

Characterization of exosomes derived from *Toxoplasma gondii* and their functions in modulating immune responses

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Introduction: Exosomes are nanograde membrane-bound vesicles secreted from most cell types through the fusion of multivesicular bodies with plasma membranes. Some of these exosomes are well defined, and are known to have immunomodulatory properties as well as play critical roles in intercellular communications. In this study, we characterized the exosomes derived from *Toxoplasma gondii* and their functions in aspect of immune responses.

Methods: *T. gondii* exosomes were isolated and identified using electron microscopy, nanoparticle tracking analysis, and Western blotting. The viability of macrophage RAW264.7 cells affected by exosomes was evaluated using a Cell Counting Kit (CKK-8). Then the uptake of *T. gondii* exosomes by RAW264.7 cells was detected by labeling with fluorescent dye PKH67. After exosomes stimulation, in vitro the production of interleukin (IL)-12, tumor necrosis factor (TNF)- α , interferon (IFN)- γ and IL-10 in RAW264.7 cells were investigated using enzyme-linked immunosorbent assay (ELISA). In immunized BALB/c mice, the antibodies, cytokines as well as the percentage of CD4+ and CD8+ T cells were determined using ELISA and flow cytometric analysis. Protective efficacy was evaluated by challenging intraperitoneally with tachyzoites of *T. gondii*.

Results: We successfully isolated and characterized the exosomes derived from *T. gondii*. Functionally, the viability of macrophage RAW264.7 cells was significantly affected by exosomes at a high concentration (160 $\mu\text{g}/\text{mL}$). The production of IL-12, TNF- α and IFN- γ in macrophage cells were increased, and the level of IL-10 was decreased. Furthermore, BALB/c mice immunized with *T. gondii* exosomes showed both humoral and cellular immune responses and also exhibited a prolonged survival time.

Conclusion: *T. gondii* exosomes could modulate macrophage activation in vitro and trigger humoral and cellular immune responses and partial protection against acute parasite infection in mice, which suggested that exosomes may serve as a potential candidate against toxoplasmosis.

Keywords: *Toxoplasma gondii*, exosomes, macrophage, mouse, immune response

Introduction

Toxoplasma gondii is an obligate intracellular apicomplexan parasite which infects all warm-blooded mammals as well as birds and causes zoonotic toxoplasmosis.^{1,2} Approximately one-third of the world populations are infected with *T. gondii* and the majority of those infected are benign or asymptomatic. Even more serious, *T. gondii* infection can cause severe diseases in immunocompromised individuals with HIV/AIDS or those who are being treated with chemotherapy.^{2,3} Furthermore, *T. gondii* infection causes congenital disease and abortions in human and domestic animals.^{4,5} Currently, treatment of toxoplasmosis depends primarily on chemotherapy. However, available drugs like sulfadiazine sodium have serious side effects, and toxoplasmosis

may recur at any time.² Although there is a commercial vaccine against *T. gondii* infection in sheep, which is effective in inducing the incidence of abortion,⁶ the vaccines against human toxoplasmosis are still unavailable.

Exosomes are tiny membrane-bound vesicles, approximately 30–100 nm in size, secreted from most cell types through the fusion of multivesicular bodies with plasma membrane. The vesicles contain a diverse suite of biomolecules including proteins, lipids, and nucleic acids, and some of them are known to have immunomodulatory properties.^{7,8} Moreover, as vehicles for delivery of cargos to recipient cells, exosomes are thought to play critical roles in intercellular communications.^{9,10} To date, numerous exosomes have been identified in both hematopoietic and non-hematopoietic cells, tumor cells, and microbes. Although the properties of exosomes vary depending on their biogenesis, their functions remain largely enigmatic.

In the past few years, several protozoan parasites including *Leishmania* species, *Trypanosoma* species, *Plasmodium falciparum*, and *Trichomonas vaginalis* have been found to release exosomes and/or microvesicles.⁸ Studies show that some exosomes secreted by these parasites have many physiological functions and are involved in many crucial biological activities. For instance, *Leishmania* exosomes could modulate the innate and adaptive immune responses in C57BL/6 mice by affecting monocytes and dendritic cells.¹¹ In addition, these exosomes could deliver effector cargoes to the target cells during parasite early invasion via the host immunosuppression.¹² Exosomes secreted by *T. vaginalis* have been found to play important roles in material exchanges and parasite adherence.¹³ A recent study by Wowk et al disclosed the first proteomic profiling of *T. gondii* extracellular vesicles (EVs) containing a broad range of canonical exosome proteins, however, the biological functions of exosomes from *T. gondii* are still largely unknown.¹⁴

Herein, we described the isolation and characterization of exosomes from *T. gondii* RH and ME49 strain in their biophysical and biological properties. Functionally, we found that *T. gondii* exosomes could modulate macrophage activation in vitro. Furthermore, *T. gondii* exosomes could trigger both humoral and cellular immune responses, and arouse partial protective immunity against acute *T. gondii* infection in BALB/c mice.

Materials and methods

Ethical statement

All animal care procedures were conducted in strict accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China. The study was approved by the

Institutional Animal Care and Use Committee of Shandong University under Contract LL201602044, and all possible efforts were made to minimize distress on animals and the numbers of mice required for each experiment. Humane endpoints are considered to avoid the animals pain or suffering via euthanasia. Mice were monitored daily for signs of toxoplasmosis, such as fatigue, difficulty in feeding and severe ascites.

Animal, parasite and cell

Specific-pathogen-free (SPF) female BALB/c mice 6–8 weeks old were purchased from the Animal Centre of Shandong University, People's Republic of China. All the mice were allowed access to feeding and tap water ad libitum and were handled in strict accordance with good animal practice according to the animal ethics procedures.

The highly virulent tachyzoites of *T. gondii* RH strain were preserved in our laboratory at the School of Medicine, Shandong University, Shandong, People's Republic of China. The low virulent tachyzoites of *T. gondii* ME 49 strain were generously provided by the Department of Parasitology, Military Medical Sciences of the Military Veterinary Institute, Jilin, People's Republic of China. These two strains were used to prepare *T. gondii* exosomes and soluble tachyzoite antigens (STAg).

