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# A live attenuated RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant of *Toxoplasma gondii* induces strong protective immunity against toxoplasmosis in mice and cats

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## Abstract

**Background** *Toxoplasma gondii* is an obligate intracellular apicomplexan parasite and is responsible for zoonotic toxoplasmosis. It is essential to develop an effective anti-*T. gondii* vaccine for the control of toxoplasmosis, and this study is to explore the immunoprotective effects of a live attenuated vaccine in mice and cats.

**Methods** First, the *ompdc* and *uprt* genes of *T. gondii* were deleted through the CRISPR-Cas9 system. Then, the intracellular proliferation and virulence of this mutant strain were evaluated. Subsequently, the immune responses induced by this mutant in mice and cats were detected, including antibody titers, cytokine levels, and subsets of T lymphocytes. Finally, the immunoprotective effects were evaluated by challenge with tachyzoites of different strains in mice or cysts of the ME49 strain in cats. Furthermore, to discover the effective immune element against toxoplasmosis, passive immunizations were carried out. GraphPad Prism software was used to conduct the log-rank (Mantel-Cox) test, Student's *t* test and one-way ANOVA.

**Results** The RH $\Delta$ *ompdc* $\Delta$ *uprt* were constructed by the CRISPR-Cas9 system. Compared with the wild-type strain, the mutant notably reduced proliferation ( $P < 0.05$ ). In addition, the mutant exhibited virulence attenuation in both murine (BALB/c and BALB/c-nu) and cat models. Notably, limited pathological changes were found in tissues from RH $\Delta$ *ompdc* $\Delta$ *uprt*-injected mice. Furthermore, compared with nonimmunized group, high levels of IgG (IgG1 and IgG2a) antibodies and cytokines (IFN- $\gamma$ , IL-4, IL-10, IL-2 and IL-12) in mice were detected by the mutant ( $P < 0.05$ ). Remarkably, all RH $\Delta$ *ompdc* $\Delta$ *uprt*-vaccinated mice survived a lethal challenge with RH $\Delta$ *ku80* and ME49 and WH6 strains. The immunized sera and splenocytes, especially CD8<sup>+</sup> T cells, could significantly extend ( $P < 0.05$ ) the survival time of mice challenged with the RH $\Delta$ *ku80* strain compared with naive mice. In addition, compared with nonimmunized cats, cats immunized with the mutant produced high levels of antibodies and cytokines ( $P < 0.05$ ), and notably decreased the shedding numbers of oocysts in feces (95.3%).

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**Conclusions** The avirulent RH $\Delta$ *ompdc* $\Delta$ *uprt* strain can provide strong anti-*T. gondii* immune responses, and is a promising candidate for developing a safe and effective live attenuated vaccine.

**Keywords** *Toxoplasma gondii*, Orotidine-5'-monophosphate decarboxylase, Phosphoribosyltransferase, Live attenuated vaccine, Immunization, Mouse, Cat

## Background

*Toxoplasma gondii* is an intracellular protozoan parasite and can infect nearly all warm-blooded animals including humans [1]. It is estimated that approximately one-third of the world's population is infected with *T. gondii* [2]. Humans acquire *T. gondii* infection usually through ingestion of tissue cysts in raw or undercooked meat, oocysts in food or water, or congenitally via the placenta [3, 4]. Although *T. gondii* usually appears as a latent infection in people with normal immunity, it can cause serious complications in individuals with suppressed immune systems such as AIDS patients or people with malignant tumors [5, 6]. Pregnant women infected with *T. gondii* may experience miscarriage and stillbirth, and the fetus will have deformities or severe intellectual disability after birth [7]. The infection of intermediate animals including cattle, sheep and pigs may result in huge economic losses to the farm and potential health risks to humans [8].

Prevention or treatment of toxoplasmosis is difficult due to the complex life cycle and elaborate immune escape system of the parasite to establish chronic infection in most hosts [3, 9]. So far, toxoplasmosis is mainly treated with drugs such as pyrimethamine and sulfadiazine. However, these drug treatments are only effective in the acute infection stage and may cause serious side effects and promote the development of drug-resistant strains [10–12]. Therefore, novel drugs and effective treatments for *T. gondii* infection should be discovered and developed.

Vaccination is a promising and effective long-term approach for disease control and prevention [13]. The current anti-toxoplasmosis vaccines mainly include protein vaccine, DNA vaccine, live vector vaccine and live attenuated vaccine [13]. Among the existing vaccines against *T. gondii*, live attenuated vaccines provided the best protection with robust cellular and humoral responses to toxoplasmosis in murine models [14]. Moreover, the only commercial *T. gondii* vaccine (Toxovax<sup>®</sup>) is also a live attenuated vaccine developed through continuous subculture from S48 *T. gondii* tachyzoites [15, 16]. However, due to the potential risk of virulence recovery, the vaccine can't be used in humans and is mainly used in sheep. In contrast, genetic deletions may completely prevent virulence regression compared to naturally attenuated strains [7]. Recently, several studies have demonstrated the stability and protective effect of

gene knockout live attenuated vaccines against *T. gondii*. For example, Li et al. developed a double gene deletion mutant of *gra17* and *npt1* in the RH strain of *T. gondii*, which can protect mice from acute, chronic and congenital toxoplasmosis [17].

Pyrimidine plays an important role in parasite proliferation. Thus, as the precursor of all pyrimidines, uridine monophosphate (UMP) is one of the most important nucleotides in RNA for *T. gondii* [18, 19]. Through de novo biosynthesis or salvage pathways, *T. gondii* can acquire adequate pyrimidine and UMP for propagation [20]. The de novo pyrimidine biosynthesis pathway mainly functions under normal circumstances, while the salvage pathway only functions when the de novo biosynthesis pathway is blocked [21]. Studies have shown that when the de novo pyrimidine biosynthesis pathway of *T. gondii* is disrupted, it has little effect on the ability of *T. gondii* to invade host cells, but its proliferation and virulence are significantly weakened [20]. Orotidine-5'-monophosphate decarboxylase (OMPDC) is the terminal enzyme belonging to the de novo biosynthesis pathway, and studies have found that after knocking out the *ompdc* gene of *T. gondii*, the mutant strain lost its replication ability and virulence [22, 23]. According to these studies, the pyrimidine auxotrophic strain of *T. gondii* can be used as an alternative live attenuated vaccine. However, we cannot ignore the fact that *T. gondii* with *ompdc* gene knockout can still proliferate weakly in vitro [24]. As mentioned above, uracil phosphoribosyltransferase (UPRT) is a key enzyme in the UMP salvage pathway [25], which could contribute to parasite proliferation when the de novo biosynthesis pathway is blocked. Thus, to completely block the uracil synthesis pathway, here we generated a mutant with double gene deletion of *ompdc* and *uprt* in the RH $\Delta$ *ku80* $\Delta$ *hxp**uprt* strain by a clustered regularly interspaced short palindromic repeats (CRISPR)/cas9 system and evaluated the immune protection of this strain against toxoplasmosis.

## Methods

### Animals and ethics statement

Six- to eight-week-old BALB/c and BALB/c-nu mice were purchased from the Center of Laboratory Animal of Hangzhou Medical College and 3-month-old cats were purchased from a local breeder. All cats were tested

serologically and found to be free of *T. gondii* and viruses, including feline calicivirus, coronavirus, feline immunodeficiency virus, feline leukemia virus, and feline parvovirus. All animals were raised under standard conditions according to the Animal Management Regulations of the People's Republic of China. Animal experiments were approved by the Animal Care and Use Committee of Hangzhou Medical College (2018–027).

#### Parasite and cell culture

Tachyzoites of the ME49, WH6, RH $\Delta$ ku80 $\Delta$ hxgp $r$ t, RH $\Delta$ ku80 $\Delta$ up $r$ t::HXGPRT and RH $\Delta$ ku80 $\Delta$ up $r$ t $\Delta$ hxgp $r$ t strains were maintained in human foreskin fibroblasts (HFFs) in our laboratory in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, MA, USA) supplemented with 5% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, MA, USA), penicillin (100 units/ml; Thermo Fisher Scientific, MA, USA), and streptomycin (100  $\mu$ g/ml; Thermo Fisher Scientific, MA, USA). The RH $\Delta$ ku80 $\Delta$ up $r$ t $\Delta$ omp $d$ c::HXGPRT strain was additionally treated with 250  $\mu$ mol/L uracil and 200  $\mu$ mol/L UMP (Sigma-Aldrich, MO, USA) [20]. HFFs were cultured in high-glucose DMEM supplemented with 10% FBS at 37 °C, and 5% CO<sub>2</sub>. In all experiments, freshly egressed tachyzoites were filtered with 5- $\mu$ m polycarbonate membranes to remove host cell debris.

#### Preparation of soluble *T. gondii* antigens (STAg)

Soluble *T. gondii* antigens were prepared as previously described [26]. In brief, suspensions of *T. gondii* RH $\Delta$ ku80 tachyzoites were collected in phosphate buffered saline (PBS), subjected to repeated freeze and thaw cycles, and then sonicated on ice at 80 W/s. The prepared product was centrifuged at 14,000 $\times$ g for 30 min at 4 °C. The supernatant was filtered through 0.22  $\mu$ m sterile nitrocellulose filters. The STAg concentration was determined by the Bradford kit (Beyotime, Shanghai, China), and aliquots were stored at -80 °C until use.

