with body weight and clinical signs monitored for 30 days. The parental PLK strain caused 100% mortality (Figure 2A) with severe body weight losses (Figure 2B) and clinical symptoms (Figure 2C), whereas infection with the mutant strain surprisingly proved to be unfatal to mice (Figure 2A), indicating that Δgra9 generation in type II PLK strain severely attenuated virulence in mice.

#### Δgra9 Infection Shows Different Cytokine Productions and Relatively High T. gondii-Specific IgG Levels Compared With Wild-Type Infection in Mice

Lethal type II strain infections are associated with extremely elevated pro-inflammatory cytokine levels in the serum, including IFN-γ and IL-12 (Mordue et al., 2001). In order to elucidate the differences in immune response caused by infection with Δgra9 or WT strains, mice were infected with a non-lethal dose of $10^3$ parasites. Sera were collected at 7 or 30 dpi to measure the changes in immune response. Levels of pro-inflammatory cytokines IFN-γ and IL-12, as well as anti-inflammatory cytokine IL-10, were significantly elevated at 7 and 30 dpi in both Δgra9 and WT infections compared with control (Supplementary Figures S1A–C). However, Δgra9-infected mice showed 39.17–54.05% lower serum cytokine levels compare with WT infections at 7 dpi, although not statistically significant (Supplementary Figures S1A–C). This suggests that loss of gra9 in PLK parasites led to milder cytokine productions resulting in complete mouse survival. Conversely, similarly high T. gondii-specific IgG levels were induced at 30 dpi after either PLK or mutant parasite
**FIGURE 1** | Disruption of gra9 in type II PLK strain reduces cyst formation in vitro and in vivo. (A) The bradyzoite differentiation from wild-type or Δgra9 with GFP tachyzoites under bradyzoite-inducing conditions in vitro. DBA-FITC (green) was used for cyst wall staining and anti-SAG1 (red) antibody was used for tachyzoite marker. Both strains formed cysts, as cysts were stained by DBA-FITC. The blue fluorescence showed the nuclear DNA staining by DAPI. (B) Conversion rates in vitro. Bradyzoite differentiation rates were counted in at least 100 vacuoles at 24, 48, 72, and 96 post-induced hours. The data are presented as the mean ± SEM of at least three independent experiments (***P < 0.0001; one-way ANOVA plus Dunnett’s multiple comparisons test). (C) The cyst number in the mouse brains of Δgra9 infection. The dose of 10^3 WT or Δgra9 tachyzoites were injected each mouse and were monitored for 35 days, and then the number of cysts in survival mouse brains were determined (n = 4; **P < 0.01; Student’s t-test).

**FIGURE 2** | Δgra9 generation in PLK strain severely attenuates the virulence in mice. A lethal dose of 10^5 parasites of wild-type or Δgra9 were infected into female BALB/c mice by intraperitoneal infection (n = 6), and mice were monitored for 30 days to note daily observations of body weight and clinical signs. (A) Survival rates (***P < 0.001; Log-rank (Mantel-Cox) test). (B) Body weight%. (C) Clinical scores. The scores varied from 0 (no signs) to 10 (all signs).

infection (Supplementary Figure S1D). At the cellular level, cytokine productions by splenocytes after T. gondii antigen stimulation was determined at 35 dpi, found that high levels of cytokine IFN-γ and IL-10 in Δgra9-infected splenocytes were induced quickly, which were slightly higher than the WT (Supplementary Figures S1E,F). Together, these results suggest
that Δgra9 infection induces balanced cytokine productions and relatively high T. gondii-specific IgG levels compared with WT infection in mice, which relates to the severely attenuated virulence of Δgra9 strain.