HEK293T cell line (Shanghai Institutes for Biological Sciences [SBIS], Shanghai, People's Republic of China) and RAW 264.7 macrophage cell line (SIBS) were maintained in complete medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin, at 37°C, in a humidified chamber with 5% CO₂.

Preparation of STAg

Parasites were maintained by serial passage in confluent monolayers of human foreskin fibroblasts (HFF) under standard *T. gondii* culture conditions. As previously described, egressed parasites were harvested for the STAg preparation.¹⁵ In brief, the pelleted tachyzoites were resuspended in 0.1 M phosphate buffered saline (PBS; pH 7.4) and freeze–thawed three times. After that, the lysate was sonicated on ice at 60 W/s and centrifuged at 10,000×g for 30 min. The supernatant containing STAg for protein quantification was determined via the Bradford method and stored at –80°C until use.

Isolation of exosomes

Approximately, 100 mL of *T. gondii* culture media was performed for isolating exosomes using ExoEasy Maxi Kit (Qiagen, Hilden, Germany). Briefly, the collected tachyzoites of *T. gondii* were washed three times with sterile PBS, and centrifuged at 5,000×g for 10 min, and resuspended in sterile

PBS. Then, the parasites were cultured in Dulbecco's Modified Eagle's Medium (DMEM) without serum for 24 h. After that, 100 mL of supernatants was filtered through 0.22 μm membrane to remove the cell debris, potential membrane fragments from lysed cells and large vesicles (>500 nm). The filtrates were concentrated using a filter unit of Vivaflow 100 kDa MWCO PES (EMD Millipore, Billerica, MA, USA) to 10 mL. Then, concentrated *T. gondii* culture media were precipitated for exosomes using ExoEasy Maxi Kit according to the manufacturer's instruction. To assess the concentration and size of exosomes, the exosome-sample was evaluated by nanoparticle tracking analysis (NTA) using the NanoSight NS300 (Malvern Instruments, Malvern, UK), and the sample was aliquoted (50 $\mu\text{g}/\text{vial}$) and stored at -80°C until use.

Electron microscopy

Exosomes to be examined by transmission electron microscopy (TEM) were isolated and coated on to a formvar/carbon grid as previously described.¹⁶ Briefly, the exosomal samples were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer at pH 7.3 for 3 h at room temperature. Then, the grids were air dried and visualized using a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan) at 100 kV.

Western blotting

The exosomal samples (20 μg) were boiled at 100°C for 5 min, subsequently separated in 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) for 30 min and transferred to a polyvinylidene difluoride (PVDF) membrane for 110 min. Then, the membrane was blocked with 5% non-fat skim milk for 2 h at room temperature and probed with monoclonal antibody anti-P30 (Abcam, Cambridge, UK), anti-CD63 (Abcam) and anti-Hsp 70 (Abcam), respectively, at 4°C overnight. Two different HRP-conjugated antibodies were used: Goat anti-mouse IgG antibody (1:10,000; Vazyme, Nanjing, People's Republic of China) and goat anti-Rabbit IgG antibody (1:7,500; EarthOx, San Francisco, CA, USA) as secondary antibody. Finally, after washing three times with phosphate buffered saline with Tween-20 (PBST), the membrane was soaked in DAB Reagents (Boshide Biotech Co, Wuhan, People's Republic of China) for signal development.

Uptake of *T. gondii* exosomes by macrophages

To examine whether *T. gondii* exosomes can be taken up by macrophages, the exosomes were labeled with the green fluorescent dye PKH67 (Sigma-Aldrich Co., St Louis, MO, USA) according to the manufacturer's directions with minor

modifications. Briefly, the *T. gondii* exosomes were diluted and suspended in sterile PBS to a final concentration of 100 $\mu\text{g}/\text{mL}$. PKH67 dye (1.6 μL) was diluted in 500 μL Diluent C (PKH67 solution). Then, 500 μL PKH67 solution and 200 μL diluent exosomes were mixed in a 1.5 mL centrifugation tube. Samples were mixed gently for 5 min at 37°C , and 800 μL 1% bovine serum albumin (BSA) was added to bind the excess PKH67 dye. The PKH67-stained exosomes were washed three times in sterile PBS to remove excess dye and collected by ExoEasy Maxi Kit. Finally, PKH67-labeled exosomes were resuspended in DMEM. Macrophages were cultured on coverslips in 24-well plates at a mean of 3×10^5 cells/well. After 48 h incubation at 37°C in an atmosphere of 5% CO_2 with complete medium, the slides were washed three times in PBS to eliminate the influence of serum exosomes, and each medium containing 10 μL of the PKH67-labeled exosomes or the same volume of the PKH67-PBS control was added into each well. Then, cells and *T. gondii* exosomes were cultured for 2 h, and the coverslips were gently washed with PBS and fixed with 4% paraformaldehyde solution for 15 min at room temperature and washed three times with PBS. For the fluorescence microscopy, nucleus staining was performed using a Pro-Long Gold Antifade Reagent with DAPI (4',6-diamidino-2-phenylindole; Solarbio, Beijing, People's Republic of China). Finally, the coverslips were removed from the wells and placed on slides for viewing on a fluorescence microscope (Carl Zeiss Meditec AG, Jena, Germany), operated by Axiovision software (Carl Zeiss Meditec AG).

Macrophage viability assay

The cell viability in vitro was evaluated using a Cell Counting Kit (CCK-8) purchased from Dojindo Laboratories, Kumamoto, Japan according to manufacturer's directions. Briefly, exosomes were diluted to a concentration of 10 $\mu\text{g}/\text{mL}$, 20 $\mu\text{g}/\text{mL}$, 40 $\mu\text{g}/\text{mL}$, 80 $\mu\text{g}/\text{mL}$ and 160 $\mu\text{g}/\text{mL}$, respectively, in sterile PBS. The macrophage RAW264.7 cells were plated in 48-well plates (5×10^6 cells/well), and incubated at 37°C for 24 h under 5% CO_2 . The cells were stimulated with 100 μL exosomes at different concentrations or PBS alone, and incubated under the same conditions for an additional 24 h. After incubation, 10 μL of CCK-8 reagent was added to each well and incubation continued for another 4 h. Finally, the absorbance was measured at 450 nm using an automatic enzyme analyzer (Allsheng, Hangzhou, People's Republic of China).