#### Construction of RH $\Delta$ up $r$ t and RH $\Delta$ omp $d$ c $\Delta$ up $r$ t mutant strains

The primers involved in this experiment are listed in Additional file 1: Table S1. The mutant strains were constructed using the CRISPR/Cas9 approach based on the RH $\Delta$ ku80 $\Delta$ hxgp $r$ t strain according to Shen B's protocol [27]. Briefly, the hxgp $r$ t and omp $d$ c targeting CRISPR plasmids were generated by replacing the up $r$ t targeting guide RNA in pSAG1::CAS9-U6::sgUPRT with an hxgp $r$ t and omp $d$ c single-guide RNA by site-directed mutagenesis (New England Biolabs, MA, USA). All plasmids were verified by DNA sequencing prior to use. First, the up $r$ t CRISPR plasmid and HXGPRT homologous template were electro transfected into RH $\Delta$ ku80 $\Delta$ hxgp $r$ t tachyzoites.

The up $r$ t-deleted parasites were screened with 25  $\mu$ g/ml xanthine (Sigma-Aldrich, MO, USA) and 25  $\mu$ g/ml mycophenolic acid (Sigma-Aldrich, MO, USA), and single-cloned by limiting the dilution. RH $\Delta$ ku80 $\Delta$ up $r$ t::HXGPRT single positive clones were identified by PCR and qRT-PCR. Second, the hxgp $r$ t CRISPR plasmid and up $r$ t5'UTR-3'UTR homologous template were electro transfected into RH $\Delta$ ku80 $\Delta$ up $r$ t::HXGPRT tachyzoites. The RH $\Delta$ ku80 $\Delta$ up $r$ t $\Delta$ hxgp $r$ t strain was screened with 10  $\mu$ mol/L 5-fluorodeoxyuracil (Sigma-Aldrich, MO, USA) and identified by PCR. Finally, the omp $d$ c CRISPR plasmid and HXGPRT homologous template were electro transfected into RH $\Delta$ ku80 $\Delta$ up $r$ t $\Delta$ hxgp $r$ t tachyzoites. The RH $\Delta$ ku80 $\Delta$ up $r$ t $\Delta$ omp $d$ c::HXGPRT strain was screened and identified in the same way as described above. The PCRs were carried out in a volume of 25  $\mu$ l containing 12.5  $\mu$ l of 2 $\times$  Taq PCR Master Mix (TIANGEN, Beijing, China), 1  $\mu$ l of each primer (10  $\mu$ mol/L), 1  $\mu$ l genomic DNA template, and 9.5  $\mu$ l of sterile distilled water by the conditions with an initial melting step at 98 °C for 3 min, followed by 30 cycles with each cycle at 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

Total RNA of *T. gondii* tachyzoites was extracted using the TRIzol reagent (Invitrogen, CA, USA). The cDNA was synthesized using a First Strand cDNA Synthesis Kit (ReverTra Ace - $\alpha$ -, Toyobo, Osaka, Japan). qRT-PCR was carried out in a volume of 20  $\mu$ l containing 10  $\mu$ l of 2 $\times$  Real time PCR Master Mix (Toyobo, Osaka, Japan), 0.4  $\mu$ l of each primer (10  $\mu$ mol/L), 1  $\mu$ l cDNA template, and 8.2  $\mu$ l of sterile distilled water and amplification was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, CA, USA). The relative mRNA levels were calculated using the comparative  $\Delta$ Ct method using the formula  $2^{-\Delta\Delta C_t}$ .

#### Parasite intracellular replication assay

An indirect immunofluorescence assay (IFA) was used to detect the intracellular proliferation of parasites. Equal amounts of RH $\Delta$ ku80 $\Delta$ omp $d$ c $\Delta$ up $r$ t and RH $\Delta$ ku80 strains were inoculated in plates filled with HFF. The wells inoculated with RH $\Delta$ ku80 $\Delta$ omp $d$ c $\Delta$ up $r$ t strains were cultured with or without 250  $\mu$ mol/L uracil and 200  $\mu$ mol/L UMP for 24 h and 48 h, and the cells were fixed with 4% paraformaldehyde (Beyotime, Shanghai, China) solution for 30 min. The cells were incubated with rabbit anti-GRA7 polyclonal antibody and Alexa Fluor488-conjugated goat anti-rabbit IgG (Abcam, Oxford, UK). The intracellular parasites at different stages of proliferation (i.e., 1, 2, 4, 8, 16 or more than 16 tachyzoites) were counted from 100 parasitophorous vacuoles under fluorescence microscopy (Nikon eclipse 80i, Tokyo, Japan). The experiment was performed in three independent biological repeats.

### Parasite plaque assay

HFF monolayers grown in six-well plates were infected with 500 tachyzoites of RH $\Delta$ ku80 or RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt strains in each well. RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt strains were cultured with or without 250  $\mu$ mol/L uracil and 200  $\mu$ mol/L UMP. After 7 days, the cells were fixed with 4% paraformaldehyde (Beyotime, Shanghai, China) and stained with crystal violet. Finally, the number and size of plaques were analyzed.

### Evaluation of the infectivity of the mutant parasites in mice

Tachyzoites of the RH $\Delta$ ku80 and RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt strains were washed and resuspended in PBS. Four groups of mice were injected intraperitoneally (i.p.) with 0.1 ml of PBS,  $1 \times 10^2$  tachyzoites of the RH $\Delta$ ku80 strain or  $1 \times 10^5$  or  $1 \times 10^6$  tachyzoites of the RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt strain (5 BALB/c mice per group). Moreover, serial doses ( $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$  tachyzoites) of the RH $\Delta$ ompdc $\Delta$ uprt strain or  $1 \times 10^2$  tachyzoites of RH $\Delta$ ku80 strain were i.p. injected into immunodeficient mice (5 BALB/c-nu mice per group). Survival of mice was monitored daily for 30 days.

### Evaluation of the infectivity of the mutant parasites in cats

Two groups of cats were injected intramuscularly (i.m.) with 0.1 ml of PBS or  $1 \times 10^7$  tachyzoites of the RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt strain, faecal samples were collected daily from 1 day post infection (dpi) to 10 dpi and monitored for *T. gondii* oocysts. The oocysts were purified as described previously [28]. Briefly, first, 1 g of feces was weighed and mixed with an appropriate amount of water and centrifuged to collect the sediment. Next, the sediment was mixed with 10 times the volume of sucrose solution with a specific gravity of 1.15 and centrifuged ( $1500 \times g$ ). Finally, approximately 5 ml of the supernatant was mixed with 45 ml of water and centrifuged ( $1500 \times g$ ); the sediment was resuspended in 1 ml of water and counted under a light microscope (Nikon Ti-S, Tokyo, Japan).

### Detection of parasite load

Six- to eight-week-old BALB/c mice (36 per group) were infected i.p. with  $1 \times 10^6$  tachyzoites of the RH $\Delta$ ku80 and RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt strains. The ascites of mice (30 per group) were collected from day 1 to day 5, and mice (6 per group) were executed on the fourth day to collect liver and lung tissues. Genomic DNA was sacrificed by using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Amplifications of genomic DNA were carried out with primers targeting the repeated 529 bp gene of *T. gondii*. The qPCR system was as described above and the amplification was performed on a CFX96

Touch™ Real-Time PCR Detection System (Bio-Rad, CA, USA). The parasite burden was subsequently determined through a standard curve of the 529 bp gene.

### Immunization and challenge in mice

Mice (30 per group) were intraperitoneally immunized with  $1 \times 10^6/100 \mu$ l RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt strain tachyzoites or an equivalent amount of PBS, once every two weeks, for a total of three immunizations. Mouse sera were collected from the tail vein at 0, 2, 4, and 6 weeks. After centrifugation at  $4000 \times g$  for 5 min, the sera were collected and stored at  $-20 \text{ }^\circ\text{C}$  until further use. Two weeks after the last immunization, mice were intraperitoneally injected with  $1 \times 10^3$  tachyzoites of the RH $\Delta$ ku80 strain,  $1 \times 10^3$  bradyzoites of the ME49 strain, or  $1 \times 10^3$  tachyzoites of the WH6, and the survival rate was recorded daily.

### Immunization and challenge in cats

Cats (6 per group) were intramuscularly immunized with  $1 \times 10^7/100 \mu$ l RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt strain tachyzoites or an equivalent amount of PBS once every three weeks for a total of two immunizations. Sera samples were collected at 0, 2 and 4 weeks and stored at  $-20 \text{ }^\circ\text{C}$  until further use. One week after the last immunization, oral administration of 200 ME49 cysts was carried out in immunized and control cats. According to the above method, feces were collected and purified for oocyst counting for 15 consecutive days after the challenge.