**Δgra9 Vaccination Induces Full T. gondii-Specific Immune Response**

The above findings reveal the fact that Δgra9 strain attenuated acute virulence and affected the formation of cysts. To evaluate the potential as a good vaccine of this mutant strain and test the immunogenicity derived from Δgra9 vaccination, we designed an immunization scheme within 105 days using mouse models (Figure 3A). At the outset, the chosen immune dose was 10³ Δgra9 tachyzoites each mouse by i.p. Results of our preliminary experiments showed that the lowly clinical score was observed in 10³ Δgra9-infected mice, whereas 10⁴ and 10⁵ Δgra9-infected or 10³, 10⁴, and 10⁵ PLK-infected mice showed severe clinical signs (Supplementary Figure S2). At 30 dpi, induced T. gondii-specific IgG in sera of 10³, 10⁴, and 10⁵ Δgra9- or 10³ and 10⁴ PLK-infected mice were of similarly high levels (Supplementary Figure S3). These represent similar immunogenicity and suggest that Δgra9 vaccination of 10³ tachyzoites was a safe and effective immune dose. After vaccination of 10³ Δgra9 tachyzoites, the immunogenicity of Δgra9 was tested through detection of specific anti-T. gondii IgG and IgG subclasses (IgG1 and IgG2a) levels in vaccinated mouse sera at 30 and 70 dpi. Unvaccinated (naïve) mice were used as control. The ELISA results showed that Δgra9 vaccination induced a significantly higher T. gondii-specific IgG level at 30 dpi, and maintained a similarly high level at 70 dpi (Figure 3B). Nest study was to test the levels of IgG subclasses, the results showed that compared to unvaccinated mice, the level of IgG2a in vaccinated mice was significantly higher at 30 and 70 dpi (Figure 3B). To test IgG1 level in mice, although the level was lower at 30 dpi compared with 70 dpi, it was also increased to significantly higher in the vaccinated mice both 30 and 70 dpi than unvaccinated mice (Figure 3B). These suggest that Δgra9 vaccination elicits a mixed Th1/Th2 immune response both 30 and 70 dpi. Opposite to the stable IgG levels, cytokine levels changed over time. Relatively higher levels of pro-inflammatory IFN-γ and IL-12, or anti-inflammatory IL-10 were recorded from vaccinated mice compared with unvaccinated mice at 30 dpi, while levels decreased at 70 dpi (Figures 3C–E). Collectively, these results reveal that Δgra9 vaccination provided a benign humoral and cellular immune response in mice and proved to induce effective immunogenicity.

To assess the immunological memory in Δgra9 vaccinated mice, splenocytes were harvested from vaccinated or unvaccinated mice at 70 dpi, and stimulated with total Toxoplasma soluble antigen (TSA) prepared from fresh wild-type (PLK) tachyzoites. As shown in Figures 4A,B, the significantly high levels of pro-inflammatory cytokine IFN-γ, as well as anti-inflammatory cytokine IL-10, were stimulated by TSA compared with no stimulation or no vaccination. Interestingly, although the significantly decreased levels of stimulated IFN-γ (16,787.5 pg/ml) and IL-10 (5,347.5 pg/ml) at 70 dpi were observed compared with their levels of IFN-γ (207,667.7 pg/ml) and IL-10 (10,210.4 pg/ml) at 30 dpi in the Δgra9 vaccinated splenocytes (Supplementary Figures S1E,F), there were obviously high levels at both time points. These suggest that Δgra9 vaccination could activate the ability of the immune cells to quickly and specifically recognize the Toxoplasma antigen to produce corresponding immune cytokines in short-term and long-term immunization.