Macrophage cytokine assay

The macrophages were obtained as described above and cultured in triplicate in 48-well plates (5×10^6 cells/well).

Then, macrophages were incubated with 1×10^5 parasites, 10 μg *T. gondii* exosomes, 10 μg BSA, and 50 μL sterile PBS, respectively. After 24 h incubation, the cell-free supernatants were collected and analyzed for interleukin-10 (IL-10), IL-12, interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α). The assays were determined by specific ELISA assay kits (Bio-Swamp, Wuhan, People's Republic of China) according to the manufacturer's directions.

Immunization and challenge

To assess the immunogenicity of *T. gondii* exosomes, BALB/c mice were randomly assigned into four groups (14 per group). Before inoculation, *T. gondii* exosomes were diluted and suspended in sterile PBS to a final concentration of 100 $\mu\text{g}/\text{mL}$. Three groups of mice were injected intramuscularly with 100 μL *T. gondii* exosomes from RH strain (Exo-R), ME 49 strain (Exo-M) or PBS, respectively. Another group of 14 mice were set as blank controls without injection. All groups of mice were immunized three times on days 0, 14 and 28 using the same dosages. Blood was collected through retro-orbital plexus puncture before each immunization and 2 weeks after the final immunization (on day 42), and stored at -20°C until used for serological tests.

Two weeks after the last immunization (on day 42), four mice per group were sacrificed and their splenocytes were aseptically harvested for cytokine detection and flow cytometric analysis. The other ten mice in each group were challenged with 1×10^3 tachyzoites of *T. gondii* RH strain (PBS or Exo-R group) or ME-49 strain (Blank control or Exo-M group).

Antibody assay

The levels of antibody against *T. gondii* (total IgG, IgG1, IgG2a) in mouse sera were determined using ELISA assay as previously described.¹⁶ Briefly, STAg was diluted to 12.5 $\mu\text{g}/\text{mL}$ in 50 mM carbonate buffer (pH 9.6) and divided into a 96-well enzyme panel with 50 μL per well at 4°C overnight. Then, the plates were washed three times with PBST. The serum samples were diluted in 1% PBST (1:100) and 50 μL placed into each well. Plates were incubated at 37°C for 60 min with gentle shaking. After washing three times, the wells were incubated with 100 μL diluted enzyme-labeled second antibody (IgG-HRP, IgG1-HRP or IgG2a-HRP, 1:1,000 dilution) (Proteintech, Chicago, IL, USA) for 60 min at 37°C . The reaction was stopped by adding 50 μL stop solution 15 min later and the absorbance was measured at 450 nm using an automated ELISA reader (Allsheng).

Cytokine assays from splenocytes

To assay cytokine production levels, splenocytes were prepared and determined by ELISA as previously described.¹⁵ Briefly, the culture supernatants of splenocytes from each group 2 weeks after the final immunization were harvested and tested for IL-4 at 24 h, IL-10 and IL-12 at 72 h, and IFN- γ at 96 h, using specific ELISA assay kits (Bio-Swamp) according to the manufacturer's directions.

Flow cytometry assay

To evaluate the subsets of CD4⁺ and CD8⁺ T cells in the spleens of immunized mice, flow cytometry assay was performed as previously described.¹⁵ Briefly, 10^6 splenocytes were suspended in 100 μL PBS prior to incubation with anti-mouse-CD3e-PE-cy5.5 (eBioscience, San Diego, CA, USA), anti-mouse-CD4-FITC (eBioscience) and anti-mouse-CD8-PE (eBioscience) at 4°C for 30 min without light. After washing with 2 mL PBS, cells were resuspended in PBS and analyzed with a FL500 flow cytometer (Beckman Coulter Inc., Brea, CA, USA). List mode data files were analyzed using FlowJo7.6 software. The total lymphocytes gate was defined by forward and side scatter in mouse splenocytes. Then, the CD3⁺ T lymphocytes gate was defined by side scatter and anti-mouse-CD3e-PE-cy5.5. The ratio of CD3⁺CD4⁺ T cells/CD3⁺CD8⁺ T cells was calculated in at least three independent experiments that provided similar results.

Statistical analysis

All statistical analyses in this study, including proliferation assay, antibody response, cytokine assay, and flow cytometry assay were compared between groups by using one-way analysis of variance. Survival time for the mice was compared using the Kaplan–Meier method. All the analyses were carried out using SPSS 11.5 software at 95% confidence level. For all analyses, $p < 0.05$ was considered significant. Every experiment was done in triplicate and three independent experiments on different days were done as biological replicates.

Results

Isolation and characterization of exosomes from *T. gondii*

To characterize the exosomes secreted by *T. gondii*, about 80 μg of vesicles were obtained from 8×10^6 *T. gondii* tachyzoites in 100 mL of culture media. TEM analysis showed that these vesicles had intact continuous bilayer membranes, about 10–150 nm in diameter (Figure 1A). Furthermore, the size distribution of vesicles was determined