### Measurement of antibody responses in mice and cats

Antibody levels of IgG, IgG1, and IgG2a of mice or IgG of cats were detected by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtiter plates were coated with 100  $\mu$ l (10  $\mu$ g/ml) STAg (diluted in PBS) and incubated at  $4 \text{ }^\circ\text{C}$  overnight. Then the plates were washed five times with PBS containing 0.05% Tween 20 (PBST) and blocked with PBST containing 5% non-fat milk powder for 1 h at  $37 \text{ }^\circ\text{C}$ . The plates were washed five times with PBST. Then, 100  $\mu$ l of sera samples diluted in PBST (1:100) containing 5% non-fat milk powder were added to the wells and incubated at  $37 \text{ }^\circ\text{C}$  for 1 h. The plates were washed five times with PBST, then 100  $\mu$ l of diluted horseradish-peroxidase-conjugated goat anti-mouse IgG (Abcam, Oxford, UK, 1:10,000), anti-mouse IgG1 (Abcam, Oxford, UK, 1:5000), IgG2a (Abcam, Oxford, UK, 1:5000) or horseradish-peroxidase-conjugated goat anti-cat IgG (Abcam, Oxford, UK, 1:5000) was added for incubation for 1 h at  $37 \text{ }^\circ\text{C}$ . After washing five times, 100  $\mu$ l of 3,3',5,5'-tetramethylbenzidine (TMB) chromogen solution (Beyotime, Shanghai, China) was added to each well and the plates were incubated at  $37 \text{ }^\circ\text{C}$  for

15 min. After adding 100  $\mu$ l of stop solution for TMB substrate (Beyotime, Shanghai, China), the absorbance was measured by an ELISA plate reader at 450 nm.

### Cytokine assay

Mice (5 per group) were sacrificed two weeks after the last immunization, and the spleens were aseptically removed to prepare a single-cell suspension. Briefly, the spleen was placed on a 70  $\mu$ m cell filter mesh and then added into a 50 ml centrifuge tube by using a 5 ml syringe plunger to grind the spleen. Hank's solution was added dropwise while grinding, and the mesh was rinsed with Hank's solution after grinding to obtain a single cell suspension. The supernatant was discarded after centrifugation, and then 5 times the cell volume erythrocyte lysing solution was added to the cell. Afterwards, the mixture was gently mixed by pipetting and lysed for 2 min. After centrifugation again, the supernatant was discarded, and the cells were resuspended in high-glucose DMEM containing 20% FBS and counted on a bovine abalone counter. Splenocytes ( $1 \times 10^6$ ) from different groups of mice were seeded in sterile 96-well cell culture plates with a final volume of 100  $\mu$ l. The culture supernatant was added to STAg at a final concentration of 10  $\mu$ g/ml and the supernatant was collected for 24 h, 72 h, 96 h. The levels of secreted interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-12 (IL-12) and interferon- $\gamma$  (IFN- $\gamma$ ) were measured by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) using a BD™ Cytometric Bead Array (CBA) kit (BD Biosciences, Franklin Lakes, NJ, USA).

### Lymphocyte proliferation assay

Two weeks after the last immunization, the spleens of three mice from each group were prepared and resuspended as described above. Splenocytes ( $1 \times 10^5$ ) from different groups of mice were added to 96-wellplates and stimulated with STAg (10  $\mu$ g/ml) or DMEM high-glucose medium of equal volume (negative control). Moreover, no cells were added, and only the wells of medium were added as a blank control. Then, the splenic lymphocytes were incubated at 37 °C for 96 h with 5% CO<sub>2</sub>. Cell Counting Kit 8 (CCK-8, Solarbio, Beijing, China) (10  $\mu$ l) was added according to the instructions and incubated for 4 h. Subsequently, the absorbance was measured by an ELISA plate reader at 450 nm to illustrate lymphocyte proliferation. The cell proliferation activity was calculated using the following formula: cell proliferation activity =  $(OD_{450} \text{ STAg} - OD_{450} \text{ Blank}) / (OD_{450} \text{ Control} - OD_{450} \text{ Blank})$ .

### Flow cytometry analysis of T cell subsets

To analyze the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes,  $1 \times 10^6$  splenocytes were prepared as described above and suspended in 100  $\mu$ l PBS. After incubation with fluorochrome-labelled mAbs including FITC-CD3, APC-Cy7-CD4 and PE-CD8 (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 15 min in the dark, the cultures were washed with 2 ml PBS. After centrifugation, the samples were suspended in 500  $\mu$ l PBS and fluorescence profiles were analyzed on a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) by FlowJo software (BD Biosciences, Franklin Lakes, NJ, USA, version 10.8.1).

### Analysis of hematoxylin–eosin (HE) staining of the liver, spleen, and lung

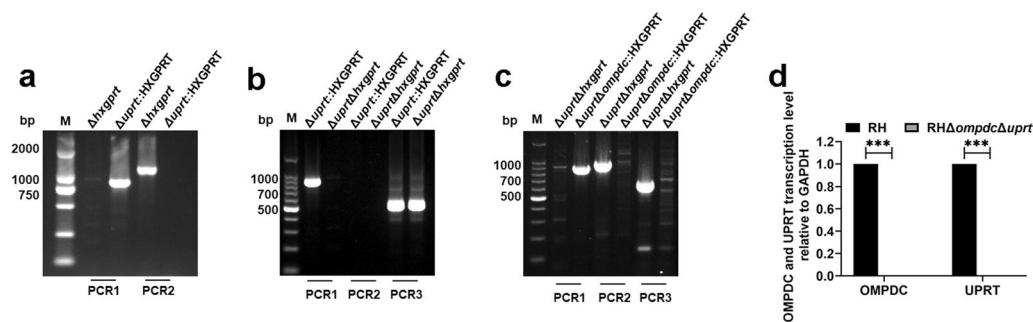
The liver, spleen, and lung tissues of mice from each group (3 mice per group) were removed and soaked in 4% paraformaldehyde (Beyotime, Shanghai, China) at room temperature for 24 h, dehydrated with ethanol, cleared with xylene, embedded in wax and sliced with a slicer. Sections were then stained with hematoxylin and eosin (H&E) as described previously [29].

### Passive immunization of sera and splenocytes from RH $\Delta$ ompdc $\Delta$ uprt-vaccinated mice

Splenocyte suspensions without erythrocytes were prepared as described above, CD19<sup>+</sup> B cells, CD8<sup>+</sup> T cells or CD4<sup>+</sup> T cells were purified (>90% purity) using Miltenyi Mouse positive selection kits (Miltenyi Biotec, Cologne, Germany). Naïve mice received splenocytes ( $1 \times 10^7$ ), CD19<sup>+</sup> B cells ( $2 \times 10^6$ ), CD4<sup>+</sup> T cells ( $2 \times 10^6$ ) or CD8<sup>+</sup> T cells ( $2 \times 10^6$ ) from RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt-vaccinated mice, or splenocytes ( $1 \times 10^7$ ) from naïve mice via tail vein injection. After cell transfer for 24 h, mice were intraperitoneally injected with  $1 \times 10^3$  tachyzoites of the RH $\Delta$ ku80 strain (5 mice per group) [30]. Two weeks after the last immunization, sera from immunized mice were collected as positive sera. In addition, sera from naïve mice were collected as negative sera. Subsequently, BALB/c mice (5 per group) were i.p. challenged with  $1 \times 10^3$  RH $\Delta$ ku80 tachyzoites, and injected with positive or negative sera (200  $\mu$ l/mouse) via the tail vein from day 0 to day 4. Survival of mice was monitored daily and the parasitic load in mouse (5 per group) peritoneal fluid was detected one day after the end of sera treatment.

### Statistical analyses

Statistical analyses in this study were performed with GraphPad Prism version 8 (GraphPad Software Inc, CA, USA). The log-rank (Mantel–Cox) test was used to



**Fig. 1** CRISPR/Cas9-mediated generation of the *ompdc-uprt* deletion mutant in the *Toxoplasma gondii* type I RH strain. **a** Diagnostic PCR of the RH $\Delta$ *uprt*::HXGPRT clone. PCR1 is used to detect drug gene and PCR2 is used to detect *uprt* gene **b** Diagnostic PCR confirming the removal of *hxgp*. PCR1 is used to detect *hxgp* gene, PCR2 is used to detect *uprt* gene and PCR3 is used to detect internal reference 529 bp gene **c** Diagnostic PCR of the RH $\Delta$ *uprt* $\Delta$ *ompdc*::HXGPRT clone. PCR1 is used to detect drug gene, PCR2 and PCR3 is used to detect *ompdc* gene **d** qRT-PCR of the RH $\Delta$ *uprt* $\Delta$ *ompdc*::HXGPRT clone. \*\*\* $P < 0.001$  by Student's *t* test. CRISPR clustered regularly interspaced short palindromic repeats, PCR polymerase chain reaction, HXGPRT hypoxanthine–guanine phosphoribosyltransferase, qRT-PCR quantitative reverse transcription polymerase chain reaction

compare the survival curves. Independent Student's *t*-test was conducted to compare two groups and one-way ANOVA was conducted to compare  $\geq 3$  groups. Quantitative variables were presented as the means  $\pm$  standard deviations (SD). In all analyses,  $P < 0.05$  was considered statistically significant.

