Novel roles of dense granule protein 12 (GRA12) in *Toxoplasma gondii* infection

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ABBREVIATIONS: ALD, anti-aldolase; FBS, fetal bovine serum; GRA, dense granule; GRAs, dense granule proteins; GBPs, guanylate binding proteins; HFFs, human foreskin fibroblasts; IVN, intravacuolar network; Igra6, immunity-related GTPase a6; IRGs, immunity-related GTPase; NHEJ, non-homologous end joining; PBS, phosphate buffered saline; PMSF, phenylmethanesulfonyl fluoride; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; ROP, rhoptry

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ABSTRACT: Dense granule protein 12 (GRA12) is implicated in a range of processes related to the establishment of *Toxoplasma gondii* infection, such as the formation of the intravacuolar network (IVN) produced within the parasitophorous vacuole (PV). This protein is thought to be important for *T. gondii*-host interaction, pathogenesis, and immune evasion, but their exact role remains unknown. In this study, the contributions of GRA12 to the molecular pathogenesis of *T. gondii* infection were examined *in vitro* and *in vivo*. Deletion of GRA12 in type I RH and type II Pru *T. gondii* strains although did not affect parasite growth and replication *in vitro,* it caused a significant reduction in the parasite virulence and tissue cyst burden *in vivo. T. gondii* Δ*gra12* mutants were more vulnerable to be eliminated by host immunity, without the accumulation of immunity-related GTPase a6 (Igra6) onto the PV membrane. The ultrastructure of intravacuolar network (IVN) in Δ*gra12* mutants appeared normal, suggesting that GRA12 is not required for the IVN biogenesis. Combined deletion of GRA12 and ROP18 induced more severe attenuation of virulence compared to single Δ*gra12* or Δ*rop18* mutant strains, suggesting that GRA12 may act synergistically with ROP18 in controlling *T. gondii* virulence. Collectively these findings indicate that although GRA12 is not essential for parasite growth and replication *in vitro*, it contributes to the virulence and growth of *T. gondii* in mice.

KEY WORDS: *Toxoplasma gondii* ·dense granule protein 12 ·rhoptry protein 18 ·intravacuolar network ·virulence ·immunity-related GTPases

Toxoplasma gondii, an intracellular protozoan parasite of veterinary and human importance, causes toxoplasmosis, which can have serious health consequences in pregnant women in immunocompromised humans (1, 2). *T. gondii* is capable of infecting virtually any type of nucleated cell in warm-blooded animals and has been reported to infect up to one-third of the global human population (1, 2). The manipulation of host cells to enable invasion and evasion of the host immune defenses, and remodeling the host cellular environment, are examples of mechanisms that this parasite can employ to sustain its intracellular survival (3-5). This parasite also influences host cell signaling and gene expression, alters vesicle trafficking and host cell cytoskeleton, and rearranges host cell organelles, such as the endoplasmic reticulum, lysosome and mitochondria (5, 6), mainly through the secretion and export of *T. gondii* effector proteins including more than 50 protein kinases and pseudokinases (7, 8).

T. gondii lytic cycle, which is essential for parasite survival within its host and proliferation of infection, starts with an active invasion of the parasite into the host cell, a process that is mediated by the sequential secretion of proteins from three specialized secretory organelles, namely micronemes, rhoptries and dense granules (9). Initially, the micronemes secrete a number of proteins containing adhesive domains, which facilitate parasite attachment to the host cell surface. Second, the rhoptries secrete a large number of proteins from the neck region that enable host cell penetration and vacuole formation (10). Additionally, invasion initiates the formation of a nonfusogenic parasitophorous vacuole (PV), a structure that separates the parasite from the hostile host intracellular environment (11,12). The PV membrane (PVM) forms the physical interface between parasite and host cytoplasm, and creates a niche for parasites replication and survival (13). Disruption of the PVM by host immune defenses causes parasite death (14). As a counter mechanism, the parasite secretes effector proteins to maintain the structural stability of PVM (4).

PVM-localized proteins secreted from two major organelles: the dense granules (GRAs) and

the rhoptries (ROPs), which act as effector proteins and subvert host cellular functions by either interacting with the PVM to protect *T. gondii* against host deleterious defense and clearance mechanisms, or crossing the PVM and translocating to nucleus to regulate gene expression (4, 5, 11, 15, 16). The kinase ROP18 and pseudokinase ROP5 function together to inactivate host immunity-related GTPase (IRGs) by phosphorylation, thereby preventing degradation of the PV (17-19). Recent studies showed that ROP17 and GRA7 are associated with ROP5/ROP18 complex to target host IRGs reducing parasite clearance and thus promoting parasite survival in mice (20-22). In addition, ROP5 and ROP18 also modulate the innate immune loading of guanylate binding proteins (GBPs) in mice, as is the pseudokinase ROP54 (23, 24). GRA15 localizes to the host cytosolic face of the PVM where it activates the host NF-κB pathway in a strain-dependent manner to induce a protective immune response against the parasite (25). GRA18 traffics across the PVM to reach the cytoplasm of infected host cells where it forms complexes with host components of the β-catenin destruction complex to regulate host gene expression in a β-catenin-dependent fashion (26). GRA16 and GRA24 are injected into the host cytosol and traffic to the host nucleus to regulate expression of specific sets of genes (27, 28).