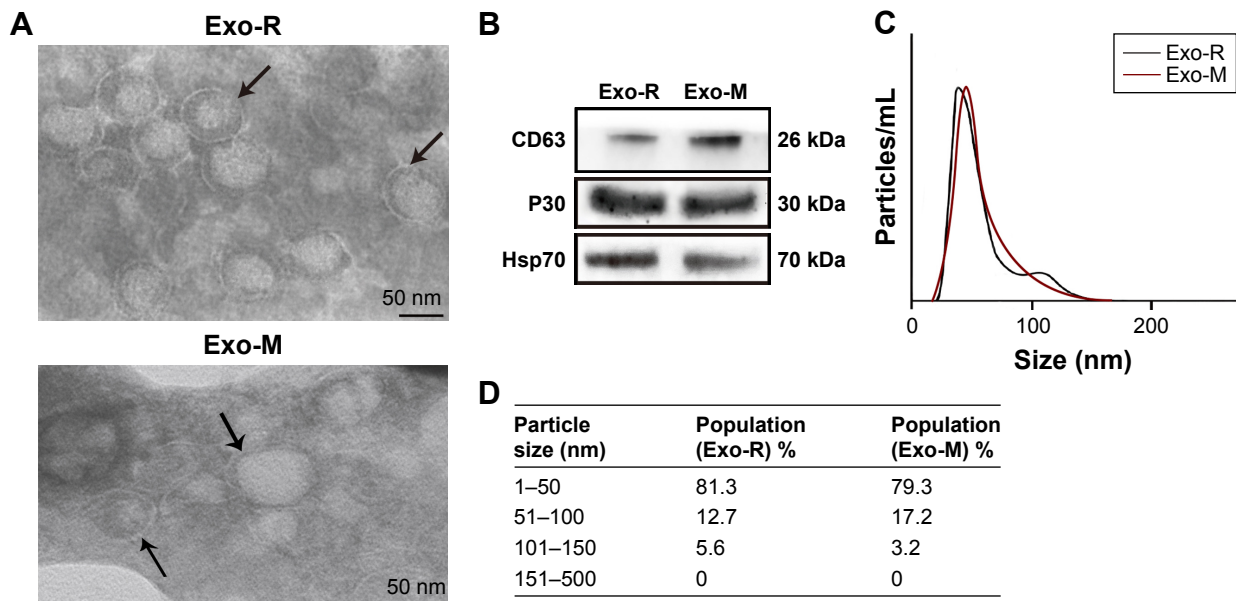


Figure 1 Characterization of *Toxoplasma gondii*-derived exosomes.

Notes: (A) Representative TEM images of vesicles derived from *T. gondii* RH strain or ME49 strain showing a range of exosomal morphologies (original magnification, $\times 100,000$; scale bar = 50 nm). Bilayer membranes are arrowed. TEM was performed at least three times. (B) Western blotting analysis of *T. gondii*-derived vesicles with anti-CD63, anti-P30 (anti-SAG1) and Hsp70. (C) Size distribution of purified vesicles derived from *T. gondii* using nanoparticle tracking analysis and showing a mean diameter of 50 nm. (D) Table from NanoSight NS300 analysis of percentage of purified vesicles derived from *T. gondii* in various size ranges.

Abbreviations: Exo-R, exosomes from RH strain; Exo-M, exosomes from ME 49 strain; TEM, transmission electron microscopy.

using nanoparticle tracking analysis. As shown in Figure 1C and D, the size of the vesicles peaked at a mean diameter of 50 nm, which is the typical morphological characteristic of exosomes. Western blotting results showed that the vesicles were positive for CD63, Hsp70 and P30 staining, which are specific markers for the exosomes (Figure 1B).

Uptake of *T. gondii* exosomes by macrophages

To determine whether *T. gondii* exosomes can be taken up by host cells, *T. gondii* exosomes were labeled with PKH67 dye (green fluorescence) and incubated with macrophage RAW264.7 cells in vitro. As shown in Figure 2, in the experimental group, the green fluorescence was observed in the cytoplasm of cells (Figure 2A), indicating that a large number of exosomes were taken up by the macrophages, compared with the PKH67-PBS control, or the PBS control (Figure 2B and C).

Effect of *T. gondii* exosomes on the cell viability of macrophage RAW264.7 cells in vitro

To determine the appropriate concentration of the exosomes used in the in vitro experiments, we investigated the influence of *T. gondii* exosomes on the viability of RAW264.7 cells using CCK-8 assay. As shown in Figure 3, the *T. gondii*

exosomes did not affect the cell viability at concentrations lower than 80 $\mu\text{g/mL}$. However, the cell viability was significantly reduced at the concentration of 160 $\mu\text{g/mL}$ ($p < 0.05$ vs untreated cells). Thus, the appropriate concentration of *T. gondii* exosomes in vitro for RAW264.7 cells was below 80 $\mu\text{g/mL}$.

T. gondii exosomes affecting macrophage cytokine production in vitro

To examine the cytokine variations during *T. gondii* exosomes stimulation, supernatants of the RAW264.7 cells were collected. As shown in Figure 4, the production of IL-12, TNF- α and IFN- γ was significantly increased after *T. gondii* exosomes stimulation ($p < 0.05$ vs PBS or BSA control), which was comparable to that in the parasite infection group (Figure 4B–D). Meanwhile, the level of IL-10 was significantly down-regulated ($p < 0.05$ vs PBS or BSA control), which is shown in Figure 4A.

Serum antibody responses in BALB/c mice to *T. gondii* exosomes

As shown in Figure 5, significantly high levels of total IgG against *T. gondii* were detected in the sera from mice immunized with Exo-M or Exo-R, 4 and 6 weeks after the first inoculation, compared with that of the controls ($p < 0.05$ or $p < 0.01$). However, there were no significant differences in

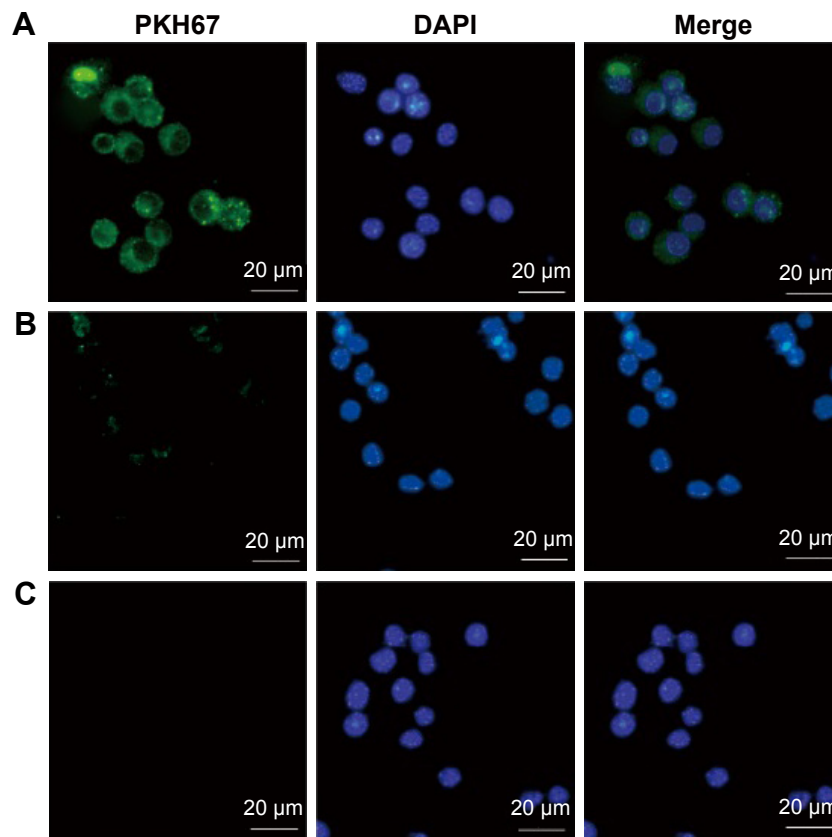


Figure 2 Uptake of *Toxoplasma gondii* exosomes by macrophages.