## Results

### Generation of the RH $\Delta$ *ompdc* $\Delta$ *uprt* Strains Using the CRISPR/Cas9 System

The HXGPRT marker was inserted into the *uprt*-specific guide RNA-targeted sequence region (Additional file 1: Fig. S1a). The single and stable RH $\Delta$ *ku80* $\Delta$ *uprt*::HXGPRT clone was verified by PCR detection (Fig. 1a). Under the effects of sgRNA of *hxgp* and enzyme Cas9, the HXGPRT marker was deleted (Additional file 1: Fig. S1b), and PCR results indicated that a single RH $\Delta$ *ku80* $\Delta$ *uprt* $\Delta$ *hxgp* strain was successfully selected (Fig. 1b). Subsequently, the *ompdc* gene was replaced by the HXGPRT marker in the RH $\Delta$ *ku80* $\Delta$ *uprt* $\Delta$ *hxgp* strain to yield the RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant (Additional file 1: Fig. S1c), and the stable mutant clone was determined by PCR and qPCR assays (Fig. 1c, d). In summary, the double gene knockout RH $\Delta$ *ku80* $\Delta$ *uprt* $\Delta$ *hxgp* strain was successfully generated.

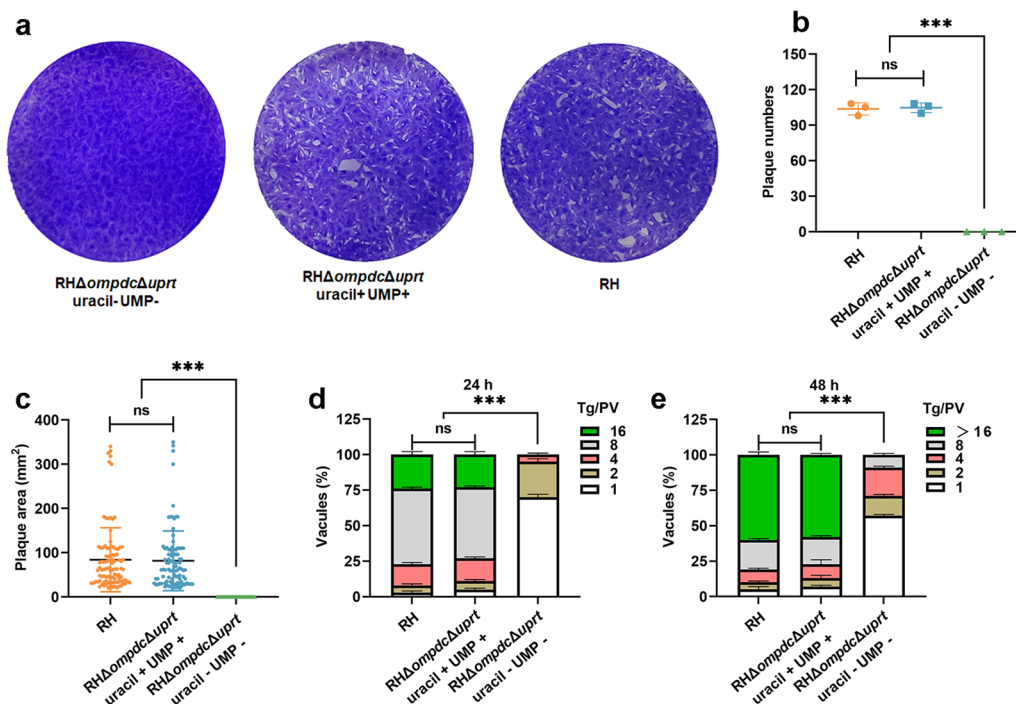
### The RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant exhibited reduced cellular replication ability

To explore the biological characteristics of the RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant, invasion, parasite replication, and plaque assays were carried out. Plaque assay results showed that in the presence of uracil and ump, the mutant could grow as normally as the wild type RH $\Delta$ *ku80* strain to form plaques, while plaques were rarely observed when the uracil and ump were removed

from the culture medium of the RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant (Fig. 2a–c). In addition, the parasite replication assay showed that at 24 h and 48 h post infection, the number of RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant tachyzoites per parasitophorous vacuole (PV) was notably lower ( $P < 0.05$ ) than that of wild type RH $\Delta$ *ku80* tachyzoites in the absence of uracil and ump, and most PVs of this mutant consisted of 1 or 2 tachyzoites indicating a nearly quiescent condition of cellular proliferation (Fig. 2d, e, Additional file 1: Fig. S2). However, the number of RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant tachyzoites per PV presented a similar level to that of the wild type once supplied with uracil and ump, indicating the replication restoration. Furthermore, the results of the invasion assay showed limited differences between the wild type strain and the RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant regardless of whether they were supplied with or without uracil and ump (Additional file 1: Fig. S3), suggesting that the deletion of *ompdc* and *uprt* had no effect on invasion.

### The virulence of the RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant was severely attenuated in mice

The survival of mice was monitored daily, and the results showed that mice injected with the wild-type strain all died within 9 days, while the survival rates of RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant-injected mice were 100%, even when challenged with an infectious high dose of  $10^6$  tachyzoites (Fig. 3a, b), indicating that the virulence of this mutant was significantly attenuated. Subsequently, ascites samples from different groups of mice were collected from 1 to 5 dpi for parasite examination. The parasite number in wild type-infected mice increased remarkably day by day, while that of RH $\Delta$ *ompdc* $\Delta$ *uprt*-injected mice notably decreased ( $P < 0.05$ ) every day (Fig. 3c). Similarly, a large number of *T. gondii* tachyzoites were detected in the liver and lung tissues of RH $\Delta$ *ku80*-injected mice,



**Fig. 2** Deficiency of *ompdc* and *uprt* in the RH strain severely reduced parasitic proliferation in vitro. **a** Plaque assay comparing the growth of the RHΔku80 strain and RHΔku80ΔompdcΔuprt strain with or without the addition of 250 μmol/L uracil and 200 μmol/L UMP. **b–c** Number and size of the plaques. **d–e** Intracellular proliferation of the RHΔku80 strain and RHΔku80ΔompdcΔuprt strain with or without the addition of 250 μmol/L uracil and 200 μmol/L UMP. The number of parasites in each parasitophorous vacuole (PV) was determined at 24 h and 48 h. These results are from three independent trials. \*\*\* $P < 0.001$  by Student's *t* test and one-way ANOVA, ns not significant. UMP uridine monophosphate, PV parasitophorous vacuole

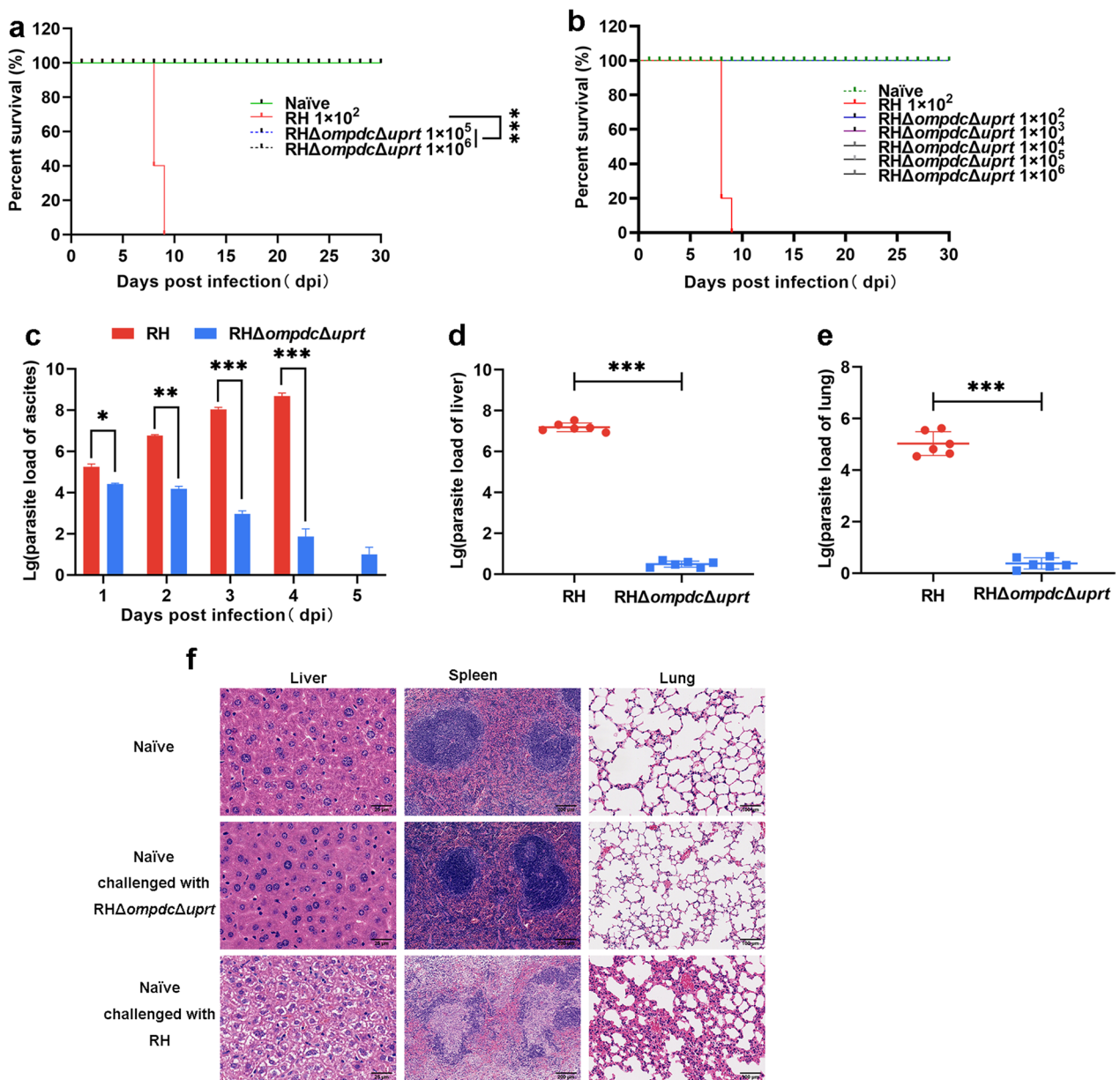
while limited tachyzoites were detected by qPCR in these tissues from RHΔompdcΔuprt-injected mice on the 4th day post infection (Fig. 3d, e). These results demonstrated a significant reduction ( $P < 0.05$ ) in parasite burden caused by the RHΔompdcΔuprt mutant. Next, the histological sections of liver, spleen, and lung tissues were subjected to HE staining for pathological examination. As shown in Fig. 3f, few changes were observed in tissue sections from RHΔompdcΔuprt-injected mice compared with naïve mice, while in wild type infected mice, large amounts of necrotic cells and absent lymphoid follicles were appeared in spleen tissue, obvious cellular separation was displayed in liver, and notably thicker alveolar walls were observed in lung tissues.

