

Toxoplasma gondii-Derived Exosomes: A Potential Immunostimulant and Delivery System for Tumor Immunotherapy Superior to Toxoplasma gondii

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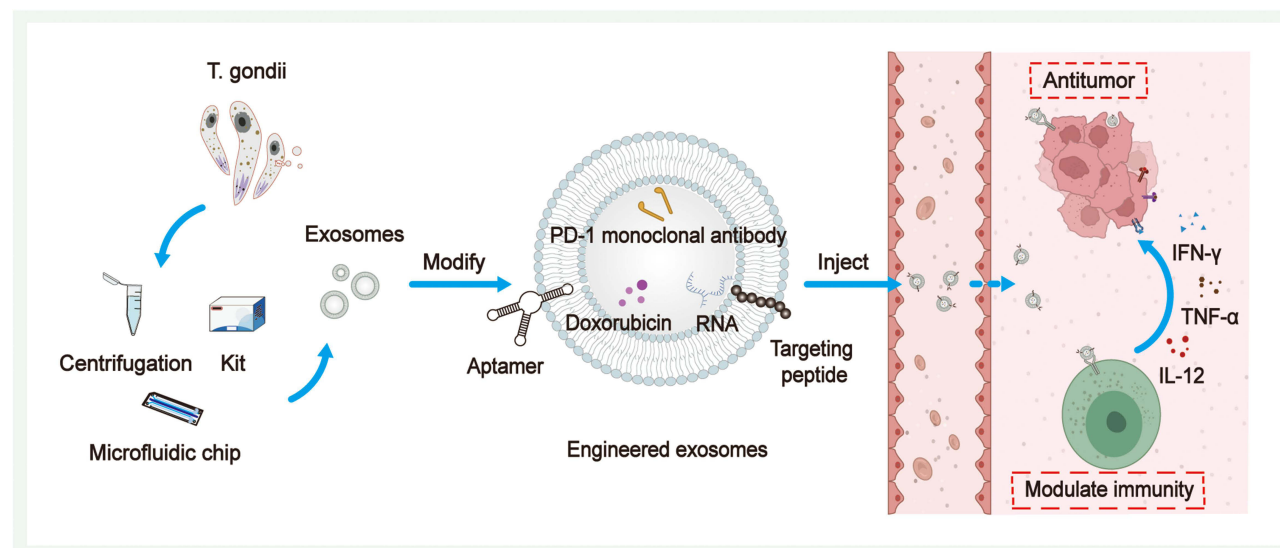
Abstract: Immunotherapies such as immune checkpoint blockade (ICB) therapy and chimeric antigen receptor T-cell (CAR-T) therapy have ushered in a new era of tumor treatment. However, most patients do not benefit from immunotherapy due to limitations such as narrow indications, low response rates, and high rates of adverse effects. *Toxoplasma gondii* (*T. gondii*), a specialized intracellular protozoan, can modulate host immune responses by inhibiting or stimulating cytokines. The ability of *T. gondii* to enhance an organism's immune response was found to have a direct anti-tumor effect and enhance the sensitivity of patients with tumors to ICB therapy. However, the application of *T. gondii* for tumor therapy faces several challenges, such as biosafety concerns. Exosomes, a subtype of extracellular vesicle that contains active components such as proteins, nucleic acids, and lipids, have become effective therapeutic tools for various diseases, including tumors. Parasites, such as *T. gondii*, mediate the communication of pathogens with immune cells and modulate host cellular immune responses through exosomes. Growing evidence indicates that *T. gondii*-derived exosomes mediate communication between pathogens and immune cells, modulate host immune responses, and have great potential as new tools for tumor therapy. In this review, we highlight recent advances in isolation and identification techniques, profiling analysis, host immunomodulatory mechanisms, and the role of *T. gondii*-derived exosomes in tumor immunotherapy. Additionally, we emphasize the potential of *T. gondii*-derived exosomes as delivery platform to enhance anti-tumor efficacy in combination with other therapies. This review proposes that *T. gondii*-derived exosomes may serve as a novel tool for tumor immunotherapy owing to their ability to activate host immune function and properties such as high modifiability, stability, and low toxicity. This work will assist in promoting the application of parasite exosomes in tumor therapy.