Notes: Ten micrograms of the (A) PKH67-labelled *T. gondii* exosomes, or (B) PKH67-PBS control, or (C) PBS control alone were added to the macrophages and incubated at 37°C for 2 h. In the fluorescence microscopy pictures, PKH67 was used to label the exosomes (green) and DAPI was used to detect the nucleus of the macrophages (blue). Results are representative of three independent experiments.

Abbreviations: PBS, phosphate buffered saline; DAPI, 4',6-diamidino-2-phenylindole.

the total IgG levels between Exo-M and Exo-R groups, as well as that between the PBS and Blank controls (Figure 5A).

The levels of serum IgG1 and IgG2a against *T. gondii* were also detected 2 weeks after the last inoculation. The results

showed that an obvious predominance of IgG2a over IgG1 was observed in the immunized mice ($p < 0.05$) (Figure 5B and C), suggesting that a Th1-type cell immune response was elicited by immunization with *T. gondii* exosomes.

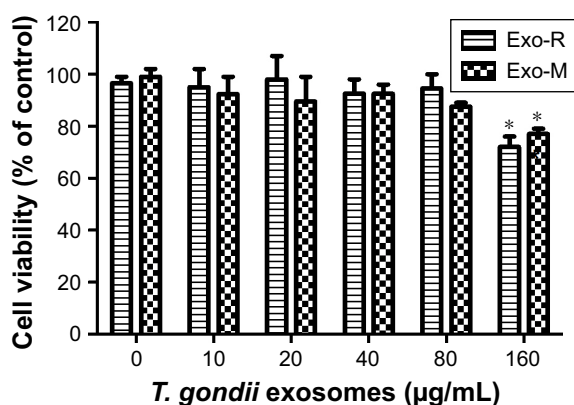


Figure 3 Effect of *Toxoplasma gondii* exosomes on the cell viability of macrophage RAW264.7 cells in vitro.

Notes: RAW264.7 cells were cultured with Exo-R or Exo-M (0–160 µg/mL) for 24 h. Cell viability was determined by the CCK-8 assay. * $p < 0.05$ compared with 0 µM exosomes. One of three experiments with similar results is shown.

Abbreviations: Exo-R, exosomes from RH strain; Exo-M, exosomes from ME 49 strain; CCK-8, Cell Counting Kit.

Cellular immune responses in BALB/c mice to *T. gondii* exosomes

The cytokines produced by splenocytes from immunized mice were evaluated by ELISA. As shown in Table 1, there were significantly high levels of both IFN- γ and IL-12 ($p < 0.05$) in the Exo-M and Exo-R groups compared with those in the PBS or Blank control groups. However, there were no significant differences in the levels of IL-4 and IL-10 among all the groups.

The percentages of T cell subsets CD4⁺ and CD8⁺ in the spleen of immunized mice were evaluated using flow cytometry techniques. As shown in Figure 6, the percentage of CD8⁺ T cells in the Exo-M or Exo-R groups was higher than that in the PBS or Blank control groups ($p < 0.05$). However, there was no significant difference in the percentage of CD4⁺ T cells between the Exo-M and Exo-R groups.

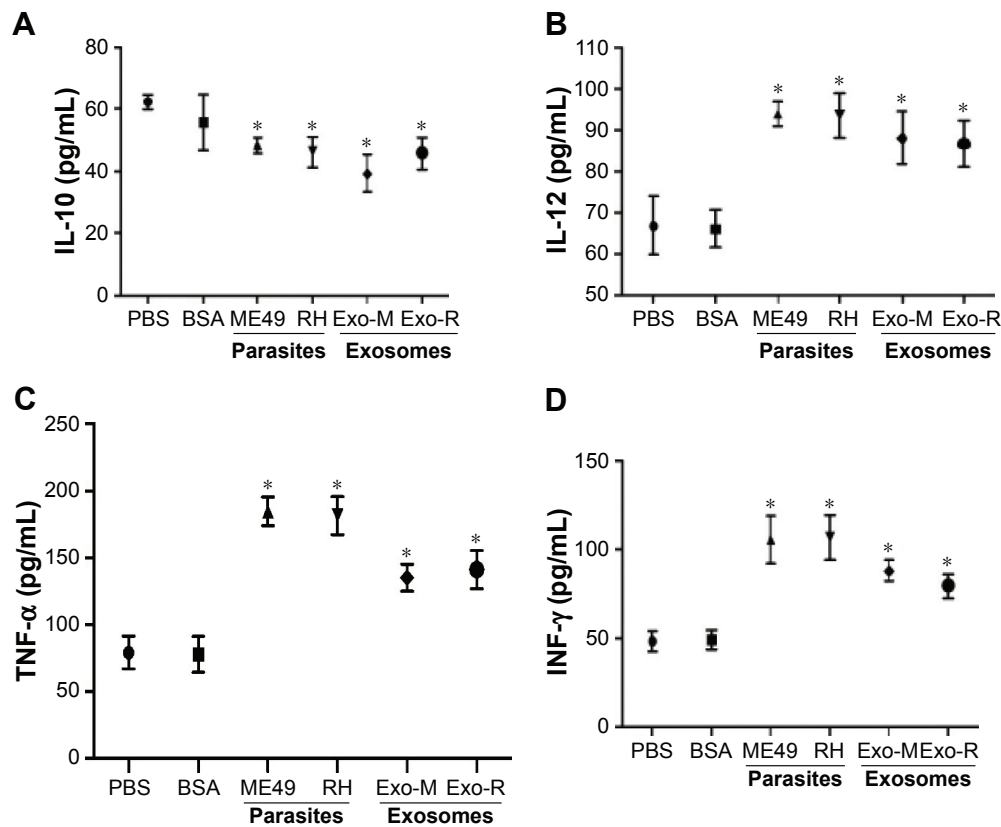


Figure 4 Macrophage cytokine productions in vitro by *Toxoplasma gondii* exosomes.