#### Robust humoral and cellular immune responses were elicited by vaccination with the RHΔompdcΔuprt mutant in mice

Significantly high levels of anti-*T. gondii* IgG were detected in vaccinated mice, and the IgG titer was increased remarkably ( $P < 0.05$ ) after every vaccination (Fig. 4b), indicating that a robust humoral response was induced. The levels of IgG subclasses (IgG1 and IgG2a) were tested to characterize the immune response type.

Both the levels of IgG1 and IgG2a were significantly higher ( $P < 0.05$ ) in RHΔompdcΔuprt-vaccinated mice than in control mice (Fig. 4c). In addition, the level of IgG2a was notably higher than that of IgG1, indicating that vaccination with RHΔompdcΔuprt in mice elicited a Th1/Th2 mixed and Th1-biased immune response.

After immunization with the RHΔompdcΔuprt mutant, a significant increase ( $P < 0.05$ ) in the percentage of CD3<sup>+</sup>CD4<sup>+</sup> T cells by 2 weeks post vaccination was observed compared with that of the controls (Fig. 4d, e). Additionally, the percentage of CD3<sup>+</sup>CD8<sup>+</sup> T cells in vaccinated mice was increased to a much higher degree ( $P < 0.05$ ) (Fig. 4f, g). In parallel, the rest of splenocytes from vaccinated and unvaccinated mice were cultured in vitro for further stimulation with STAg. The cytokine levels in the splenocyte supernatant were then detected by flow cytometry. Consistent with the levels of IgG1 and IgG2a, the proinflammatory cytokine levels, including Th1-type cytokines (IL-2, IL-12, IFN-γ) and Th2-type cytokines (IL-10, IL-4) of the immunized mice were notably higher (IL-2:  $24.27 \pm 3.35$  pg/ml; IL-12:  $83.83 \pm 14.15$  pg/ml; IFN-γ:  $26.61 \pm 2.68$  ng/ml; IL-10:  $1380 \pm 357.7$  pg/ml; IL-4:  $92.22 \pm 21.46$  pg/ml) than those of the control mice

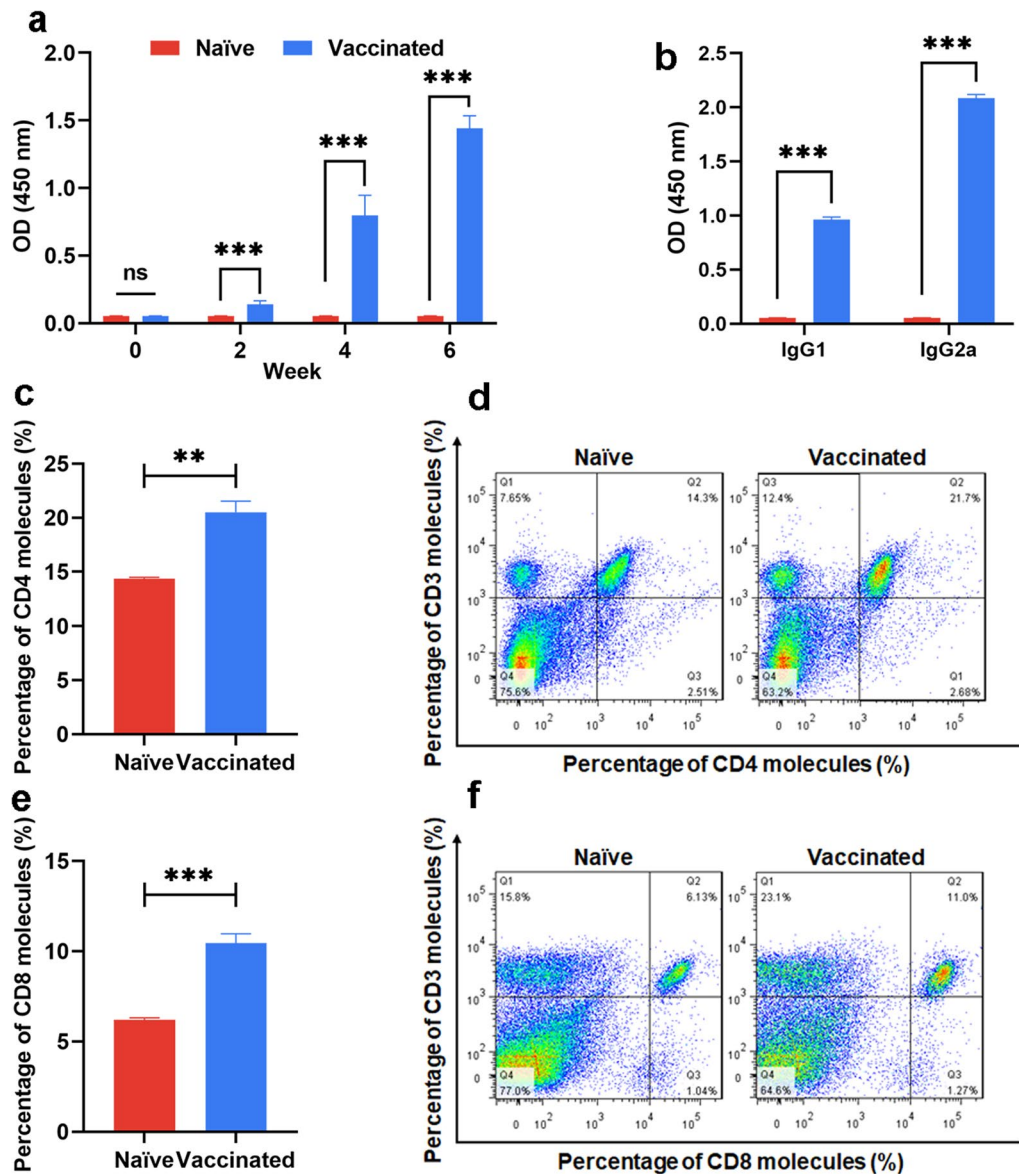


**Fig. 3** The virulence of the RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt mutant was severely attenuated in mice. **a** Tachyzoites of RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt or RH $\Delta$ ku80 tachyzoites were injected intraperitoneally (i.p.) into BALB/c mice ( $n = 5$ ) and monitored for more than 30 d. **b** Tachyzoites of RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt or RH $\Delta$ ku80 tachyzoites were injected i.p. into BALB/c- $\nu$  mice ( $n = 5$ ) and monitored for more than 30 d. **c** The parasite numbers in ascitic fluid of BALB/C mice infected with the  $1 \times 10^6$  RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt strain and RH $\Delta$ ku80 strain from 1 to 5 days. **d–e** The parasite burden of liver and lung tissues infected with the RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt strain and RH $\Delta$ ku80 strain for 4 days. **f** Tissue damage in the spleen, liver and lung of naïve mice inoculated with the RH $\Delta$ ku80 strain and defective strain respectively. The 529 bp gene was detected by qPCR to demonstrate the number of *T. gondii* tachyzoites in each sample. \*\*\* $P < 0.001$  by Student's t test and log-rank (Mantel–Cox) test. i.p. intraperitoneally, *T. gondii* *Toxoplasma gondii*, qPCR quantitative polymerase chain reaction

( $P < 0.05$ ) (Fig. 5a–e). The results also showed a quick and robust proliferation ( $P < 0.05$ ) of splenocytes once stimulated by the STAg (Fig. 5f), indicating the efficient cellular immune response induced by the

immunization of the mutant. Of note, IL-12 and IFN- $\gamma$  which are the key factors in cellular immune clearance of tachyzoites were also found significantly increased ( $P < 0.05$ ) in sera samples of immunized mice and