Keywords: *Toxoplasma gondii*, exosomes, tumor, immune regulation, immunotherapy

Introduction

Tumors are serious threats to human health and are important obstacles to increasing human life expectancy.¹ Traditional tumor treatments focus on directly killing tumors through physical removal, chemotherapeutic drugs, or radiation,^{2,3} which have poor efficacy in advanced tumors and side effects on vital organs.⁴ In recent years, immunotherapies have emerged rapidly and have provided new pathways for tumor treatment.⁴ Immunotherapies, including cancer vaccines,⁵ chimeric antigen receptor T-cell (CAR-T) therapy,⁶ nonspecific immunomodulator therapy,⁷ and immune checkpoint blockade (ICB) therapy,^{8,9} intervene in multiple stages of tumor immune evasion by activating host effector T cells and enhancing anti-tumor immune responses, thereby achieving treatment of or even curing tumors.¹⁰ However,

Graphical Abstract



immunotherapies are in the early stages of development and have several shortcomings. The low clinical response rate leaves a large number of patients without benefit.^{11,12} The tumor location is an important factor affecting anti-tumor strategies,¹³ but current tumor immunotherapies are generally not organ-specific, leading to various adverse effects, including central system complications,¹⁴ cytotoxicity,¹⁵ and inflammation.¹⁶ Therefore, new strategies for implementing tumor immunotherapy to improve efficacy and safety remain to be explored.

Toxoplasma gondii (*T. gondii*) is an obligate intracellular eukaryotic parasite able to infect humans and other warm-blooded animals. *T. gondii* invades organisms and repeatedly proliferates in infected cells, leading to massive host cell destruction and causing disease.^{17,18} In recent years, the role of *T. gondii* in modulating host immunity and anti-tumor activity has attracted widespread attention. Numerous studies have indicated that *T. gondii* parasitizes the host and induces vigorous immune responses, which have anti-tumor effects.¹⁹ This effect results from the ability of *T. gondii* to activate antigen processing, antigen presentation, and tumor antigen-specific CD8⁺ T cells.^{20,21} Despite numerous studies confirming the potential of *T. gondii* for immunomodulatory and anti-tumor applications, several serious obstacles to live *T. gondii* use as a clinical tumor therapeutic tool exist. First, *T. gondii* must be cultured in living cells to maintain its activity and must be isolated and extracted before use, which greatly increases the time and capital costs. Secondly, directly using live parasites and products for treatment involves biosafety concerns and ethical issues. Live replicating *T. gondii* strains are unlikely to be suitable for tumor therapy, as tumor patients are immunocompromised and thus more vulnerable to infection. Once a nosocomial *T. gondii* infection occurs, it may lead to a variety of adverse effects in the patients. Symptoms such as dizziness and vomiting in milder cases, and retinal necrosis in more serious cases.²² In contrast, non-replicating attenuated *T. gondii* strains are more likely to be used for tumor therapy.²³ However, the attenuated *T. gondii* strains are significantly inhibited in their ability to replicate by special processing, making them difficult to produce in large quantities. Both of their treatments are often invasive, with unpredictable risks of nosocomial *T. gondii* infection.²⁴ Therefore, safer, more convenient, and more cost-effective tumor immunotherapy strategies based on *T. gondii* need to be developed.