Notes: The supernatants of RAW264.7 cells were collected and assayed by ELISA for (A) IL-10, (B) IL-12, (C) TNF- α , and (D) INF- γ at 24 h. * $p < 0.05$ compared with PBS or BSA. One of three experiments with similar results is shown.

Abbreviations: Exo-R, exosomes from RH strain; Exo-M, exosomes from ME 49 strain; IL, interleukin; TNF, tumor necrosis factor; INF, interferon; PBS, phosphate buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay.

Protective immunity of *T. gondii* exosomes in mice

The survival curves of mice after lethal challenge infections are shown in Figure 7. Non-immunized control mice were all dead within 8 days after *T. gondii* infection. However, the mice immunized with Exo-M were dead within 23 days post ME49 strain challenge, which showed a

significantly prolonged survival time ($p < 0.05$). The mice immunized with Exo-R were all dead within 17 days post RH strain challenge, which also showed a significantly prolonged survival time compared with the control group ($p < 0.05$). But the difference in survival time of immunized mice between the Exo-M and Exo-R groups was not statistically different.

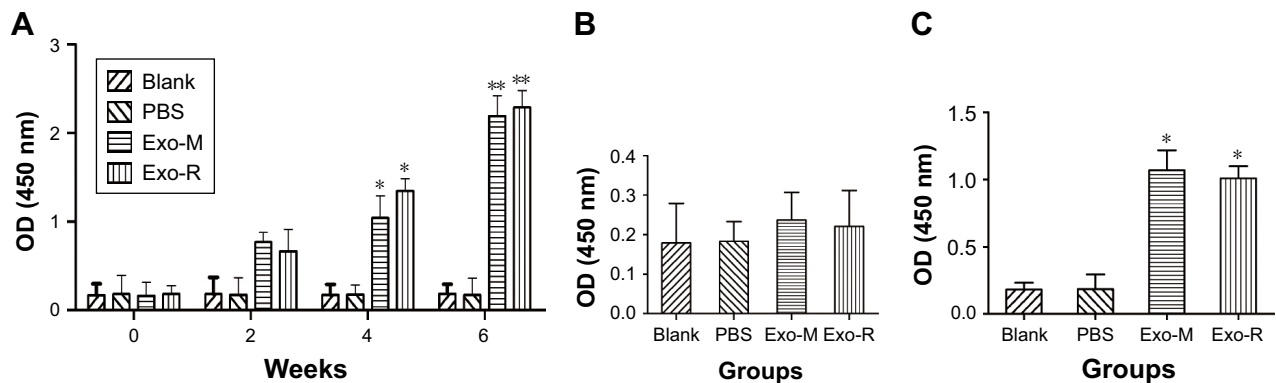


Figure 5 Antibody responses in immunized BALB/c mice.

Notes: (A) Total IgG antibody against *Toxoplasma gondii* in the sera of BALB/c mice immunized with *T. gondii* Exo-R or Exo-M, PBS and Blank control on weeks 0, 2, 4, 6 after the first inoculation. (B) IgG1, and (C) IgG2a against *T. gondii* in the sera of the immunized BALB/c mice 2 weeks after the last inoculation. Results are expressed as the mean OD $450 \pm$ SD and are representative of three individual experiments. $n = 14$ animals per group. * $p < 0.05$ or ** $p < 0.01$ compared with PBS or Blank control.

Abbreviations: Exo-R, exosomes from RH strain; Exo-M, exosomes from ME 49 strain; PBS, phosphate buffered saline.

Table 1 Cytokine production by splenocytes of immunized BALB/c mice (n=4 animals per group)

Groups	Production of cytokines (pg/mL)			
	IFN- γ	IL-4	IL-10	IL-12
Blank	160.4 \pm 7.38	93.9 \pm 2.04	74.0 \pm 9.62	97.1 \pm 3.8
PBS	163.0 \pm 5.27	91.1 \pm 3.08	76.2 \pm 5.70	99.5 \pm 5.4
Exo-M	903.4 \pm 13.27**	96.25 \pm 6.69	78.4 \pm 2.79	199.3 \pm 8.9*
Exo-R	879.0 \pm 6.81**	92.43 \pm 3.78	81.9 \pm 4.50	204.6 \pm 3.2*