**Δgra9 Vaccination Confers Potent for Protection Against Acute and Chronic T. gondii Challenges**

Based on the findings that Δgra9 has strong immunogenicity and immunological memory, we then preformed the second challenges with 10³ type I (RH) or 10⁵ type II (PLK) tachyzoites by i.p. or 50 cysts (PLK) by oral administration to vaccinated mice at 70 dpi. All challenged mice were monitored for another 30 days for tachyzoite or 35 days for cyst infections, and recorded daily body weights, clinical signs, and survival rates. For both RH (Figure 5A) and PLK (Figure 5B) tachyzoite challenges, Δgra9 vaccination elicited strong protection following 100% survival rates of mice and no obvious signs or body weight changes were observed during the 30-day challenge period (Supplementary Figure S4). However, for unvaccinated mice challenged with the lethal dose of RH or PLK tachyzoites, the clinical signs and body weight changes were initially observed at day 4 of RH or day 2 of PLK infections until the signs developed to most severe at 6-8 days (Supplementary Figure S4), resulting in 100% mortality rates for unvaccinated mice within 8 days post-challenges (Figures 5A,B). As expected, all Δgra9-vaccinated mice survived when infected with 50 cysts (PLK strain), whereas only 50% of unvaccinated mice survived (Figure 5C). While the 50 cysts-challenges to vaccinated or unvaccinated mice led to decreased body weights (%) in both groups during the whole period of 35 days, clinical signs were observed only in unvaccinated mice starting from day 8 post-challenge infection (Supplementary Figure S4). Altogether, Δgra9 vaccination confers strong protective immunity against acute and chronic toxoplasmosis.

**Δgra9 Vaccination Rapidly Clears Challenging Parasites and Blocks Cyst Formation in New Challenges**

To further understand how Δgra9 vaccination provided strong protection in mice suffering both acute and chronic T. gondii challenges, peritoneal fluids and sera in challenged mice were collected at 7 days tachyzoite post-challenges or 14 days cyst post-challenges (time-points when we observed the most serious signs in unvaccinated mice) to determine cytokine productions, as well as parasite burdens in peritoneal fluids. In naïve mice, RH infections resulted in rapid proliferation with 87,305 parasites in 50 ng DNA, whereas PLK infections caused higher parasite burdens of 2.74 × 10⁷ at 7 days post-challenge infection (Figure 6A). However, we could not detect any parasites using a qPCR test in both RH- and PLK- challenged Δgra9-vaccinated mice, suggesting that Δgra9 vaccination promoted the activity to rapidly clear infecting parasites.
Immunization scheme and study design. (A) Immunization scheme. The immune dose of $10^3$ Agra9 tachyzoites each mouse was injected into female BALB/c mice ($n = 6$ each group) by intraperitoneal injection and that day was designated as 0 day post-vaccination. The immunogenicity of Agra9 vaccination was tested via the detection of specific anti- $T. gondii$ IgG levels and cytokine productions in sera at 30 and 70 dpv. At 70 dpv, vaccinated or unvaccinated mice were secondly challenged by acute or chronic $T. gondii$ infection to assess the protection. Within the vaccination and challenge period of total 105 days, the daily body weights, clinical signs, and survival rates of all mice were recorded in detail, and peritoneal fluid or serum samples were collected to evaluate immune response at the limited sampling periods as shown in (A). (B) The specific anti- $T. gondii$ IgG and IgG subclasses (IgG1 and IgG2a) levels in vaccinated mice at 30 or 70 dpv. Unvaccinated naïve mice with the same ages were used as control (****$P < 0.0001$; Student’s t-test). (C–E) Cytokine productions in sera at 30 and 70 dpv. The levels of IFN-$\gamma$ (C), IL-12p70 (D), or IL-10 (E) were determined by ELISA kits. –, unvaccinated mice; +, vaccinated mice (*$P < 0.05$; **$P < 0.01$; ns, not significant; Student’s t-test).

Toxoplasmosis, parasites were not detected in any mouse peritoneal fluid at 14 dpi (data not shown), but markedly reduced number of cysts in vaccinated survivor mouse brain was noted by day 35 compared with unvaccinated mice (Figure 6B), which are similar to that level of cyst formation of vaccinated but no cyst challenged mice (Figure 1C). This suggests that Agra9 vaccination blocks cyst formation in new challenged cysts.

Subsequently, the immune responses were also tested during the limited time-points, levels of cytokines (IFN-$\gamma$, IL-12, and IL-10) in both peritoneal fluid and serum were substantially induced in unvaccinated mice relative to the extremely low levels in Agra9-vaccinated mice, especially the IFN-$\gamma$ levels (Figure 7A). More importantly, all mice remained with high levels of $T. gondii$-specific IgG (Figure 7B). Taken together, Agra9 vaccination provided efficient and safe immune protection to kill challenging parasites, resulting in host survival.