Exosomes are nanoscale membrane-bound vesicles formed by phospholipid bilayers encapsulating various components,²⁵ that include membrane proteins, metabolic enzymes, cytoplasmic and nuclear proteins, signaling molecules, a variety of nucleic acids,²⁶ lipids, and metabolites.^{27,28} Exosomes interact with receptor cells by directly signaling through ligand/receptor interactions or enter receptor cells and regulate receptor cell function by membrane fusion,

endocytosis, and macropinocytosis.^{29,30} Exosomes of different origins play distinct roles in normal physiological processes such as the immune response, cell proliferation, and inflammation, as well as in various stages of disease, such as tumors.^{31,32} Exosomes naturally, abundantly, and stably exist in a series of body fluid samples, contain specific substances derived from parental cells such as tumor cells, and their detection is simpler and more specific than that of traditional tumor markers. Thus, exosomes show great potential in tumor body fluid biopsy techniques.³³ Exosomes are also characterized by low toxicity and immunogenicity, and artificially modified engineered exosomes have become a focus of attention as loaded tumor-targeted drug delivery vehicles and cancer vaccines.^{34,35} Recently, research has shown that parasites such as *T. gondii* can secrete exosomes, facilitating communication with host cells.^{36,37} *T. gondii*-derived exosomes can regulate host immune responses by inducing macrophage polarization and stimulating T cells.^{38,39} Compared with live *T. gondii*, *T. gondii*-derived exosomes have significant advantages in terms of biosafety and convenience. Thus, *T. gondii*-derived exosomes have great potential as novel tools for tumor treatment.

Hence, in this paper, we highlight recent advances in isolation, profiling analysis, and host immunomodulatory mechanisms of *T. gondii*-derived exosomes and the role of *T. gondii*-derived exosomes in tumor immunotherapy. Additionally, we emphasize the potential of *T. gondii*-derived exosomes as delivery platform to enhance anti-tumor efficacy in combination with other therapies. This review will assist in promoting the application of parasite exosomes in tumor therapy.

Immunomodulatory and Anti-Tumor Effects of *Toxoplasma gondii*

Toxoplasma gondii

T. gondii is one of the most common parasites worldwide, infecting nearly one-third of the global population. *T. gondii* invades organs such as the heart through the circulatory system, resulting in chronic infection, and is able to cross biologically restrictive barriers such as the blood–brain barrier (BBB) and placental barrier to enter immune-privileged sites.^{40,41} Although *T. gondii* infection is asymptomatic or presents with recurrent ocular lesions, lymphadenopathy, etc, in most immunocompetent individuals, immunocompromised individuals can develop life-threatening central nervous system infections after repeated chronic infections.^{40,42} Studies have consistently shown the immunomodulatory and anti-tumor functions of *T. gondii*. In the 1970s, researchers reported the phenomenon of growth restriction of solid tumors in mice infected with *T. gondii*,^{43,44} which attracted widespread attention. Subsequently, studies on *T. gondii* and its products have increased, further revealing the immunomodulatory and anti-tumor effects of *T. gondii*.

Toxoplasma gondii-Mediated Immune Regulation

T. gondii triggers innate immune responses mediated by macrophages, dendritic cells, neutrophils, monocytes, and natural killer (NK) cells during the acute phase of host infection.⁴⁵ Subsequent generation of specific immune responses against *T. gondii* infection relies on cells such as NK cells, CD4⁺ T cells, and CD8⁺ T cells, which produce interferon- γ (IFN- γ).^{42,46} Innate immunity serves as the first defense of the host after infection with pathogens. As an important part of innate immunity, Toll-like receptors (TLRs) recognize microbial components of pathogen-associated molecular patterns (PAMPs) and initiate subsequent immune responses.⁴⁷ After infection of the host by *T. gondii*, Profilin-like proteins, which can be recognized by TLR11 and TLR12 of the TLR family in mice, are released and ultimately induce the production of interleukin 12 (IL-12) in mouse dendritic cells.^{48,49} A significant reduction in IL-12 production by dendritic cells can be observed in *T. gondii*-infected mouse models after knocking out important molecules in TLR-related pathways.⁵⁰ TLR11 and TLR12 are not expressed by human genes; however, human TLR7 and TLR9 can recognize RNA and DNA derived from *T. gondii*, respectively,⁵¹ and may accordingly induce host cells to produce high levels of proinflammatory cytokines, including IL-12 and TNF- α . IL-12 produced by dendritic cells, macrophages, etc, induces massive proliferation of NK cells,⁵² CD4⁺ T cells, and CD8⁺ T cells against *T. gondii* infection (Figure 1).^{53,54} In addition to these cells, antigen-presenting cells such as monocytes also play an important role in *T. gondii* infection. Upon recognition of *T. gondii* antigens by these antigen-presenting cells, genes encoding chemokines, such as CCL2 and CXCL2, are induced, resulting in massive production of chemokines that drive neutrophils and monocytes toward infected sites.⁵⁵ Furthermore, innate lymphoid cells Group 1 (ILCs1), including NK cells, produce Th1-type cytokines,