Within the PV, the intravacuolar network (IVN) of membranous tubules of 20-50 nm diameter unfolds throughout the vacuolar space, and the tubules is topologically contiguous with the host cytosol (29, 30). The IVN is decorated by parasite secretory proteins secreted from the dense granules. The GRA2 and GRA6 are required for IVN biogenesis and deletion of either GRA2 or GRA6 results in a complete loss of the well-structured membranous tubules in vacuoles (31). Disruption of either GRA2 or GRA6 did not affect parasite growth in *in vitro* cell culture, but results in a dramatically reduced virulence and a lower tissue cyst burden in a mouse model *in vivo* (32-34).

GRA12, a novel dense granule protein associated with the intravacuolar membranous nanotubular network in the PV, is co-localized with GRA2 and GRA6, however its function is

unknown (35). In the present study, we investigated the functions of GRA12 by deleting this gene in type I RH and type II Pru strains. Our results showed that unlike the GRA2- or GRA6-deficient parasites, parasites that lack GRA12 grow within vacuoles with normal ultrastructure of the IVN. While the *in vitro* growth of GRA12-deficient parasites was not affected, their virulence and tissue cyst burden in mice were significantly reduced. In addition, parasites lacking the GRA12 protein were more susceptible to be eliminated by host immunity, however, immunity-related GTPase vacuole recruitment assay showed that the localization of Igra6 onto the PV of Δ*gra12* parasites was not increased. Combined deletion of GRA12 and ROP18 rendered the parasite avirulent, suggesting that GRA12 may act synergistically with ROP18 in controlling parasite virulence in mice.

MATERIALS AND METHODS

Ethics statement

The study was approved by The Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences and all animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China. All efforts were made to minimize the number of mice and their suffering. Animal experiments were performed with 7- to 8-week-old female Kunming or C57BL/6 mice obtained from Lanzhou University Laboratory Animal Center, Lanzhou, China. Mice were housed under a 12-h light/dark cycle in a climate-controlled room and had free access to sterilized water and food *ad libitum*.

Parasite culture and purification

Tachyzoites of *T. gondii* type I RH and type II Pru strains were maintained in monolayers of human foreskin fibroblasts (HFFs) cultured in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 2% fetal bovine serum (FBS), as previously described (36). To isolate tachyzoites, heavily infected HFF monolayers of late-stage vacuoles containing large number of replicating tachyzoites were scraped and lysed through a 26-gauge needle, and residual host cell material was removed by filtration through 3-μm polycarbonate membranes.

Construction of knockout strains

Mutant strains were generated using the CRISPR-Cas9 approach. All plasmids and primers used in this study are listed in **Table S1** in the supplemental materials. GRA12 or ROP18-specific CRISPR plasmids were constructed by replacing the UPRT targeting guide RNA in pSAG1-Cas9-sgUPRT with corresponding guide RNAs, as previously described (36, 37). The pGRA12::DHFR plasmid was constructed by ligating the gene of the 3' and 5' regions flanking the GRA12 amplified from *T. gondii* genomic DNA as well as the DHFR gene amplified from pUPRT-DHFR-D plasmid into pUC19 using the Gibson assembly kit. It was used as the homologous template to replace the coding region of GRA12 with DHFR. The Ble gene was amplified from pSAG1-Ble plasmid and pROP18::Ble constructed in a similar way was used to disrupt the coding region of ROP18. To generate gene knockouts in the desired strains, corresponding gene specific CRISPR plasmid and homology construct were co-transfected into freshly egressed tachyzoites, as previously described (36). Subsequently transfectants were selected with either pyrimethamine (for DHFR) or phleomycin (for Ble) as previously described (36, 38). Diagnostic PCRs, Western blotting and immunofluorescence assays were used to check the disruption of the corresponding genes.

Total RNAs were isolated from the type I RH or type II Pru strain using the TRIzol reagent (Invitrogen, USA). cDNA was synthesized using a high-capacity cDNA reverse transcription kit and random primers (Applied Biosystems, USA). The full-length *GRA12* coding sequence amplified from *T. gondii* cDNA library was fused with 3×hemagglutinin (HA) tag at 3' end by a second round PCR. To complement GRA12 at the UPRT locus, the endogenous *GRA12*

promoter was amplified to construct the PGAR12::GRA12::Ble plasmid, then the pSAG1-Cas9-sgUPRT plasmids and the PGAR12::GRA12::Ble fragment were co-transfected into Δ*gra12* clone and selected with phleomycin and 5-fluoro-2-deoxyuridine for positive and negative screening, respectively. Positive clones were identified by diagnostic PCR, western blotting and immunofluorescence assay.

Immunoblotting and immunofluorescence assays

For antibodies (Abs), rabbit anti-HA monoclonal Ab (MAb) was purchased from Cell Signaling Technology, and mouse anti-HA (MAb) and goat anti-*Toxoplasma* were purchased Abcam. Polyclonal mouse anti-ROP18 (39) and polyclonal rabbit anti-Irga6 antibodies were kindly provided by Professor Qun Liu. Polyclonal rabbit anti-aldolase, polyclonal rabbit anti-GRA12, mouse anti-SAG1 (MAb), mouse anti-GRA2 (MAb) and mouse anti-GRA5 (MAb) antibodies were kept in our laboratory. For the preparation of rabbit anti-GRA12 antibody, full-length His-tagged GRA12 expressed in *Escherichia coli* and purified protein was used to immunize rabbits.