**A Special Protection Against $T. gondii$ Infection Is Provided by the Sera of Agra9-Vaccinated Mice**

Agra9 vaccination induced a significantly high $T. gondii$-specific IgG level with low cytokine levels at 70 dpv in mouse sera as shown above. In this study, the sera from PLK:Agra9-vaccinated mice were administered into parasites infected mice with lethal dose, survival rates were recorded and parasite burdens were determined in peritoneal fluids at day 5 and 7 post-infection to evaluate parasite growth under passive immunization. The
FIGURE 4 | Δgra9 vaccination activates the ability of the splenocytes to rapidly and specifically recognize the Toxoplasma antigen to induce high-level cytokines, compared with unvaccinated. Immunological memory of mice in Δgra9 vaccination was evaluated at 70 dpv via stimulated splenocytes by total Toxoplasma soluble antigen resulting in the production of cytokines IFN-γ (A) or IL-10 (B). RPMI 1640 with 20% FBS only or 5 µg/ml concanavalin A were used as negative or positive controls, respectively. The data are presented as the mean ± SEM of at least three repeats each sample (***P < 0.0001; Student’s t-test).

FIGURE 5 | Δgra9 vaccination protects mice against T. gondii infections. Vaccinated or unvaccinated mice were challenged with 10³ type I RH (A) or 10⁵ type II PLK (B) tachyzoites by intraperitoneal injection or 50 PLK cysts (C) by oral administration at 70 dpv, and monitored for another 30 days for tachyzoite or 35 days for cyst infections to note survival rates (***P < 0.001; Log-rank (Mantel-Cox) test).

FIGURE 6 | Δgra9 vaccination rapidly clears challenging parasites and blocks cyst formation in new challenges. (A) Parasite burdens in peritoneal fluids of RH or PLK tachyzoites-challenged vaccinated mice. A quantitative PCR of the TgB1 gene using 50 ng extracted DNA from peritoneal fluids was used to confirm parasite proliferation in vaccinated mice at 7 days post-challenge infection, compared with unvaccinated challenged mice (****P < 0.0001; Student’s t-test). (B) No. of cyst in brain in PLK cysts-challenged vaccinated mice. At day 35 post-challenges, the brains were isolated from survival mice of 50 cysts challenges to estimate cyst numbers by DBA-FITC staining (**P < 0.01; Student’s t-test).

results showed that vaccinated sera gave 40% survival rates (n = 5, Figure 8A) and led to significantly lower parasite burdens in peritoneal fluids of WT parasites-infected mice both 5 and 7 dpi, compared with naïve sera (Figure 8B). As shown in Figure 8B, the numbers of parasite in control mice at 5 or 7 day post-infection were resulted in 5 or 4 times of mice injected with vaccinated sera, respectively. These suggest that the sera of Δgra9-vaccinated mice with high IgG and low cytokine levels are able to reduce parasite propagation in mice.