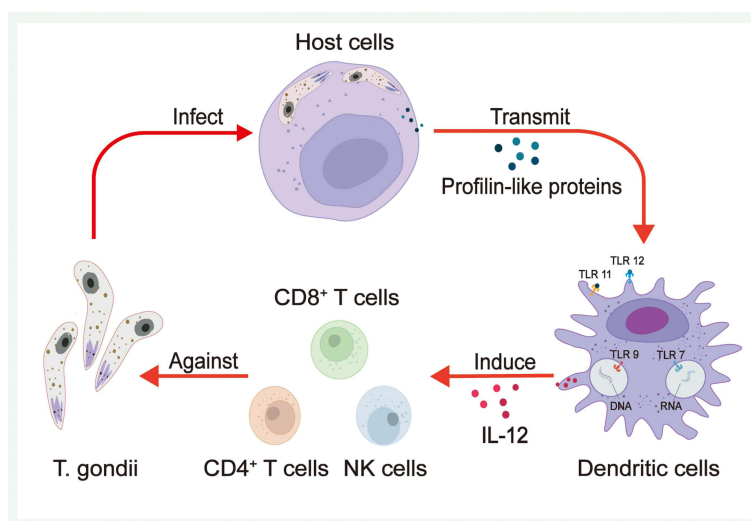


Figure 1 *Toxoplasma gondii*-mediated immune regulation. After infection of the host cells by *T. gondii*, Profilin-like proteins are released. When Profilin-like proteins are transmitted to immune cells, such as dendritic cells, they can be recognized by TLR11 and TLR12 and ultimately induce the production of interleukin 12 (IL-12). In addition, TLR7 and TLR9 can recognize RNA and DNA from *T. gondii*, respectively, and accordingly induce immune cells, such as dendritic cells, to produce pro-inflammatory cytokines, including IL-12, which induces the massive proliferation of NK cells, CD4⁺ T cells, and CD8⁺ T cells to protect against *T. gondii* infection.

such as IFN- γ and tumor necrosis factor- α (TNF- α), to counteract *T. gondii* infections via the oral route.⁵⁶ The mechanisms described above favor the organism over *T. gondii* infection, and the potential application of this host immunomodulatory capacity due to *T. gondii* infection in tumor therapy has drawn the attention of researchers.

Toxoplasma gondii and Tumor Immunotherapy

T. gondii triggers a vigorous immune response in the host to control self-replication and facilitate its survival and proliferation within host. Such vigorous immune responses can also inhibit the progression of tumors (Figure 2).⁵⁷ After