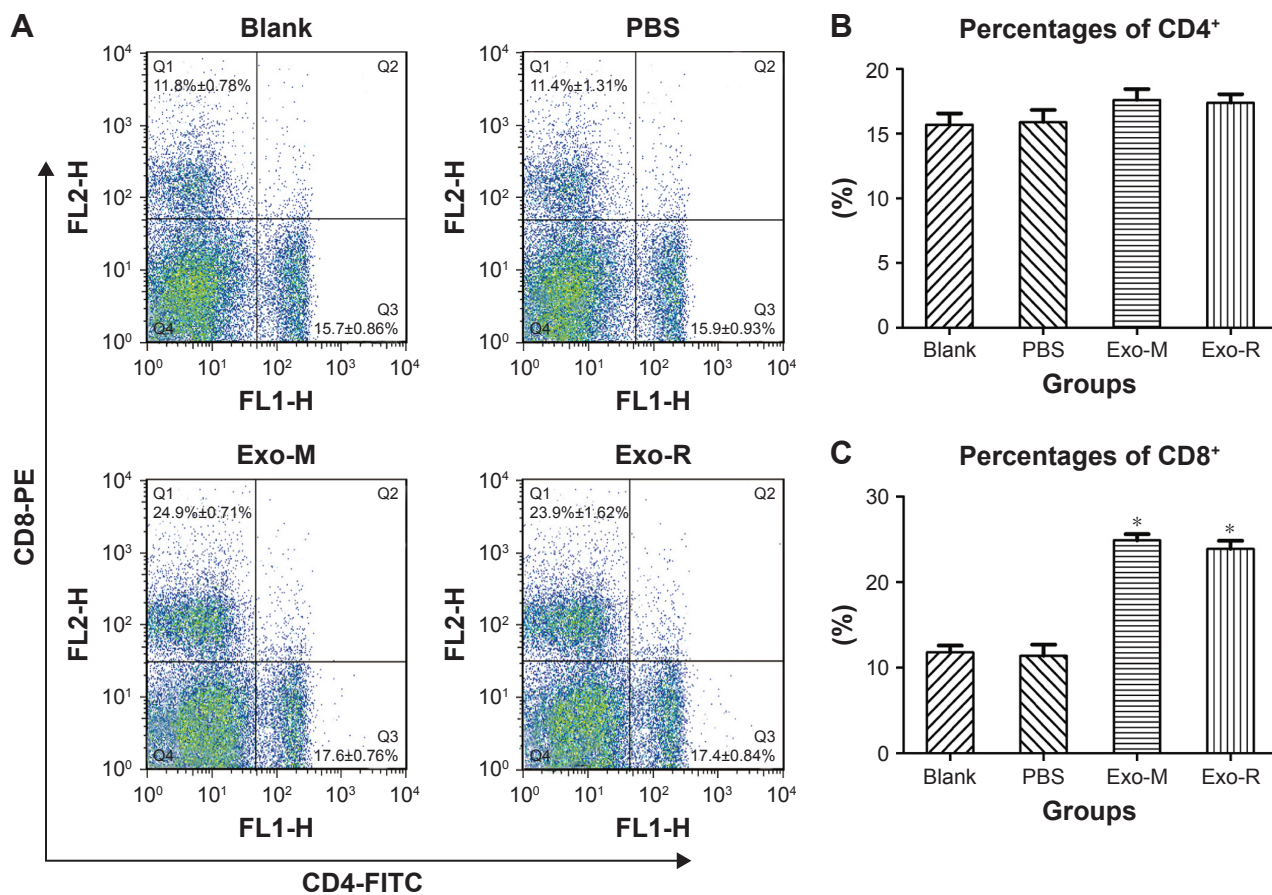
Notes: Splenocytes from mice were harvested 2 weeks after the last immunization and assayed for IL-4 at 24 h, IL-10 and IL-12 at 72 h, and IFN- γ at 96 h. Results are expressed as mean \pm SD from three independent experiments. * p <0.05 or ** p <0.01 compared with PBS or Blank control.

Abbreviations: Exo-R, exosomes from RH strain; Exo-M, exosomes from ME 49 strain; IL, interleukin; IFN, interferon; PBS, phosphate buffered saline.

Discussion

Previous studies have looked at properties and functions of exosomes associated with *T. gondii*, however, those exosomes were from *T. gondii*-infected cells, such as dendritic cells (DCs), macrophages, HFF or rat myoblast L6 cells.^{17–21}

A recent study has demonstrated that *T. gondii* EVs contain some canonical exosome proteins, however, their biological functions have not been investigated.¹⁴ In the present study, we isolated and characterized *T. gondii*-derived EVs using TEM, NTA and Western blotting analysis. Consistent with the general observation, the vesicles isolated from *T. gondii* have an intact continuous bilayer membrane, ranging from 10–150 nm with a mean size of 50 nm in diameter, which are not apoptotic bodies. Generally, exosomes are characterized based on their protein content, which indicates their endosomal origin.^{9,10} Our results showed that the vesicles were positive for CD63, Hsp70, and P30 using Western blotting. Conventionally, CD63 and Hsp70 are known as exosome markers in many cell types^{9,10} as well as in *T. gondii*, while protein P30, namely a major surface antigen 1 (SAG1) of *T. gondii*, is an extremely useful marker for identifying *T. gondii*.²² Collectively, our results confirmed that the microvesicles excreted from *T. gondii* were exosomes in

**Figure 6** Flow cytometry analysis of T cell subsets.

Notes: The total mouse spleen cells were analyzed by using anti-mouse-CD3e-PE-cy5.5, anti-mouse-CD4-FITC and anti-mouse-CD8-PE for quality control. (A) The percentages of CD4⁺ (region Q3) and CD8⁺ (region Q1) T cells in the spleen of immunized mice are shown and the results are representative of three independent experiments. (B) The percentages of CD4⁺ and (C) the percentages of CD8⁺. One of three individual experiments is shown. n=4 animals per group, * p <0.05 compared with PBS or Blank control.

Abbreviations: Exo-R, exosomes from RH strain; Exo-M, exosomes from ME 49 strain; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

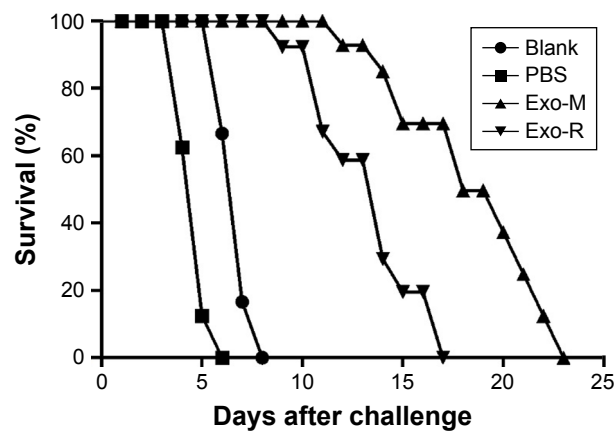


Figure 7 Survival curves of immunized mice after *Toxoplasma gondii* challenge infections.

Notes: BALB/c mice were challenged intraperitoneally with 1×10^3 tachyzoites of *T. gondii* RH strain (PBS or Exo-R group) or ME 49 strain (Blank or Exo-M group) 2 weeks after the final immunization ($n=10$ animals per group). Survival times were monitored daily and the comparison of differences between the experimental group and the control group was significant ($p < 0.05$). These data are representative of three experiments with similar results.

Abbreviations: Exo-R, exosomes from RH strain; Exo-M, exosomes from ME 49 strain; PBS, phosphate buffered saline.

terms of their biophysical and biological properties in their size, morphology, and protein components.