DISCUSSION

In recent years, active immunization is considered to be the ideal and long-term strategy to induce the host immune response against acute and chronic T. gondii infections (Loh et al., 2019). One means is to develop live attenuated vaccines, a whole parasite-based vaccine, which is the live strain with reduced replication and attenuated virulence but retaining the ability to induce an immune response against a variety of wild-type strain...
FIGURE 7 | Δgra9 vaccination provides safe and effective immune protection. The peritoneal fluids and sera in challenged mice were collected at 7 days tachyzoite post-challenges or 14 days cyst post-challenges to determine cytokine productions and T. gondii specific IgG, compared with unvaccinated but secondly challenged mouse samples. (A) IFN-γ, IL-12p70, or IL-10 levels in serum or peritoneal fluid samples. –, unvaccinated; +, vaccinated (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not significant; Student’s t-test). (B) The levels of T. gondii specific IgG. IgG levels in vaccinated challenged or unvaccinated challenged mice at 7 days tachyzoite post-challenges or 14 days cyst post-challenges were determined by an ELISA test. –, unvaccinated; +, vaccinated (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; Student’s t-test).
The sera from \(\Delta\text{gra9}\)-vaccinated mice are able to reduce parasite propagation in mice. The sera from PLK:\(\Delta\text{gra9}\)-vaccinated mice were administered into \(10^5\) PLK parasites infected mice at 0 and 3 day post-infection, survival rates were noted and parasite burdens were determined in peritoneal fluids at day 5 and 7 post-infection to evaluate parasite growth under passive immunization. The four mice for naïve sera as negative control, and five mice for PLK:\(\Delta\text{gra9}\)-vaccinated sera as test group. (A) Survival rates (*\(P < 0.05\); Log-rank (Mantel-Cox) test). (B) Parasite burdens (***\(P < 0.0001\); Student’s t-test).
II cyst challenge and reduction of cyst formation. Interestingly, IFN-γ was cytokine which had significantly different levels between vaccinated and unvaccinated mice at day 14 post-cyst-challenge, suggesting that IFN-γ is also central to the development of immunity against T. gondii cyst infection, consistent with Δgra9-vaccination-induced immune response for protection of cyst challenge.

CONCLUSION

In conclusion, the present study demonstrated that loss of gra9 in type II PLK strain dramatically attenuated virulence and significantly reduced the formation of cysts in animals. Δgra9 vaccination effectively elicited immune responses which conferred absolute protection from subsequent lethal type I RH, type II PLK wild-type tachyzoites or type II PLK cysts challenge infections in mice, suggesting that the mutant Δgra9 of T. gondii type II PLK strain is a potential live-attenuated vaccine candidate against acute and chronic toxoplasmosis. However, the current study is limited to the mouse model; thus, the effectiveness and safety should likewise be extensively investigated in animals of veterinary and economic importance, including but not limited to definitive hosts cats, susceptible sheep and pigs and other meat-producing animals. Future studies should assess its effectiveness against other Toxoplasma strains, particularly infections with local endemic strains.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The Ethics Statement of Animal Experiments was reviewed and approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine, Japan (permission numbers: 190246).

AUTHOR CONTRIBUTIONS

JL and XX conceived and designed the experiments. JL, EG, HG, ML, YL, and SJ performed the experiments. JL, IZ, YG, and WZ analyzed the data and wrote the manuscript. PA, MR, MT, BB, AR, and TM critically revised the manuscript. All authors read and approved the final version of the manuscript.

REFERENCES

Abdelbaset, A. E., Fox, B. A., Karram, M. H., Abd Ellah, M. R., Bzik, D. J., and Igarashi, M. (2017). Lactate dehydrogenase in Toxoplasma gondii controls virulence, bradyzoite differentiation, and chronic infection. PLoS One 12:e0173745. doi: 10.1371/journal.pone.0173745

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.619335/full#supplementary-material

Supplementary Figure 1 | Δgra9 infection induces mild cytokine productions and relatively high T. gondii-specific IgG levels in mice compared with Wild-type PLK infection. A non-lethal dose of 10^3 parasites was chosen to infect BALB/c mice, and the sera were collected at day 7 or 30 post-infection to measure immune response. Naive mouse sera were used as controls. The levels of pro-inflammatory cytokines IFN-γ (A) and IL-12p70 (B) or anti-inflammatory cytokine IL-10 (C) were assessed by an ELISA test (n = 4; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; Control vs parental or Δgra9 at 7 dpi; 5P < 0.05, control vs parental or Δgra9 at 30 dpi; one-way ANOVA plus Tukey-Kramer post hoc analysis). (D) The T. gondii-specific IgG levels were measured at day 7 or 30 post-infection compared with naive mice (**P < 0.0001; Student’s t-test). (E,F) Cytokine productions by splenocytes after T. gondii antigen stimulation. At 35 dpi, the splenocytes were harvested from parental or Δgra9 infected mouse spleens, and 3 × 10^6 viable splenocytes were cultured in vitro and stimulated by T. gondii antigen to induce cytokine IFN-γ (E) or IL-10 (F) productions. For the negative and positive controls, the same number of splenocytes was cultured at same time and stimulated with RPMI 1640 with 20% FBS only or 5 µg/ml concanavalin A, respectively (**P < 0.0001; Student’s t-test).