For Western blotting assay, purified tachyzoites were treated with RIPA lysis buffer and phenylmethanesulfonyl fluoride (PMSF) on ice prior to resolution on SDS-PAGE and transferred to a nitrocellulose membrane. The primary antibodies used in this study were rabbit anti-Aldolase (1:500), rabbit anti-GRA12 (1:450), and mouse anti-ROP18 (1:250).

For immunofluorescence assays (IFA), infected confluent HFF monolayers were fixed with 4% paraformaldehyde in Phosphate Buffered Saline (PBS) before being permeabilized with 0.2% Triton X-100 and blocked with 3% Bovine Serum Albumin in PBS (BSA-PBS). The samples were then incubated with primary rabbit anti-GRA12 (diluted 1:250), rabbit anti-HA (diluted 1:1000), rabbit anti-Irga6 (diluted 1:500), mouse anti-GRA5 (diluted 1:1000), mouse anti-HA (diluted 1:1000), mouse anti-SAG1 (diluted 1:1000), or mouse anti-GRA2 (diluted 1:500) for overnight, washed five times, and then secondary antibodies were added for 1 h. After washing five times with PBS, samples were imaged with a Leica confocal microscope system (TCS

SP52, Leica, Germany).

In vitro plaque assays

HFF monolayers grown on 6-well plates were infected with 200 freshly egressed tachyzoites per well for 7 or 9 days. The infected cell cultures were washed with PBS and fixed with 4% paraformaldehyde and then stained with crystal violet and imaged on a scanner to analyze the relative size and number of plaques formed by the growing parasites, as previously described (40).

Parasite intracellular replication assay

Confluent HFF monolayers grown on 6-well plates were infected with 10^5 freshly egressed parasites per well for 1 h followed by several washing to remove unbound tachyzoites. Then, the pates were incubated for further 23 h in order to allow parasite growth. Subsequently, the cultures were fixed and stained with mouse anti-SAG1 followed by goat-anti mouse IgG conjugated to Alexa Fluor 488. Numbers of parasites per vacuole were determined by counting at least 200 vacuole and data were expressed as the percentage of vacuoles containing different numbers of tachyzoite (40).

Transmission electron microscopy

Monolayers of HFFs were infected with the wild-type RH or RHΔ*gra12* strain for 20 h and then were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at ambient temperature. Samples were washed in cacodylate buffer and post-fixed in 1% osmium tetroxide for 1 h and processed as described previously (31). Images were obtained with a HITACHI HT7700 electron microscope under 80 kV.

In vitro bradyzoite differentiation

Bradyzoite differentiation was induced by alkaline treatment of infected HFF cell monolayers

as previously described (41). Tachyzoites were allowed to infect HFFs and grown under normal conditions for 1 h, before the medium was replaced with differentiation medium (alkaline media with pH = 8.2, ambient CO_2). The medium was replaced every day to maintain high pH. Subsequently, the samples were subject to immunofluorescent analysis. All parasites were stained with goat anti-*Toxoplasma* and bradyzoites were stained with rabbit anti-BAG1. The percentage of bradyzoite differentiation was determined by dividing the number of TgBAG1 positive vacuoles by that of anti-*Toxoplasma* positive vacuoles. The experiments were repeated three times independently.

Animal infection experiments

For virulence assay, tachyzoites purified from freshly lysed HFF cells were used to infect mice by intraperitoneal (i.p.) injection. In parallel, the accurate number of infectious parasites infected with mice was determined by performing plaque assays. For immunosuppression, mice were treated with dexamethasone (dexamethasone 21-phosphate disodium salt) in drinking water (30 mg/liter) two days before infection (42). Drinking water was replaced second day with water containing freshly prepared dexamethasone. All animals were monitored daily for clinical signs and mortality for 30 days. Blood collected from mice that survived at day 30 were tested by enzyme linked immunosorbent assay (ELISA) to confirm *T. gondii* infection. To assess the cyst burden, mice were euthanized at day 30 post infection. Whole brains were homogenized in PBS and the number of brains cyst was performed by examining dilutions of *Dolichos biflorus* lectin-stained brain homogenates as previously described (43).

For *in vivo* competition assay, C57BL/6 mice were infected with a mixed aliquot of ~30% Δ*gra12*C and ~70% Δ*gra12*. *In vitro* plaque assays were used to confirm the ratio of the mixed inoculum. On days 4 and 7, mice were euthanized and peritoneal lavage fluids were collected with DMEM containing 2% FBS. The peritoneal lavage fluid cells containing replicating tachyzoites were force lysed through a 26-gauge needled to release parasites. Confluent HFFs

monolayers were infected with the parasites for 24 h and the ratios of Δ*gra12*C and Δ*gra12* parasite vacuoles were determined by IFA stained with mouse anti-SAG1 and rabbit anti-HA.