Increasing evidence has indicated that exosomes and their cargo can be transferred from one cell to another, resulting in the cell-to-cell communication via various functional biomolecules including proteins, bioactive lipids, and RNA, which can alter recipient cell functions.^{23–25} For instance, exosome-like vesicles from *T. gondii*-infected HFF cells could attach and deliver their contents to uninfected cells, thereby leading to altered functions in the uninfected cell.²⁰ In addition, exosomes secreted by *T. gondii*-infected L6 cells could change the host cell proliferation and alter the host cell cycle.²¹ Similarly, our results demonstrated that *T. gondii* exosomes can be taken up by macrophages in vitro, suggesting the possible relevance of cell-to-cell communication based on exosomes. Importantly, exosomes were also shown to modulate in vitro cytokine productions. Previous study has revealed that exosomes from macrophages infected with different intracellular pathogens can stimulate macrophage activation in vitro, and that these exosomes contain PAMPs as an important mechanism of immune surveillance.¹⁹ Additionally, parasite-derived exosomes have been shown to promote immune cell activation, and induce cytokine production.^{11,13} Silverman et al have investigated the properties of exosomes from *Leishmania donovani* in terms of affecting innate and adaptive immune responses, resulting in high IFN- γ , high IL-10, and low TNF- α in human monocytes.¹¹ Twu et al have reported that *T. vaginalis*

exosomes specifically modulate the production of IL-8 rather than IL-6 production in human cervical epithelial cells.¹³ Herein, we demonstrated that *T. gondii* exosomes induced production of IL-12, IFN- γ and TNF- α in macrophages, implying exosomes could stimulate macrophage activation via the production of proinflammatory cytokines. Moreover, as target cells, macrophages may be unsatisfactory since DCs are very important in activating T cells, more than macrophages. Future studies should consider those issues and address the molecular mechanisms by which *T. gondii* exosomes modulate host immune responses.

Currently, exosomes are under intensive exploration to realize their potential as therapeutic targets and diagnostic biomarkers. Previous studies have shown that exosomes can mediate immune responses including immune activation and immune suppression, and potentially trigger inflammation.^{11,26,27} Therefore, the potential use of exosomes as vaccines has aroused much interest. Mice treated with exosomes derived from DCs pulsed with *T. gondii* antigens were shown to elicit humoral and cellular immune response and protect the mice against subsequent parasite infection.^{17,18} Moreover, CBA/J mice immunized with SRDCs exosomes plus *T. gondii* antigens were induced protective immune responses against *T. gondii* infection in a congenital model.²⁸ Consistent with these studies, our data showed that *T. gondii* exosomes could stimulate mice producing both humoral and cellular immune responses, and partial protective immunity against toxoplasmosis.

An ideal vaccine against toxoplasmosis should be sufficiently immunogenic to induce humoral and cellular immune responses, in particular, a potent Th1 type immune response.²⁹ This Th1 immune response, defined by the production of IFN- γ and IL-12, is characteristic of infection with many intracellular pathogens including *T. gondii*. IFN- γ is a kind of critical cytokine in resisting *T. gondii* infection and promoting multiple intracellular mechanisms to kill the parasite and inhibit its replication.³⁰ IFN- γ also stimulates the generation of IgG2a and suppresses the synthesis of IgG1. In addition, IL-12 is a major cytokine for inducing Th1 type immune response and controlling the acute infection by *T. gondii* tachyzoites.³¹ In the present study, we demonstrated that mice immunized with *T. gondii* exosomes produced high levels of IFN- γ , IL-12, and IgG2a. However, there were no significant differences in the levels of IL-4 and IL-10 among all groups. These results indicated that *T. gondii* exosomes induced a dominant Th1 type immune response in mice, since IL-4, IL-10 and IgG1 were associated with Th2 type immune response. One possible mechanism of action

might be attributable to the exosomes trafficking. It has been proved that exosomes can mediate antigen presentation and are implicated in the transfer of biological material from one cell to another.³² Therefore *T. gondii* exosomes could exchange the antigenic information between cells in the immune system and regulate the dynamic balance between Th1 and Th2 immune response.

Owing to the characteristics of the intracellular parasite, a strong CD8⁺ cytotoxic T lymphocyte (CTL) response plays an important role in the control of development and spreading of *T. gondii* infection.^{33,34} Our data showed that the percentage of CD8⁺ T cells was significantly increased in the immunized mice, suggesting that *T. gondii* exosomes might be presented by major histocompatibility complex (MHC) I molecules to CD8⁺ T cells. This was consistent with our previous observation in T cells' subsets activated by the recombinant DNA vaccine against *T. gondii*.^{16,35} Moreover, humoral immune response resulting in IgG antibodies seems to be necessary for preventing and controlling *T. gondii* infection.³⁶ This is because specific antibodies can inhibit the attachment of the parasites to host cells, as well as promote macrophages to kill antibody-coated parasites.³⁶

As far as the evaluation of protective potency of *T. gondii* exosomes was concerned, the inbred BALB/c mice were challenged intraperitoneally with 1×10^3 tachyzoites of *T. gondii* RH strain (Type I) and ME 49 strain (Type II). However, none of vaccinations provided complete protection against *T. gondii* infection, since all immunized mice died. Although the survival time of immunized mice between the two groups was different, there was no significant difference, which might be the virulence efficiency of *T. gondii* strains. It is known that RH strain of *T. gondii* is a highly virulent strain with 100% lethal dosage (LD100)=1 in mice while the ME49 strain is a low virulent strain (50% lethal dosage [LD50] $> \text{or} = 1,000$) in mice. Besides, many parameters might hinder the evaluation of protection against *T. gondii*, including the dose of inoculums, the inoculation route, and the mouse strain.³⁷ Future studies should consider the above factors as well as the tissue histology and parasite quantification, and also address the properties of exosomes and immunization strategy. Nonetheless, the *T. gondii* exosomes may be an effective alternative vaccine candidate against infectious disease.

Conclusion

In conclusion, we isolated the exosomes from *T. gondii* and identified the parasite-derived exosomes in their biophysical and biological properties. Functionally, we found that *T. gondii* exosomes could modulate macrophage activation in vitro. Furthermore, *T. gondii* exosomes triggered both

humoral and cellular immune responses, and aroused partial protective immunity against acute parasite infection in the mice model. Therefore, this study suggested that exosomes may serve as a potential vaccine candidate or therapeutic agent against toxoplasmosis.

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Disclosure

The authors report no conflicts of interest in this work.

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