Supplementary Figure 2 | Clinical symptoms of mice during parasite infection. The different dose of 10^3, 10^4, and 10^5 Δgra9- or 10^3, 10^4, and 10^5 T. gondii-specific IgG in sera from PLK-parasites were injected into mice, and clinical signs of infected mice were noted within 30 days post-infection. The clinical scores varied from 0 (no signs) to 10 (all signs). No-infected mice were used as control.

Supplementary Figure 3 | Δgra9 vaccination protects mice against T. gondii infections. Vaccinated or unvaccinated mice were challenged with 10^3 type I RH or 10^4 type II tachyzoites by intraperitoneal injection or 50 PLK cysts by oral administration at 70 dpv, and monitored for another 30 days for tachyzoite or 35 days for cyst infections to note daily body weights (A–C) and clinical signs (D–F) in detail.

Alday, P. H., and Doggett, J. S. (2017). Drugs in development for toxoplasmosis: advances, challenges, and current status. Drug Des. Devel. Ther. 11, 273–293.

Dunay, I. R., Gajurel, K., Dhakal, R., Liesenfeld, O., and Montoya, J. G. (2018). Treatment of toxoplasmosis: historical perspective, animal models, and current clinical practice. Clin. Microbiol. Rev. 31:e0057–17. doi: 10.1128/CMR.0057-17