Accumulation of immunity-related GTPase to the parasite containing vacuole

Mouse macrophage RAW 264.7 cells were activated by treatment with 0.1 ng/mL LPS (*Escherichia coli* 055:B5; Sigma-Aldrich) and 100 U/mL murine recombinant IFN-γ (rIFN-γ; R&D Systems) for 8 h, as previously described (20). Activated cells were infected with freshly harvested tachyzoites for 2 h, after which cultures were fixed and stained with mouse anti-GRA5 for staining the PV and rabbit anti-Irga6 for immunity-related GTPase localization. The number of Irga6-containing positive vacuoles was determined by counting at least 10 fields in three biological experiments with three replicates.

Statistical analysis

All experiments with three or more independent experiments were analyzed by two-tailed, unpaired Student *t* test for comparing means between two groups and one-way analysis of variance and for comparing means between ≥3 groups. P value < 0.05 was considered statistically significant.

RESULTS

Genetic removal and complementation of *GRA12* in type I RH and type II Pru strains

To determine the function(s) of GRA12, the *gra12* locus was disrupted by CRISPR-Cas9-mediated homologous recombination. To achieve this, a knockout construct consisting of the *gra12* flanking regions surrounding the dihydrofolate reductase (DHFR) selectable marker was used to replace the GRA12-coding region (**Fig. 1*A***). After transfection of *gra12* gene special CRISPR plasmid and the linear knockout construct into corresponding parental strains (RH or

Pru), transfectants were selected with pyrimethamine and single cloned was obtained by limiting dilution and diagnostic PCR. The absence of GRA12 was confirmed by amplification of the coding sequence at genomic level using PCR (Fig. 1*C* and ***S*1**) and at the protein level as detected by Western blotting (Fig. 1*E* and *S*1) or immunofluorescence analysis (**Fig. 2 and S2**). Δ*gra*12 mutants were then complemented with a three C-terminally hemagglutinin (HA)-tagged wild type GRA12-coding gene. This constructs were integrated into the uracil phosphoribosyltransferase (*UPRT*) locus by CRISPR-Cas9 mediated non-homologous end joining (NHEJ) (Fig. 1*B*). Integration of the GRA12 coding sequence into *UPRT* locus was confirmed by PCR detection (Fig. 1*D* and *S*1) and by restoration of protein expression by Western blotting (Fig. 1*E* and *S*1) or immunofluorescence analysis using anti-GRA12 (Fig. 2 and *S*2) or anti-HA (**Fig. *S*3**).

GRA12 is dispensable for the lytic cycle

After obtaining the knockout mutants, plaque assays were performed to assess the Δ*gra12* knockout parasites for any defects through successive lytic cycle. HFFs monolayers were infected with Δ*gra12* mutants, their parental or complemented strains, and the parasites were allowed to replicate for 7 (for type I) or 9 (for type II) days before fixation and staining of the monolayers. Repeated experiments showed that the number and sizes of plaques observed in HFF confluent monolayers generated by both RHΔ*gra12* and PruΔ*gra12* mutants were similar to that of their parental or complemented strain, suggesting that GRA12 is not required for tachyzoites to efficiently progress *in vitro* through the lytic cycle (**Fig. 3*A*, *B* and *S*4**).

To further test the parasite growth more precisely, intracellular replication assay was performed to assess the proliferation efficiency. Parasite replication was measured by infecting HFF confluent monolayers for 24 h, before fixation and the number of parasites in each PV was determined by fluorescence microscopy. Our results showed that both RHΔ*gra12* and PruΔ*gra12* mutants displayed similar replication dynamics that are comparable to their parental

strains, indicating that GRA12 is not essential for tachyzoite replication *in vitro* (Fig. 3*E* and *S*4). In addition, the IVN and PV morphology were not significantly affected by the absence of GRA12 (**Fig. 4 and *S*3**).

GRA12 is essential for acute and chronic *T. gondii* infection

To determine the consequences of GRA12 disruption on parasite virulence, C57BL/6 mice (10 mice/group) were i.p. infected with tachyzoites of Δ*gra12* knockouts, their parental or complemented strains, and the survival rate of infected mice was determined. The results for the RH-based strains (RH, RHΔ*gra12* or RHΔ*gra12*C) are shown in **Fig. 5*A***. With the infection dose of 100 tachyzoites per mouse, the survival times of mice infected with the RHΔ*gra12* strain were significantly longer than those of mice infected with the wild-type RH or the complemented strains (P <0.001). The virulence of GRA12 was also assessed in type II Pru strains. Mice were i.p. infected with doses of 5×10^2, 5×10^3, 5×10^4 and 5×10^5 tachyzoites of the Pru, PruΔ*gra12* or PruΔ*gra12*C strain and infected mice were monitored for morbidity, symptoms of infection and weight loss. Mortality of mice infected with the wild-type (parental) and the complemented strains was consistent with previously reported intermediate virulence of type II parasites, with a 50% lethal dose (LD_{50}) of ~10^3 to 10^4. All mice infected with 5×10^4 or 5×10^5 Pru and PruΔ*gra12*C died 9-13 days after infection. In sharp contrast, all mice infected with 5×10^3, 5×10^4 or 5×10^5 PruΔ*gra12* parasites remained alive 30 days (Fig. 5*B-E*). In addition, mice infected with PruΔ*gra12* didn't exhibit any signs of illness, mice infected with Pru and PruΔ*gra12*C strains showed a significant loss in body weight and deteriorated body condition (e.g., ruffled coat, hunched back and ascites) during the course of infection. Finally, all mice infected with PruΔ*gra12* survived and were protected against a lethal challenge with 1×10^3 RH tachyzoite.