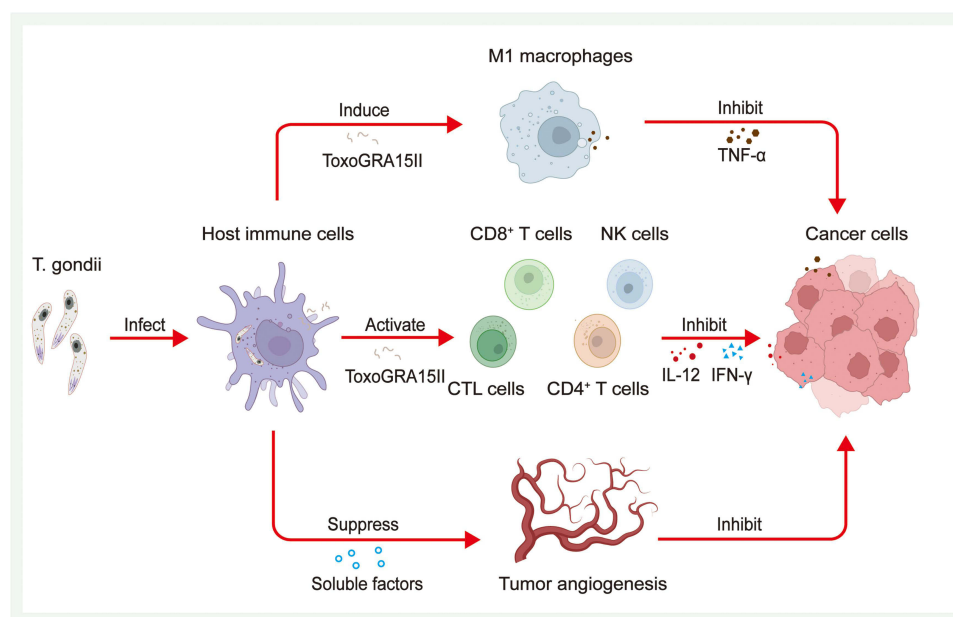


Figure 2 *Toxoplasma gondii* and tumor immunotherapy. *T. gondii* triggers a vigorous immune response in the host. Such vigorous immune responses can inhibit the progression of tumors. Research has found after infecting host immune cells, (*T. gondii* induces the production of certain soluble factors in host immune cells, which induce a strong and systematic suppression of tumor angiogenesis, leading to severe hypoxia within the tumor tissue. Furthermore, after *T. gondii* infection, substances from *T. gondii* such as ToxoGRA15II can activate the proliferation of NK cells, CD4⁺ T cells, and CD8⁺ and CTL cells, followed by inducing them to produce large amounts of IL-12 and IFN- γ . The ToxoGRA15II also can induce host macrophages to polarize to the M1 type and induce polarized M1 cells to generate large amounts of TNF- α . All of these mechanisms can inhibit tumor cells.