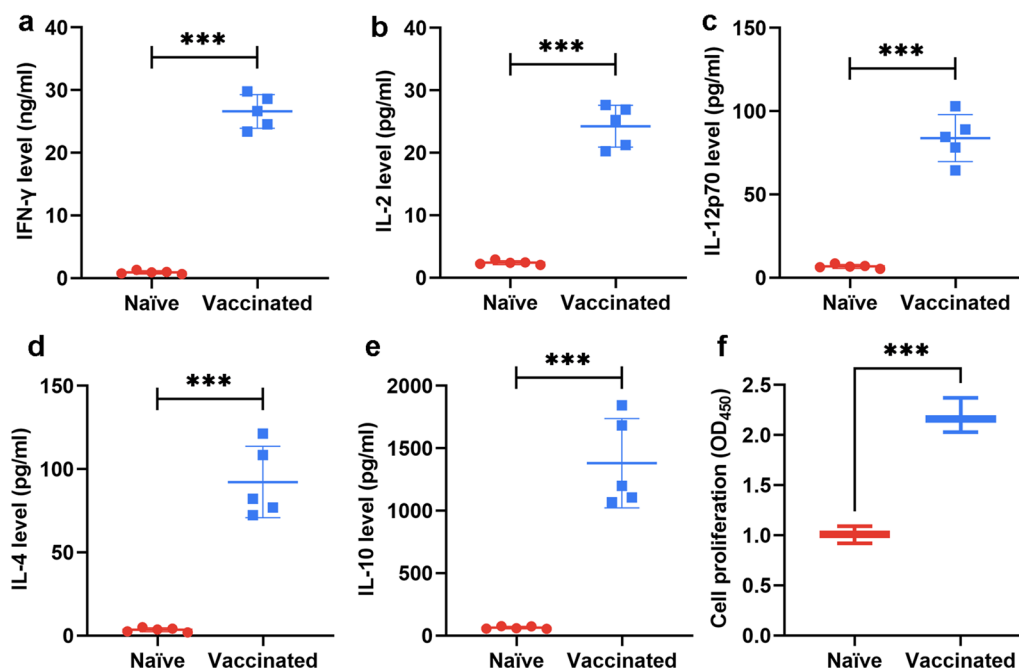
**Fig. 4** Immunization with the RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt mutant vaccine induced specific humoral and cellular responses. **a** Determination of IgG antibodies in the sera of BALB/c mice at 0, 2, 4, and 6 weeks. **b** Detection of antibody subtypes (IgG1 and IgG2a) in the sera of immunized mice 2 weeks after the last immunization. **c–f** Percentages of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells subsets in immunized BALB/c mice. The results are shown as the means  $\pm$  SD from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  \*\*\* $P < 0.001$  by Student's  $t$  test, ns not significant. SD standard deviation

lasted for nearly a week post vaccination (Additional file 1: Fig. S4).

**RH $\Delta$ ompdc $\Delta$ uprt immunization confers protection against infection with various types of *T. gondii* tachyzoites in mice**

Now that the above results demonstrated that the strong immune responses were successfully stimulated, the protective efficacy provoked by the RH $\Delta$ ompdc $\Delta$ uprt mutant was evaluated. Two weeks after the third vaccination,

naïve and vaccinated mice were challenged with lethal doses of type I RH $\Delta$ ku80 strains. All naïve mice died within 10 days, while 100% of the vaccinated mice completely survived (Fig. 6a). In addition, when challenged with type II ME49 tachyzoites or Chinese locally isolated strain WH6, RH $\Delta$ ompdc $\Delta$ uprt mutant-vaccinated mice presented 100% survival, while naïve mice all died within 13 or 14 days post infection (Fig. 6b, c). Subsequently, tissues including liver, spleen, and lung were collected for pathological examination after 6 days (RH $\Delta$ ku80) or



**Fig. 5** Pro-Inflammatory cytokines were elicited by immunization with RH $\Delta$ ku80 $\Delta$ ompdc $\Delta$ uprt. Splenocytes collected from the immunized and non-immunized (6 weeks after immunization) mice were co-incubated with STAg (10  $\mu$ g/ml). The levels of Th1 [IFN- $\gamma$  (a), IL-2 (b), and IL-12 (c)] and Th2 [IL-4 (d) and IL-10 (e)] in the culture supernatants were measured by flow cytometry. (f) The proliferative responses of splenocytes were measured by a CCK8 kit. The results are presented as the means  $\pm$  SD. ( $n = 5$ , \*\*\* $P < 0.001$  by Student's  $t$  test). STAg soluble *Toxoplasma gondii* antigens, CCK Cell counting kit, SD standard deviation

12 days (ME49) of challenged with tachyzoites. Obvious changes were observed in unvaccinated mice, such as liver cell necrosis, spleen body destruction, and lung congestion as described above, while the tissue sections of vaccinated mice showed limited changes (Fig. 6d).

#### The immune protection induced by RH $\Delta$ ompdc $\Delta$ uprt vaccination could be adoptively transferred against acute infection in mice

Mice that received naïve splenocytes or RH $\Delta$ ompdc $\Delta$ uprt-vaccinated CD4<sup>+</sup> T cells succumbed to death by 9 dpi (Fig. 6e), in contrast, the passive immunization of purified CD19<sup>+</sup> B cells, CD8<sup>+</sup> T cells, or total splenocytes from RH $\Delta$ ompdc $\Delta$ uprt-vaccinated mice survived a significantly longer ( $P < 0.05$ ) time than naïve mice (Fig. 6e). The data showed a relative but significantly longer ( $P < 0.05$ ) survival rate in passively immunized mice than in control mice (Fig. 6f). Consistent with this result, a significantly lower parasite load was observed in passively immunized mice (Fig. 6g).

#### Vaccination with the RH $\Delta$ ompdc $\Delta$ uprt mutant was determined to be safe in cats and to induce a robust immune response

As showed in Table 2S, no oocysts were found in any of the RH $\Delta$ ompdc $\Delta$ uprt-injected cats. Then, the immune

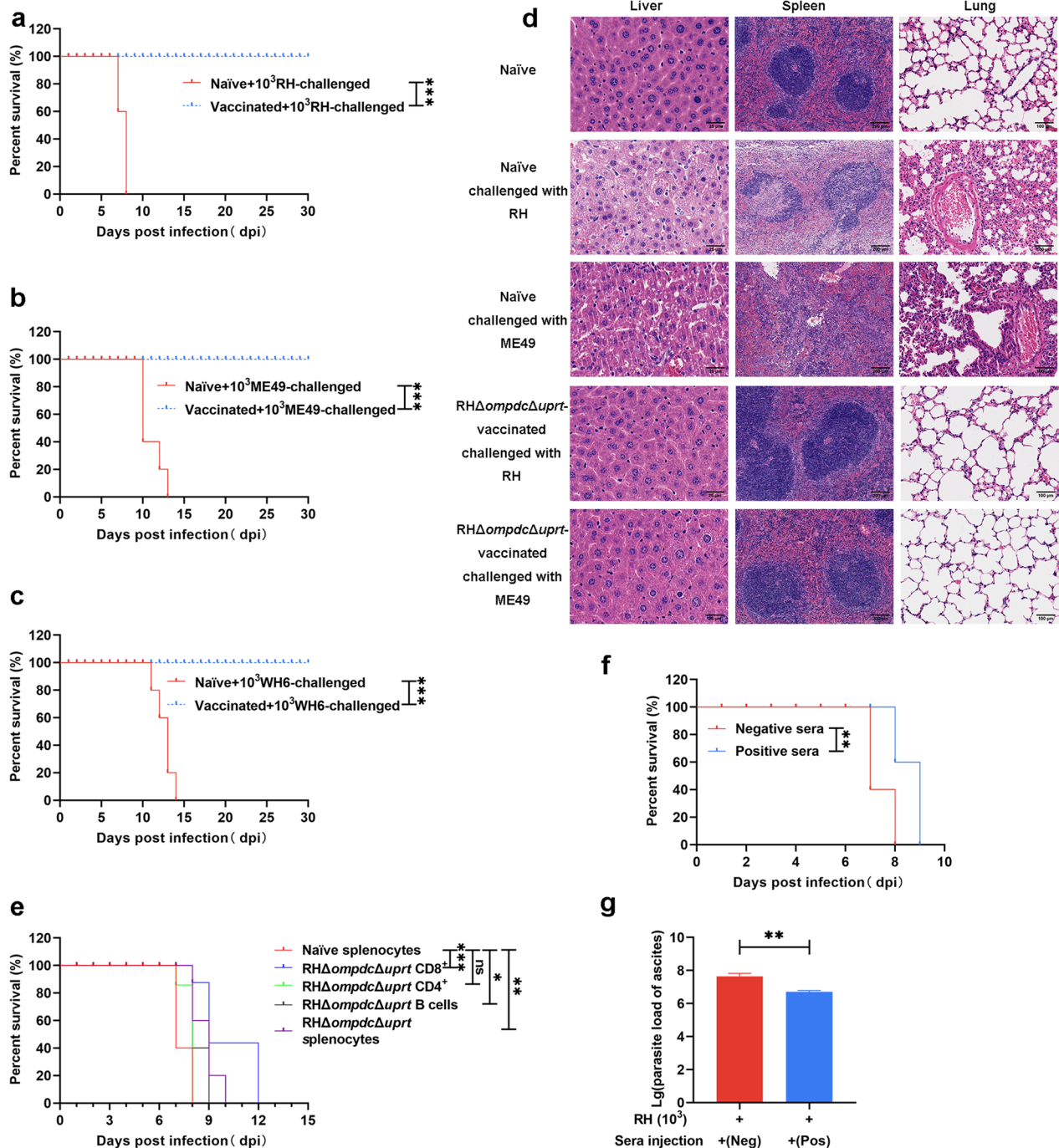
response in cats was further evaluated. After vaccination for 2 doses, sera obtained from cats were used to determine the specific antibody response. Significantly high levels of anti-*T. gondii* IgG were detected in vaccinated cats, and the IgG titer was increased remarkably ( $P < 0.05$ ) after every vaccination (Fig. 7a), indicating that a robust humoral response was induced by the mutant.