To assess the role of GRA12 during chronic infection, brain cyst burden was examined at 30 days after infection. While the parental Pru strain produced low numbers of brain cysts in

C56BL/6 mice, higher cyst burden was detected in Kunming mice. Thus, Kunming mice were infected with Pru, PruΔ*gra12* or PruΔ*gra12*C strain and the brain cyst burden was determined. Brain cyst burden in mice infected with PruΔ*gra12* was significantly lower than that in mice infected with Pru and PruΔ*gra12*C strains, even with a 1000-fold-higher inoculum of the PruΔ*gra12* strain ($P < 0.001$) (Fig. 5*F*).

To test whether the inability of PruΔ*gra12* parasites to form brain cysts *in vivo* was due to defective bradyzoite cyst formation, we examined the ability of this mutant to form bradyzoites-containing cysts *in vitro* following exposure to a pH shift (pH 8.2). PruΔ*gra12* and Pru tachyzoites infected HFF cells were maintained under 0.0% CO_2 condition for 4 days and then the expression of the bradyzoite-specific marker BAG1 was examined by immunofluorescent microscopy to evaluate bradyzoite formation efficiency. Results showed that PruΔ*gra12* parasites can form *in vitro* cysts as efficiently as Pru parasites. However, the vacuole formed by the PruΔ*gra12* parasite appears smaller than that of Pru (**Fig. 6*A***) suggesting that GRA12 is required to support parasite growth under stress conditions.

GRA12-deficient parasites are more susceptible to innate immune clearance

Competition assays were performed to determine the kinetics of Δ*gra12* mutant parasites clearance *in vivo*. C57BL/6 mice were i.p. injected with a mixture of RHΔ*gra*12 and RHΔ*gra12*C at a dose of 200 parasites per mouse (~70/30 ratio of RHΔ*gra12*/RHΔ*gra12C*) or a mixture of PruΔ*gra12*C and PruΔ*gra12* at a dose of 5×10^4 parasites per mouse (~70/30 ratio of PruΔ*gra12*/PruΔ*gra12*C). At days 4 and day 7 post-infection, the ratio of Δ*gra12* to their corresponding complemented strain collected form the peritoneum was determined by IFA. The Δ*gra12* parasites in both type I RH and type II Pru were outcompeted by their corresponding complemented parasites *in vivo* as the infection progresses (Fig. 6*B*, *C*). The decrease in the relative numbers of Δ*gra12* parasites indicated that Δ*gra12* parasites either were cleared by the innate immune response or have a slower growth rate compared to parental strains *in vivo*. To

explore these two possibilities, the morbidity kinetics of Δ*gra12* and their complemented strain in immunosuppressed mice treated with dexamethasone were examined. Immunosuppressed mice infected with either Δ*gra12* or their parental strain have similar morbidity kinetics (Fig. 6*D*), indicating that GRA12 plays key roles in the manipulation of the innate immune response in mice.

Double deletion of GRA12 and ROP18 in type I RH leads attenuates acute virulence

Since RO18 plays a key role in the virulence of type I RH in mice (18, 19), we were interested in examining whether GRA12 and ROP18 have a synergistic effect in controlling virulence of type I RH in mice. To achieve this, a RHΔ*gra12*Δ*rop18* double knockout strain was constructed by deleting *rop18* gene in the RHΔ*gra12* strain. The absence of ROP18 in the RHΔ*gra12* strain was confirmed by PCR amplification (**Fig. *S*5**) and at the protein level as detected by Western blotting (**Fig. 7*A***). Plaque assays and replication assays were performed to test whether deletion caused a growth defect as mentioned above. Results showed no gross defects in parasites growth and intracellular replication between RHΔ*gra12* and RHΔ*gra12*Δ*rop18* parasites (Fig. 7*B*, *C*). In addition, knockout of GRA12 in the RHΔ*rop18* strain caused the similar phenotype of RHΔ*gra12*Δ*rop18*.

To determine the virulence of double GRA12 and ROP18 knockout parasites, mice were infected with a dose of 100 RH, RHΔ*gra12*, RHΔ*rop18*, RHΔ*gra12*Δ*rop18* or RHΔ*rop18*Δ*gra12* tachyzoites. All of mice infected with RH, RHΔ*gra12*, RHΔ*rop18* died within 30 days. In contrast, mice infected with the RHΔ*gra12*Δ*rop18* or RHΔ*gra12*Δ*rop18* parasites survived (Fig. 7*D*). To establish the extent of this attenuation of double knockout of GRA12 and ROP18, the dose-dependent mortality was measured. Our results showed that mice can survive infection with as high as 10^3 RHΔ*gra12*Δ*rop18* or RHΔ*gra12*Δ*rop18* parasites (Fig. 7*E*).

The mechanism of control of *T. gondii* infection in mouse macrophages is primary dependent

on IFN-γ, which can upregulate of IRG to damage the PV and kill the parasites inside (14). Given that ROP18 is necessary to subvert IRG clearance and double knockout of ROP18 and GRA12 leads to more severe attenuation of virulence, it became interesting to determine whether the double mutants may be more susceptible to the IRG pathway. IFA analysis with anti-Irga6 antibodies showed the percentage of Irgba6 localization to RHΔ*gra12*Δ*rop18* or RHΔ*rop18*Δ*gra12* parasite vacuoles were similar to that in the RHΔ*rop18* vacuoles. Interestingly, the extent of Irga6 localization to RHΔ*gra12* was similar to that in the parental RH strain (Fig. 7*F*).