infecting host immune cells, the *T. gondii* Me49 strain induces the production of certain soluble factors that exert strong and systemic anti-tumor angiogenic effects, leading to severe hypoxia within the tumor tissues, which in turn inhibits the growth of B16F10 melanoma cells in experimental animal models.⁵⁸ A subsequent study found that the survival rate of Lewis lung carcinoma-loaded mice significantly increased after infection with the *T. gondii* Me49 strain. In this study, researchers not only observed significant inhibition of tumor angiogenesis in mice but also significant increases in IFN- γ -related mRNA expression levels, CD8⁺ T-cell percentages, and cytotoxic T lymphocyte (CTL) counts, suggesting that the *T. gondii* Me49 strain achieves anti-tumor effects by modulating host immunity.⁵⁹ Recently, another study found that the *T. gondii* Me49 strain can stimulate dendritic cells to produce exosomes, which achieve inhibition of tumor growth in hormonal mice by suppressing macrophage polarization toward the M2 type.⁶⁰ In addition to the *T. gondii* Me49 strain, researchers have developed the *T. gondii* nonreplicating avirulent uracil auxotroph vaccine strain (*CPS*). Uridine-5'-mono-phosphate (UMP) is necessary for nucleic acid synthesis by *T. gondii*. UMP production can be blocked by blocking *T. gondii* synthetic pyrimidines, which leads to the inability of *T. gondii* replication. Modified strains become *T. gondii* *CPS* strains characterized by low toxicity.^{23,61} After treatment with the *T. gondii* *CPS* strain, B16F10 melanoma-loaded mice showed significantly increased counts and activity of CD8⁺ T cells, while primary tumors gradually regressed.²¹ *T. gondii* *CPS* strains are not specific to melanoma, they show similar inhibitory effects on ovarian,⁶² pancreatic,⁶³ and breast cancers.⁶⁴ Immune cells such as CD8⁺ T cells and NK cells and immune-related substances such as IL-12, IFN- γ , and chemokine receptor 3 (CCR3) play key roles in this anti-tumor effect. Subsequently, the anti-tumor effect of *T. gondii* *CPS* strains was shown to be based on the secretion of rhoptry protein (ROP) or dense granule protein (GRA), and knockout of genes related to these two proteins resulted in a significant reduction of the anti-tumor effect.²⁴ Researchers also observed similar effects on the products of treated *T. gondii* as well. Back in the 1990s, researchers discovered the anti-tumor effect of *T. gondii* lysate antigen (TLA). After using *T. gondii* lysate antigen on YAC-1 lymphoma cell-loaded mice, a significant enhancement of the cytotoxic response was observed.⁶⁵ Treating mice with TLA before tumor emergence can delay tumor initiation. Based on histopathological observations, the TLA-treated group showed significant tumor necrosis and infiltration of lymphocytes and neutrophils compared with groups without TLA treatment.⁶⁶ Later, researchers found that TLA had an inhibitory effect on mouse colon cancer CT26 cells. After treating mice with TLA, they observed a significant reduction in the expression of the serum metastasis marker TIMP-1 and enhanced serum IL-12 and MyD88 signaling pathway expression in mouse macrophages.⁶⁷ Many experiments have shown that the TLA-induced maturation of dendritic cells exerts anti-tumor effects through specific immune functions.^{68,69} *T. gondii* virulence-associated dense granule protein (ToxoGRA15II) can induce host macrophage polarization to the M1 type and downregulation of matrix metalloproteinase-9 (MMP-9) and MMP-2, which in turn inhibits Hepa1-6 tumor cell metastasis and invasion.⁷⁰ ToxoGRA15II also induces polarized M1 cells to produce large amounts of TNF- α , which can inhibit liver fibrosis and sarcoidosis.⁷¹ In addition, since ToxoGRA15II is nontoxic to mammals, its potential value as a novel tool to modulate host immunity and anti-tumor is noteworthy. These studies have documented the immunomodulatory and anti-tumor effects of *T. gondii* through both cellular and animal experiments. In summary, *T. gondii* can transform tumors from “cold” to “hot” by awakening the host’s immune response and destroying the immunosuppressive tumor microenvironment, ultimately reducing the tumor size and inhibiting tumor metastasis. However, because of various obstacles such as biosafety issues and economic burdens, the clinical application of *T. gondii* requires more research support.

Immunomodulatory and Anti-Tumor Effects of *T. gondii*-Derived Exosomes

T. gondii-Derived Exosomes

Exosomes were first isolated from tumor cell lines in the 1880s and were initially described as vesicles with 5'-nucleotidase activity.⁷² Subsequent research has shown that exosomes are nanoscale vesicles containing various components, such as proteins, nucleic acids, lipids, and metabolites, with diameters ranging from 30 nm to 150 nm; these vesicles are released from intracellular multivesicular bodies (MVBs).⁷³ Exosomes are produced by various cells and are involved in a wide range of physiological activities, such as cell-to-cell communication and functional

regulation.²⁸ Exosomes play an important role in the generation, diagnosis, and treatment of tumors, infectious diseases, and cardiovascular diseases.^{74,75} Recent studies have reported that numerous parasites, such as *Schistosoma japonicum*,⁷⁶ *Leishmania protozoa*,⁷⁷ *Trypanosoma brucei*,⁷⁸ *Plasmodium falciparum*, *Neospora caninum*,⁷⁹ and many others, can communicate with host cells by secreting exosomes and delivering virulence factors, drug resistance factors, and differentiation factors, thus modulating the immune responses of hosts.^{80,81} *T. gondii* can also produce exosomes. Some researchers have successfully identified *T. gondii*-derived exosomes and analyzed them using proteomics, revealing several classical exosomal proteins.⁸² Further studies on *T. gondii*-derived exosomes using transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) revealed that *T. gondii*-derived exosomes range from 10 to 150 nm in diameter, averaging approximately 50 nm. The specific markers of *T. gondii*-derived exosomes, recombinant lysosomal associated membrane protein 3 (CD63), heat shock protein 70 (Hsp70), and surface antigen 1 (SAG1, also named P30), were also detected using protein blotting.⁸³