#### RH $\Delta$ ompdc $\Delta$ uprt-vaccinated cats notably reduced the oocyst shed number and period

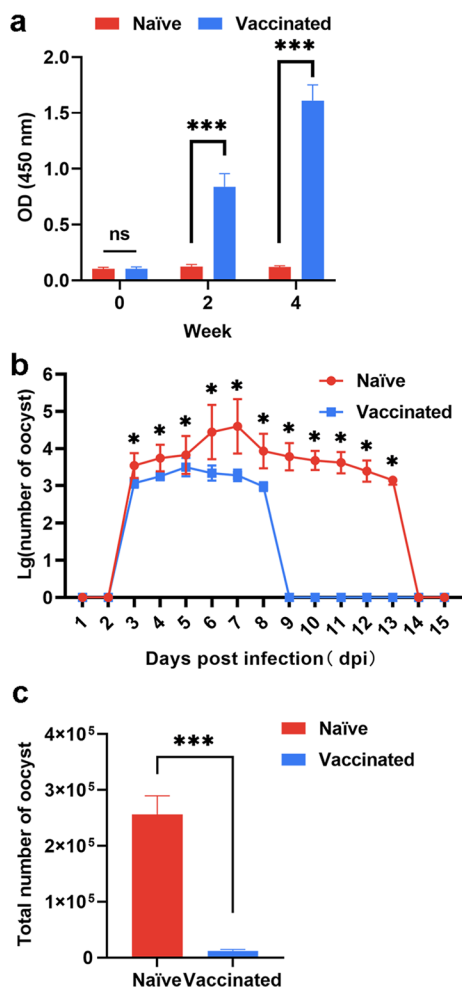
To evaluate the possibility of applying the vaccine in cats, we performed a challenge and tested the expulsion of oocysts. Oocysts were detectable at 3 dpi for all-infected cats, while RH $\Delta$ ompdc $\Delta$ uprt-vaccinated cats notably reduced ( $P < 0.05$ ) the shedding period (6 days) compared with that of naïve cats (11 days) as shown in Fig. 7b. Furthermore, the results showed a remarkable decrease ( $P < 0.05$ ) in the total number of oocysts (95.3%) in vaccinated cats compared with naïve cats (Fig. 7c).

#### Discussion

Control of *T. gondii* is a major challenge since the parasite can cross the blood–brain barrier to develop a persistent infection where most chemicals are inaccessible [31]. Currently, the combination of pyrimethamine and sulfadiazine is commonly used clinically for the treatment



**Fig. 6** Protective immunity induced by the RHΔku80ΔompdcΔuprt vaccine against *T. gondii* challenge infection in mice. **a–c** Survival curves of naïve or RHΔku80ΔompdcΔuprt immunized mice infected with RHΔku80, ME49 and WH6. Two weeks after the last immunization, mice were intraperitoneally (i.p.) challenged with  $1 \times 10^3$  *T. gondii* tachyzoites of RHΔku80, ME49 and WH6 strains (5 mice/strain), and monitored for 30 days. **d** Organ damage in vaccinated mice and naïve mice after challenge with the RHΔku80 and ME49 strains. **e** Two weeks after the last immunization, whole splenocytes, CD4<sup>+</sup>, CD8<sup>+</sup> and CD19<sup>+</sup> splenocytes were harvested, and  $1 \times 10^7$  total immune splenocytes,  $2 \times 10^6$  CD8<sup>+</sup> T cells,  $2 \times 10^6$  CD4<sup>+</sup> T cells,  $2 \times 10^6$  CD19<sup>+</sup> B cells, or  $1 \times 10^7$  total naïve splenocytes were transferred to naïve recipient mice. Twenty-four hours after transfer mice were challenged with  $1 \times 10^3$  *T. gondii* RHΔku80 i.p. and monitored for survival. **f** From day 0 to day 4 after infection, mice were treated with sera from RHΔku80ΔompdcΔuprt-vaccinated and naïve mice. **g** Parasite load detection after one day of sera treatment. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by log-rank (Mantel–Cox) test, ns not significant



**Fig. 7** Protective immunity induced by the RHΔku80ΔompdcΔuprt vaccine against *T. gondii* challenge infection in cats. **a** Determination of IgG antibodies in the sera of cats at 0, 2 and 4 weeks. **b** Daily oocyst emissions of naïve or RHΔku80ΔompdcΔuprt immunized cats orally administered 200 cysts of ME49. **c** Total oocyst emissions of naïve or RHΔku80ΔompdcΔuprt immunized cats orally administered 200 cysts of ME49. (n = 6, \*P < 0.05, \*\*\*P < 0.001 by Student's *t* test, ns not significant.)

of acute toxoplasmosis [32]. However, these drugs have limited effects on *T. gondii* cysts. It has become a popular theory that preventative strategies such as vaccination are a more effective way to provide protection against *T. gondii*. Attempts to develop vaccines over the last 60 years have acquired a few successful experimental candidates, among which live attenuated vaccines are the most promising ones. Live attenuated vaccines are non-disease cause mutants created by weakening or altering the pathogen. In this study, we applied the double gene knockout strain RHΔompdcΔuprt as a live attenuated vaccine and explored the induction of protective immunity against *T. gondii* challenge.

Undoubtedly, safety and effectiveness are the primary factors for a vaccine. Currently, there is only one vaccine called *Toxovax* on the market specifically derived from the S48 strain to reduce fetal abortion in sheep. However, this vaccine is not safe enough to use in humans due to the possibility that the strain may revert the ability to form cysts as the data show that it could cause acute infection and lead to death in murine models [33]. Recently, several studies with emphasis on gene modified mutants proved that robust protective immune responses had been elicited by these vaccines, such as RHΔgra17Δntp1 [17] and ME49Δldh [34] and the uracil auxotroph mutants (ΔcpsII and Δompdc) [20, 23]. With defective de novo UMP biosynthesis activity, uracil auxotroph mutants lose the ability to propagate in vivo and induce long-term protection against acute and chronic *T. gondii* infection. However, we cannot ignore the fact that their protection is not always 100%. As reported by Peng [24], the Δompdc strain supplied without uracil could still replicate relatively slow but never stop way in vitro, indicating that the salvage pathway may play a compensatory role. Although they concluded that in ompdc disrupted strains, the potential salvage of host cell uracil and nucleosides is not sufficient to support a significant rate of parasite replication in vitro [23], safety cannot be guaranteed when this live attenuated vaccine is used in meat producing animals or cats. Thus, according to the UMP synthesis and salvage pathways, we specifically deleted the ompdc and uprt through a CRISPR/Cas9 system to completely block the production or intake of UMP. Consistent with the predicted model, the RHΔompdcΔuprt strain lost viability in vitro, as confirmed by parasite replication and plaque assays. Furthermore, no deaths were observed when the RHΔompdcΔuprt strains were subjected to BALB/c or BALB/c-nu mice, demonstrating that this strain is safe in immunodeficient individuals. As documented and evaluated here, the tachyzoites could be found in ascitic fluids as early as 1 dpi in acute infection mice and parasite loads in tissues such as liver and lung increased notably [35]. However, we found a rather small number of parasites in ascitic fluids and tissues, which means that the RHΔompdcΔuprt strain is avirulent to mice. Additional visual evidence is that limited pathological changes or tachyzoites were found in histological sections from RHΔompdcΔuprt strain injected mice. To our knowledge, once infected by *T. gondii*, mouse tissues display obvious changes, such as clear cellular separation in the liver indicating hepatocellular dysfunction, thicker alveolar walls in the lung representing interstitial pneumonia, and plenty of necrotic cells in the spleen with absent lymphoid follicles [36]. Thus, this avirulent RHΔompdcΔuprt strain is nearly harmless to mice. In addition, the daily analysis of cat feces after vaccination

with the RH $\Delta ompdc\Delta uprt$  strain revealed no oocysts, indicating that this attenuated mutant also lost the ability of sexual reproduction compared with previous results [37]. Consequently, we conclude that the attenuated strain is unable to reproduce in mouse and cat models with limited harm or side effects.