DISCUSSION

The family of *T. gondii* dense granule proteins has largely been identified by traditional antibody production and organelle isolation strategies, and more recently by bioinformatics and proximity-based protein labeling techniques approaches (44, 45). These studies have indicated that *T. gondii* genome encodes more than 40 GRA proteins. While the functions of most of GRA proteins are not studied elaborately, analyses of a few of these family members have indicated that GRAs play crucial roles in vacuole remodeling and nutrient uptake (46), and immune evasion although they are not essential for parasite growth *in vitro* (47). Previous immunofluorescence and immuno-electron microscopy showed that GRA12 behaving similarly to both GRA2 and GRA6 is secreted into the PV and is associated with the mature membranous IVN (35). Consistent with previous data, we found that GRA12 was secreted in the PV, co-localized with GRA2 detected using polyclonal rabbit anti-GRA12 or anti-HA antibody. Together, these studies indicated that GRA12 is a dense granule protein that was associated with membranous nanotubular network.

The IVN serves diverse functions, such as nutrient acquisition through trafficking of host-derived vesicles (48), uptake of soluble host proteins (49), protection from antigen presentation

(50), and facilitates *T. gondii* effector protein localization to the PVM (5), protecting the parasite from clearance by host defense mechanism. Several GRA proteins (GRA2, GRA4, GRA6 and GRA9) are located at the intravacuolar membranous nanotubular network (51), however, only GRA2 and GRA6 are required for IVN biogenesis and parasites lacking either GRA2 or GRA6 grow in PV without the well-structured membranous tubules (31). Like GRA4 and GRA9 (52), deletion of GRA12 did not affect the normal ultrastructure of the IVN. Recently, a secreted kinase WNG1 identified in *T. gondii* as an important regulator of tubular membrane biogenesis, and can phosphorylate GRA proteins involved in IVN biogenesis (53). WNG1-dependent phosphorylation of the GRA proteins is important for the proper formation of the IVN. In that study, GRA2 and GRA6 reported to be phosphorylated by WNG1, whereas GRA12 protein in which phosphorylation was not significantly reduced between the parental and RHΔ*wng1* strains (53). These studies suggest that GRA12 is not required for the IVN biogenesis.

Like most GRAs, disruption of GRA12 in both type I RH and type II Pru strains do not affect the parasite's growth and replication *in vitro.* However, GRA12-deficient parasites exhibited attenuated virulence in the mouse model. In type I strain, mice infected with a low dose of RHΔ*gra12* tachyzoites survived with a significantly longer time compared to mice infected with the wild type RH or RHΔ*gra12*C tachyzoites. In type II strains, all mice inoculated with a high dose (up to 5×10^5 per mice) of PruΔ*gra12* tachyzoites survived and did not exhibit any toxoplasmosis-related symptoms, while all mice infected with high dose of Pru or PruΔ*gra12*C tachyzoites died within 7-11 days. These results collectively indicate that disruption of GRA12 lead to attenuated virulence in both type I and type II *T. gondii* strains, however disruption of GRA12 in type II strain led to more severe attenuation than that in type I strain. Mice infected with type II GRA12 mutant had an extremely lower numbers of cysts than those infected with Pru and PruΔ*gra12*C strains. *In vitro* bradyzoite differentiation showed that PruΔGRA12 parasites have the ability to form *in vitro* cysts as efficiently as Pru strains although the slower

growth of the PruΔ*gra12* mutants under alkaline medium in combination without CO_2. Taken together, these data suggest that attenuation of PruΔ*gra12* parasite virulence during acute infection may contribute to the less PruΔ*gra12* brain cyst burden detected in mice with chronic infection.

In vivo competition assays showed that the decrease in the relative numbers of Δ*gra12* mutant parasites may have contributed to the longer survival time of mice infected with Δ*gra12* mutant parasites. On the other hand, mice treated with dexamethasone and infected with Δ*gra12* mutant or Δ*gra12* mutant complement strains showed similar death kinetics, suggesting that the decrease of GRA12 mutant parasites was linked to immunological control mechanism other than the slow growth *in vivo* and that Δ*gra12* mutants were more susceptible to be cleared by mice immunity. This suggest that GRA12 may manipulate host immune response to promote the parasite virulence, optimal parasite growth under stress conditions and establishment of chronic infection.

During *T. gondii* infections, host cells upregulate a family of IRGs that target the parasite-containing PVM to clear the parasite by damaging the PV, leading parasite degradation (16). To determine how GRA12 modulates *T. gondii* virulence, the localization of Irga6 in the RH and RHΔ*gra12* mutants were examined. However, the localization of Irga6 between RH and RHΔ*gra12* parasites was not different, suggesting that GRA12 may not directly modulate Igra6 localization at parasite-containing vacuoles to promotes parasite infection.