El Bissati, K., Zhou, Y., Dasgupta, D., Cobb, D., Dubey, J. P., Burkhard, P., et al. (2014). Effectiveness of a novel immunogenic nanoparticle platform for...
Toxoplasma peptide vaccine in HL-A transgenic mice. Vaccine 32, 3243–3248. doi: 10.1016/j.vaccine.2014.03.092
Fox, B. A., and Bzik, D. J. (2015). Nonreplicating, cyst-destroyive type II Toxoplasma gondii vaccine strains stimulate protective immunity against acute and chronic infection. Infect. Immun. 83, 2148–2155. doi: 10.1128/IAI.02566-14
Fox, B. A., Guevara, R. B., Rommereim, L. M., Falla, A., Bellini, V., Pétre, G., et al. (2019). Toxoplasma gondii parasitophorous vacuole membrane-associated dense granule proteins orchestrate chronic infection and GRA12 underpins resistance to host gamma interferon. mBio 10:e0589-19.
Guevara, R. B., Fox, B. A., Falla, A., and Bzik, D. J. (2019). Toxoplasma gondii intravascular-network-associated dense granule proteins regulate maturation of the cyst matrix and cyst wall. mSphere 4:e0487-19. doi: 10.1128/mSphere.00487-19
Guo, H., Gao, Y., Jia, H., Moumouni, P. F. A., Masatani, T., Liu, M., et al. (2019). Characterization of strain-specific phenotypes associated with knockout of dense granule protein 9 in Toxoplasma gondii. Mol. Biochem. Parasitol. 229, 53–61.
Howe, D. K., and Sibley, L. D. (1995). Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease. J. Infect. Dis. 172, 1561–1566. doi: 10.1093/infdis/172.6.1561
Hunter, C. A., and Sibley, L. D. (2012). Modulation of innate immunity by Toxoplasma gondii virulence effectors. Nat. Rev. Microbiol. 10, 766–778. doi: 10.1038/nrmicro2855
Huskinson-Mark, J., Araujo, F. G., and Remington, J. S. (1991). Evaluation of the effect of drugs on the cyst form of Toxoplasma gondii. J. Infect. Dis. 164, 170–171. doi: 10.1093/infdis/164.1.170
Innes, E. A. (2010). A brief history and overview of Toxoplasma gondii. Zoonoses Public Health 57, 1–7. doi: 10.1111/j.1863-2378.2009.01276.x
Kirkman, L. A., Weiss, L. M., and Kim, K. (2001). Cyclic nucleotide signaling in Toxoplasma gondii bradyzoite differentiation. Infect. Immun. 69, 148–153.
Lagal, V., Dinis, M., Cannella, D., Bargieri, D., Gonzalez, V., Andenmatten, N., et al. (2015). AMA1-deficient Toxoplasma gondii parasites transiently colonize mice and trigger an innate immune response that leads to long-lasting protective immunity. Infect. Immun. 83, 2475–2486. doi: 10.1128/IAI.02666-14
Le Roux, D., Djikic, V., Morisse, S., Chauvin, C., Doré, V., Lagré, A. C., et al. (2020). Evaluation of immunogenicity and protection of the Mic-1-3 knockout Toxoplasma gondii live attenuated strain in the feline host. Vaccine 38, 1457–1466.
Leesombun, A., Boonmasawai, S., Shimoda, N., and Nishikawa, Y. (2016). Effects of extracts from Thai Piperaceae plants against infection with Toxoplasma gondii. PLoS One 11:e015616. doi: 10.1371/journal.pone.015616
Li, J., Guo, H., Galon, E. M., Gao, Y., Lee, S. H., Liu, M., et al. (2020). Hydroxylamine and carbosynthymoxaline can inhibit Toxoplasma gondii growth through an aspartate aminotransferase-independent pathway. Antimicrob. Agents Chemother. 64:e01889-19.
Li, X. Z., Wang, X. H., Xia, L. I., Weng, Y. B., Hernandez, J. A., Tu, L. Q., et al. (2015). Protective efficacy of recombinant canine aviendrine virus type-2 expressing TgROP18 (CAV-2-TgROP18) against acute and chronic Toxoplasma gondii infection in mice. BMC Infect. Dis. 15:114. doi: 10.1186/s12879-015-0815-1
Liang, Q. L., Sun, L. X., Elsheikha, H. M., Cao, X. Z., Nie, L. B., Li, T. T., et al. (2020). RHγAγα17Δnpt1 strain of Toxoplasma gondii elicits protective immunity against acute, chronic and congenital toxoplasmosis. J. Infect. Dis. 218, 768–777.
Li, J. L., Zhang, N. Z., Li, T. T., He, J. J., Elsheikha, H. M., and Zhu, X. Q. (2019). Advances in the development of anti-Toxoplasma gondii vaccines: Challenges, opportunities, and perspectives. Trends Parasitol. 35, 239–253.
Xia, N., Yang, J., Ye, S., Zhang, L., Zhou, Y., Zhao, J., et al. (2018a). Functional analysis of Toxoplasma lactate dehydrogenases suggests critical roles of lactate fermentation for parasite growth in vivo. Cell Microbiol. 20,10.
Xia, N., Zhou, T., Liang, X., Ye, S., Zhao, P., Yang, J., et al. (2018b). A lactate fermentation mutant of Toxoplasma stimulates protective immunity against acute and chronic toxoplasmosis. Front. Immunol. 9:1814. doi: 10.3389/fimmu.2018.01814
Yang, W. B., Wang, J. L., Gui, Q., Zou, Y., Chen, K., Liu, Q., et al. (2019). Immunization with a live-attenuated RHΔNPT1 strain of Toxoplasma gondii induces strong protective immunity against toxoplasmosis in mice. Front. Microbiol. 10:1875. doi: 10.3389/fmicb.2019.01875
Yarovinsky, F. (2014). Innate immunity to Toxoplasma gondii infection. Nat. Rev. Immunol. 14, 109–121. doi: 10.1038/nri3598
Zhang, N. Z., Xu, Y., Wang, M., Petersen, E., Chen, J., Huang, S. Y., et al. (2015). Protective efficacy of two novel DNA vaccines expressing Toxoplasma gondii rhomboid 4 and rhomboid 5 proteins against acute and chronic toxoplasmosis in mice. Expert. Rev. Vacc. 14, 1289–1297. doi: 10.1517/14766584.2015.1061938

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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