Effectiveness is a more crucial factor for an ideal vaccine. We noticed that the type of attenuated *T. gondii* strain for vaccine development is also of great importance. As reported by Xia et al. [34], the ME49 $\Delta ldh$  mutant induced long-term protection against type 2 and 3 strains, while short-term protection against the type 1 RH strain. Therefore, we chose the RH strain to construct the live attenuated vaccine. Consistently, this defective vaccine contributed 100% protection against the RH $\Delta ku80$ , ME49 and WH6 strains through a humoral and cellular mixed immune response in a murine model. In consideration of the data, we gathered above that RH $\Delta ompdc\Delta uprt$  would be cleared within 1 week, we proceeded with a three-dose inoculation procedure rather than a single inoculation procedure, as reported by others [14, 22, 24, 34] to achieve a higher and longer immune response. As expected, high levels of specific anti-*T. gondii* IgG antibodies were gradually induced by the RH $\Delta ompdc\Delta uprt$  strain, particularly both IgG2a and IgG1 were significantly increased indicating a mixed Th1/Th2 immune response which is consistent with other live attenuated *T. gondii* vaccines [17, 38, 39]. These specific antibodies play a protective role by neutralizing the attachment of *T. gondii*. [40–43]. In addition, the level of IgG2a was significantly higher than that of IgG1, suggesting that the immunized mice successfully induced a significant Th1-type humoral immune response. This is consistent with previous reports that IgG2a is more efficient than IgG1 in clearing *T. gondii* tachyzoites [44]. In addition, we demonstrated that the adoptive transfer of sera from RH $\Delta ompdc\Delta uprt$  immunized mice to naïve recipients provided short-term protection against virulent challenge. The protection of sera is most likely provided by the IFN- $\gamma$  and IL-12p70 cytokines since we observed that they were significantly induced by the injection of the RH $\Delta ompdc\Delta uprt$  strain and sustained for a quite long period, but on the other hand, the eventual death of mice was probably due to the depletion of cytokines succumbing to virulent challenge.

Understanding the mechanism of immunity is essential for the development of vaccines. Since immunity has been correlated with a Th1 biased inflammatory response to *T. gondii*, most vaccines attempt to achieve a higher Th1 response. Evidence from other studies particularly supports that CD8 $^+$  T cells and IFN- $\gamma$  are absolutely essential to combat *T. gondii* infection. Our analysis indicated that both CD4 $^+$  and CD8 $^+$  T cell subsets were

recruited by RH $\Delta ompdc\Delta uprt$ . Despite the fact that both CD4 $^+$  and CD8 $^+$  T cells correspond to the production of IFN- $\gamma$ , infected mice developed partial protection against RH $\Delta ku80$  challenge provided by passive immunization of purified CD8 $^+$  T cells instead of CD4 $^+$  T cells. Interestingly, we also established that adoptive transfer of purified B cells to naïve recipients provided protection against virulent challenge. However, the protective effectiveness of cells, as well as sera, from RH $\Delta ku80$ -infected mice remained unclear since these passive immunizations were difficult to carry out due to the persistent presence of parasites in these cells that could result in *T. gondii* infection. Collectively with the results of the humoral protective immune response above, we assume that protective immunity would be enhanced by memory B cells through a more efficient antigen processing and presentation producer via multi-point inoculation. Therefore, a CD8 $^+$  cells dominant and B cells required immunity was elicited in response to vaccination with RH $\Delta ompdc\Delta uprt$  for the protection of *T. gondii*.

IFN- $\gamma$  can induce various intracellular mechanisms to kill parasites or inhibit their replication and proliferation [45–47]. We observed that the expression levels of IFN- $\gamma$  in the culture supernatant of splenocytes and sera from the vaccinated mice were remarkably higher than those of the unvaccinated mice, which in turn provided effective protection against a high dose virulent challenge. Correspondingly, we found that mice vaccinated with RH $\Delta ompdc\Delta uprt$  exhibited 100% protection against type I, type II, or Chinese isolated strains, in accordance with the protective effects of the  $\Delta gra7\Delta npt1$  and  $\Delta cpsII$  strains, while those immunized with ME49 $\Delta ldh$  or ME49 $\Delta cdpk3$  [48] only provided short-term protection against the type I RH strain. In addition, we also revealed that RH $\Delta ompdc\Delta uprt$  can induce and sustain significant IL-12p70 production, indicating that the innate immune response is also activated. IL-12p70 is mainly produced by B cells and neutrophils, as reported by Gigley [39]. Infection with live attenuated tachyzoites recruits neutrophils early and acts as an innate effector cell, resulting in the early secretion of IL-12 to destroy intracellular parasites by autophagy. Our data indicate a rapid increase in IL-12p70 and a daily decrease in response to vaccination with RH $\Delta ompdc\Delta uprt$ , while IFN- $\gamma$  presents a gradually increasing pattern with a peak production level at 4 dpi. However, other studies have shown that excessive levels of Th1 inflammatory factors may also cause pathological damage to mice or even lead to the death of mice [49, 50]. At this time, IL-10 and IL-4 are needed to regulate the inflammatory response during *T. gondii* infection [51–53]. In accordance with this theory, we discovered that Th2-type cytokines (IL-4 and IL-10) were notably increased in response to RH $\Delta ompdc\Delta uprt$  infection

to balance the high production of Th1 cytokines. As the results, immunized mice developed immune profiles capable of clearing high doses of type I and type II *T. gondii* tachyzoites challenge with limited pathological change.

It is well known that as the definitive host, cats infected with *T. gondii* can excrete a large number of infective oocysts with feces lasting for about two weeks, while there are few studies on anti-*T. gondii* vaccines in cats. Given the crucial role of cats in the transmission of *T. gondii*, there is an urgent need to develop vaccines for cats. The mutant of the *T. gondii* bradyzoite (T-263) was the first report of a feline *T. gondii* vaccine [54]. By oral administration of T-263 bradyzoites obtained from brain cysts, 84% of immunized cats excreted no oocysts [54], while immunization with T-263 tachyzoites did not completely induce protective immunity against oocyst shedding [55]. We should not ignore that vaccination with bradyzoites from brain cysts is impractical in clinical use in consideration of production and cost. Later, some studies have shown that rhoptry protein vaccines could induce a 67% preventable fraction of oocysts in cats [56]. In the present study, we found that cats immunized with this live attenuated strain developed high antibody titers and showed a 95.3% reduction in oocyst shedding after challenge. Of note, all cats were successfully immunized with a significantly shorter period of oocyst shedding indicating effective protection against toxoplasmosis in cats.

Although RH $\Delta$ *ompdc* $\Delta$ *uprt* strains as attenuated live vaccine showed a protective effect against feline toxoplasmosis, they failed to reach the 100% blocking level of oocyst, which may be related to the immune dose level, immune frequency and immune interval. In the future, we expect to further enhance immune protection by optimizing the immune program.

## Conclusions

Our study shows that the attenuated *ompdc-uprt* double knockout strains from the RH strain of *T. gondii* are safe and avirulent, can protect mice from challenge with high doses of RH $\Delta$ *ku80*, ME49 and WH6 strain tachyzoites and can allow the cat to reduce the excretion of oocysts. These data suggest that the RH $\Delta$ *ompdc* $\Delta$ *uprt* mutant has the potential to be used as a candidate for a live attenuated vaccine. Although this mutant strain has great potential as a vaccine in mice, we also need to further study whether this vaccine has similar efficacy in other animals.

## Abbreviations

OMPDC Orotidine—5'—monophosphate decarboxylase  
UPRT Uracil phosphoribosyltransferase

UMP	Uridine monophosphate
HFFs	Human foreskin fibroblasts
DMEM	Dulbecco's Modified Eagle medium
i.p.	Intraperitoneally
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
IFA	Immunofluorescence assay
ELISA	Enzyme-linked immunosorbent assay
TMB	3,3',5,5'-Tetramethylbenzidine
CBA	Cytometric bead array
CCK-8	Cell counting kit 8
PV	Parasitophorous vacuole

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40249-023-01109-9>.

**Additional file 1: Table S1.** The primers used in this study. **Table S2.** This table shows the oocysts in the feces of cats from 1 to 10 days after being infected with RH $\Delta$ *ompdc* $\Delta$ *uprt* strain. **Figure S1.** The diagram of knocking out. (a) Schematic illustration of knocking out *uprt* by homologous gene replacement in RH $\Delta$ *ku80* $\Delta$ *hxgprt* strain. (b) Schematic showing deletion of *hxgprt* gene by insertion of a *uprt*5'utr-3'utr into *hxgprt* gene. (c) Diagram illustrating the deletion of *ompdc* in RH $\Delta$ *uprt* $\Delta$ *hxgprt* to make the double mutant RH $\Delta$ *uprt* $\Delta$ *ompdc*:HXGPRT by CRISPR/Cas9-mediated homologous gene replacement. **Figure S2.** Intracellular proliferation of OMPDC-UPRT deletion mutant under the fluorescence microscope. (a) 24 hours of intracellular proliferation. (b) 48 hours of intracellular proliferation. **Figure S3.** Invasion and attachment assay for RH $\Delta$ *ku80* and RH $\Delta$ *ompdc* $\Delta$ *uprt* strains. (a) The invasion and attachment of *T. gondii* RH $\Delta$ *ku80* and RH $\Delta$ *ompdc* $\Delta$ *uprt* were evaluated by indirect immunofluorescence assay. (b) The analysis of the invasion and attachment. **Figure S4.** The levels of cytokines in the serum of mice injected with RH $\Delta$ *ompdc* $\Delta$ *uprt*. The levels of IFN- $\gamma$  (a) and IL-12 (b) were measured by flow cytometer.

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## Author contributions

SL, XZ, YS and BZ designed the experiments. YS, BZ, HS, SW, JF, JD, MG, QK, DL and HD performed the experiments. YS, BZ and HS collected and analyzed the data. YS made major contributions to the writing of the manuscript. SL and XZ revised the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

The original data that supports the conclusions of this article are presented in the article or in the supplementary information.

## Declarations

### Ethics approval and consent to participate

Animal experiments were approved by the Animal Care and Use Committee of Hangzhou Medical College (2018-027).

### Consent for publication

Not applicable.

**Competing interests**

The authors declare no competing interests.

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