In *T. gondii,* the localization of IRGs to the PVM can be inhibited mainly through the ROP5/17/18 complex (21). In addition, GRA7 can bind to oligomers of Irga6, acting synergistically with ROP18 to control the localization of IRGs onto the PVM, although deletion of GRA7 has not or slightly increased the IRGs recruitment (20, 22). We wondered whether GRA12 and ROP18 act in a complex to target IRGs, likewise GRA7. Our results showed that the combined disruption of GRA12 and ROP18 in RH strain resulted in greater attenuation than

the disruption of either GRA12 or ROP18 alone. However, plaque assay and *in vivo* immunosuppressed mice infection experiments showed that the GRA12 and ROP18 double knockout strains have normal growth and replication, suggesting that the attenuation of this double knockout mutants is a consequence of immunological clearance and not due to intrinsic differences in parasite growth. The localization of Irga6 in double GRA12 and ROP18 mutant were similar to that in RHΔ*rop18*, suggesting that deletion of GRA12 in the RHΔ*rop18* strain would not further increase the percentage of Irga6 recruitment, and GRA12 may not act synergistically with ROP18 to target IRGs although they act synergistically in controlling *T. gondii* virulence. In this study, we only examined the localization of Irga6; whether GRA12 has potential roles in disarming other members of the IRG or GBP family, remains to be investigated.

In conclusion, deletion of GRA12 did not abolish the lytic cycle of *T. gondii in vitro* and did not alter the ultrastructure of the IVN, suggesting that GRA12 is unessential for sustaining the normal growth of *T. gondii in vitro and* IVN biogenesis. However, parasites lacking GRA12 exhibited a reduced virulence in mice and were more susceptible to be cleared by host immune response although the susceptibility of the Δ*gra12* parasites was not correlated with the increased Irga6 accumulation at the vacuoles of Δ*gra12* parasites. GRA12 and ROP18 double knockout strain showed considerable attenuation of virulence in mice. Our data reveal a key role for GRA12 in *T. gondii* pathogenesis and highlight a novel function for this protein in determining the clinical outcome and burden of infection in mice. Additional work on the *in vivo* functions of GRA12, especially in the modulation of host-parasite interaction is needed. More studies of virulence factors essential for *T. gondii* survival can provide a basis for future anti-*Toxoplasma* drug development.

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AUTHOR CONTRIBUTIONS

J. Wang designed the experiments; M. Bai performed the experiments; X. Zhu and H. M. Elsheikha critically revised the manuscript; Q. Liang and X. Cao provided technical support; T. Li participated in the analyzed the date.

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FIGURE LEGEND

Figure 1. Construction of GRA12deficient mutants and GRA12 complemented strains. *A*) Schematic representation of deleting *gra12* gene by CRISPR-Cas9 mediated homologous gene replacement. *B*) Complementation strategy of GRA12 into the *UPRT* locus by CRISPR-CAS9 mediated non-homologous end joining (NHEJ). The complemented gene contains a C-terminal 3×HA epitope tag and is driven by the endogenous promoter. *C*) PCR1 and PCR3 detected the 5′ and 3′ integration of the selection marker whereas the PCR2 was used to examine successful deletion of *gra12* gene. *D*) Diagnostic PCRs of the complemented clones showing the successful integration of the complemented gene into the *UPRT* locus. *E*) Western blotting confirming the loss of and restoration of GRA12 expression in the RHΔ*gra12* and RHΔ*gra12*C strains, respectively. Anti-aldolase (ALD) was used to confirm the presence of the parasite protein.

Figure 2. Immunofluorescence assays of RH, RHΔ*gra12* and RHΔ*gra12*C parasites. Rabbit anti-GRA12 shows strong staining in the network membranes in the parasitophorous vacuole of RH and RHΔ*gra12*C parasites, which was co-localized with GRA2. GRA12 signal was absent in RHΔ*gra12* strain.

Figure 3. GRA12 is not essential for lytic cycle *in vitro*. *A*) Plaque assay of parental RH, RHΔ*gra12* mutant, and complemented strains over a 7-day infection of HFFs. *B*) Quantification of relative size of plaques showed no significant differences between parental RH, RHΔ*gra12* mutant, or complemented strain. *C*) Quantification of parasite replication by counting parasites per vacuole showed RHΔ*gra12* mutant or complemented strain to have similar intracellular replication dynamics to the parental RH strain. n.s. = not statistically significant.

Figure 4. Ultrastructure of the intravacuolar network (IVN) of wildtype RH and RHΔ*gra12* strains. HFF monolayers were infected with either RH or RHΔ*gra12* tachyzoites, and after 24 h, cells were fixed and prepared for transmission electron microscopy. Representative transmission electron microscopic images of the wild type RH (*A*), and RHΔ*gra12* (*B*) illustrate

the normal ultrastructure of the IVN, close to the PVM, between two parasites.

Figure 5. GRA12 is an important virulence factor in the murine model. *A*) Survival data of C57BL/6 female mice (10/group) infected with 100 freshly egressed tachyzoites of parental RH, RHΔ*gra12* mutant, or complemented strain. *B-E*) Survival of female C57BL/6 mice infected with 5×10^2 (*B*), 5×10^3 (*C*), 5×10^4 (*D*) or 5×10^5 (*E*) parental Pru, PruΔ*gra12* mutant, or complemented strain. *F*) Brain cyst burdens were quantified from brains extracted from female Kunming mice sacrificed after 30 days of infection with parental Pru, PruΔ*gra12* mutant, or complemented strain. ** $P < 0.01$; *** $P < 0.001$.

Figure 6. GRA12 is required for optimal parasite growth under stress conditions and *in vivo*. *A*) Bradyzoites conversion assay. Parental Pru and PruΔ*gra12* mutants were used to infect HFF cells and grown for 4 days under bradyzoite-inducing conditions (pH = 8.2, 0.5% CO2). Subsequently, the samples were stained with rabbit anti-BAG1 (to stain bradyzoites) and goat anti-*Toxoplasma* (to stain the whole organism). The smaller vacuole formed by the PruΔ*gra12* strain may be attributed to the decreased fitness of the mutant parasite. Scale bars, 20 μm. *B* and *C*) *In vivo* competition assay results for Δ*gra12* and Δ*gra12* complemented parasite strains, showing a steady decrease in the percentage of Δ*gra12* vacuoles and a steady increase in the percentage of Δ*gra12C* vacuoles as the coinfection progresses. *D*) Immunosuppressed mice were intraperitoneally injected with $5x10^3$ parasites of Δ*gra12,* Δ*gra12C* or RHΔ*rop18*Δ*gra12* and became moribund with similar kinetics, suggesting that GRA12 modulates the innate immune responses.

Figure 7. Combined deletion of GRA12 and ROP 18 cause severe attenuation of acute virulence in the murine model. *A*) Western blotting confirms the loss of GRA12 or/and ROP18 protein in the knock out strains. *B*) Plaque assay of RHΔ*gra12*, RHΔ*rop18,* RHΔ*gra12*Δ*rop18* and RHΔ*rop18*Δ*gra12* strains over a 7-day infection of HFFs. *C*) Quantification of relative size of plaques showed no significant differences between RHΔ*gra12*, RHΔ*rop18,* RHΔ*gra12*Δ*rop18* and RHΔ*rop18*Δ*gra12* strains. *D*) Survival of mice infected with 100 parasites of RHΔ*gra12*,

RHΔ*rop18,* RHΔ*gra12*Δ*rop18* or RHΔ*rop18*Δ*gra12* strains. Ten mice were used for each strain. No mortality was observed in mice infected with RHΔ*gra12*Δ*rop18* or RHΔ*rop18*Δ*gra12* strain. *E*) Dose-dependent mortality of RHΔ*rop18*Δ*gra12* or RHΔ*gra12*Δ*rop18* parasites. Ten mice were used for each strain. *F*) Quantification of Irga6 localized to the parasite vacuole in activated RAW cells by using immunofluorescence staining of Irga6 on parasite-containing vacuoles in IFN-γ and LPS activated RAW macrophages at 2 h post-infection stained with rabbit polyclonal Irga6 (green), anti-GRA5 (red). Scale bars, 5 μm. *** $P < 0.001$; n.s. = not statistically significant.

Figure S1. Construction of GRA12-deficient mutant and GRA12 complemented Pru strains. *A*) PCR1 and PCR3 detect the 5′ and 3′ integration of the selection marker whereas the PCR2 was used to examine the efficiency of deletion of *gra12* gene. *B*) Diagnostic PCRs of the complemented clone showing successful integration of the complemented gene into the *UPRT* locus. *C*) Western blotting confirming the loss of and restoration of GRA12 expression in the PruΔ*gra12* and PruΔ*gra12*C strains, respectively. Anti-aldolase (ALD) was used to confirm the presence of parasite protein.

Figure S2. Immunofluorescence assays of Pru, PruΔ*gra12* and PruΔ*gra12*C parasites. Rabbit anti-GRA12 shows visible staining of the GRA12 in the network membranes in the parasitophorous vacuole of RH and RHΔ*gra12*C parasites, which was co-localized with GRA2. GRA12 signal was absent in PruΔ*gra12* parasites.

Figure S3. Immunofluorescence assays of RH, RHΔ*gra12* and RHΔ*gra12*C parasites by anti-HA. Rabbit anti-HA shows strong staining in the network membranes in the parasitophorous vacuole of RHΔ*gra12*C parasites, co-localizing with GRA2. GRA12 signal was absent in RHΔ*gra12* and RH parasites.

Figure S4. GRA12 is not essential for Pru strain lytic cycle *in vitro*. *A*) Plaque assay of parental Pru, Pru Δ*gra12* mutant, and complemented strains 9-day post-infection of HFFs. *B*)

Quantification of relative size of plaques showed no significant differences between parental Pru, PruΔ*gra12* mutant, or complemented strain. *C*) Quantification of parasite replication by counting parasites per vacuole showed that PruΔ*gra12* mutant or complemented strain had similar intracellular replication dynamics to the parental RH strain. n.s. = not statistically significant.

Figure S5. Construction of ROP18-deficient mutant and double GRA12 and ROP18 knockout strains. *A*) Schematic showing deletion of *rop18* gene by CRISPR-Cas9 mediated homologous gene replacement. *B*) Diagnostic PCRs of the RHΔ*rop18* mutant showing the successful knockout of the *rop18* gene. *C*) Diagnostic PCRs of the RHΔ*gra12*Δ*rop18* mutant showing the successful knockout of the *rop18* gene in the RHΔ*gra12* strain. *D*) Diagnostic PCRs of the RHΔ*rop18*Δ*gra12* mutant showing the successful knockout of the *gra12* gene in the RHΔ*rop18* strain.