Isolation and Identification of *T. gondii*-Derived Exosomes

Exploring exosomes is highly important for related life science research. The prerequisites for exploring exosomes are isolation and characterization techniques with high purity and yield. Exosomes derived from different sources carry distinct bioactive substances,^{28,84} and differ in size; thus, different isolation techniques are required.⁸⁵ Traditional isolation techniques include ultracentrifugation-based methods,^{86,87} size-based methods⁷⁴ and precipitation,⁸⁸ as well as immunoaffinity capture, which is based on the binding of aptamers or antibodies to specific proteins in exosomes.⁸⁹ Currently, ultracentrifugation, which has the advantages of simplicity and quantity, is the gold standard for isolating exosomes and is suitable for large samples. However, some studies have shown that ultracentrifugation may destroy exosome vesicles and interfere with subsequent research.⁹⁰ In summary, traditional exosome isolation techniques are cumbersome, require special equipment, and are costly and time-consuming. Researchers often combine these techniques in complementary manners, for example, by combining ultrafiltration with size-exclusion chromatography (SEC), which allows for the extraction of higher-purity free exosome concentrates.^{91,92} In recent years, novel technologies have emerged, providing new possibilities for exosome isolation and characterization. Microfluidic chip technology is based on a chip containing numerous microtubules that can handle microvolume fluid.⁹³ In the field of exosome isolation and identification, synthesized microfluidic chip systems combining ultracentrifugation, immunoaffinity capture methods, and other technologies have emerged.^{94,95} These methods are convenient, automated, and applicable for the analysis of minor samples due to their low throughput. Some researchers have achieved markerless detection of PD-L1 in exosomes using surface plasmon resonance (SPR) biochips.⁹⁶ Proper culture of *T. gondii* and obtaining supernatants that contain *T. gondii*-derived exosomes is essential for their isolation. Two common techniques determine the purity and yield of *T. gondii*-derived exosomes.^{82,97} First, the host cells are infected with *T. gondii* tachyzoites. After a period of coculture, *T. gondii* is separated from the cells by centrifugation. Then, activated *T. gondii* is introduced into medium without exosomes, and the supernatant is collected for subsequent isolation after the appropriate time. Second, based on the former method, without separating activated *T. gondii* from the host cells, the culture is continued, and the supernatant containing exosomes is collected for subsequent isolation (Figure 3). The latter technique enables specialized intracellular parasitism of *T. gondii* and can maintain *T. gondii* activity for a long time, resulting in a greater yield of exosomes. However, this approach results in lower purity because host cells can also secrete exosomes. For the isolation of *T. gondii*-derived exosomes from the supernatant, the most used methods include centrifugation and exosome sorting kits. Exosome sorting kits are based on polymer precipitation technology and allow easier handling, lack special equipment, as well as higher recovery rates than centrifugation.⁹⁸ After repeated centrifugation and concentration of the supernatants of *T. gondii* cocultured with cells in combination with specialized kits, exosome samples meeting the requirements for subsequent experiments can be extracted. However, problems such as lower purity due to nonexosome contamination still cannot be solved. The use of emerging technologies such as microfluidic microarrays with high purity and high recovery rates for the isolation and characterization of *T. gondii*-derived exosomes is a new direction for future research. After extracting *T. gondii*-derived exosomes, the concentration, ultrastructure, particle size, and markers of exosomes must be determined using techniques such as nanoparticle tracking and analysis (NTA), TEM, scanning electron microscopy (SEM) and Western blotting (WB) for subsequent analysis.^{99,100}

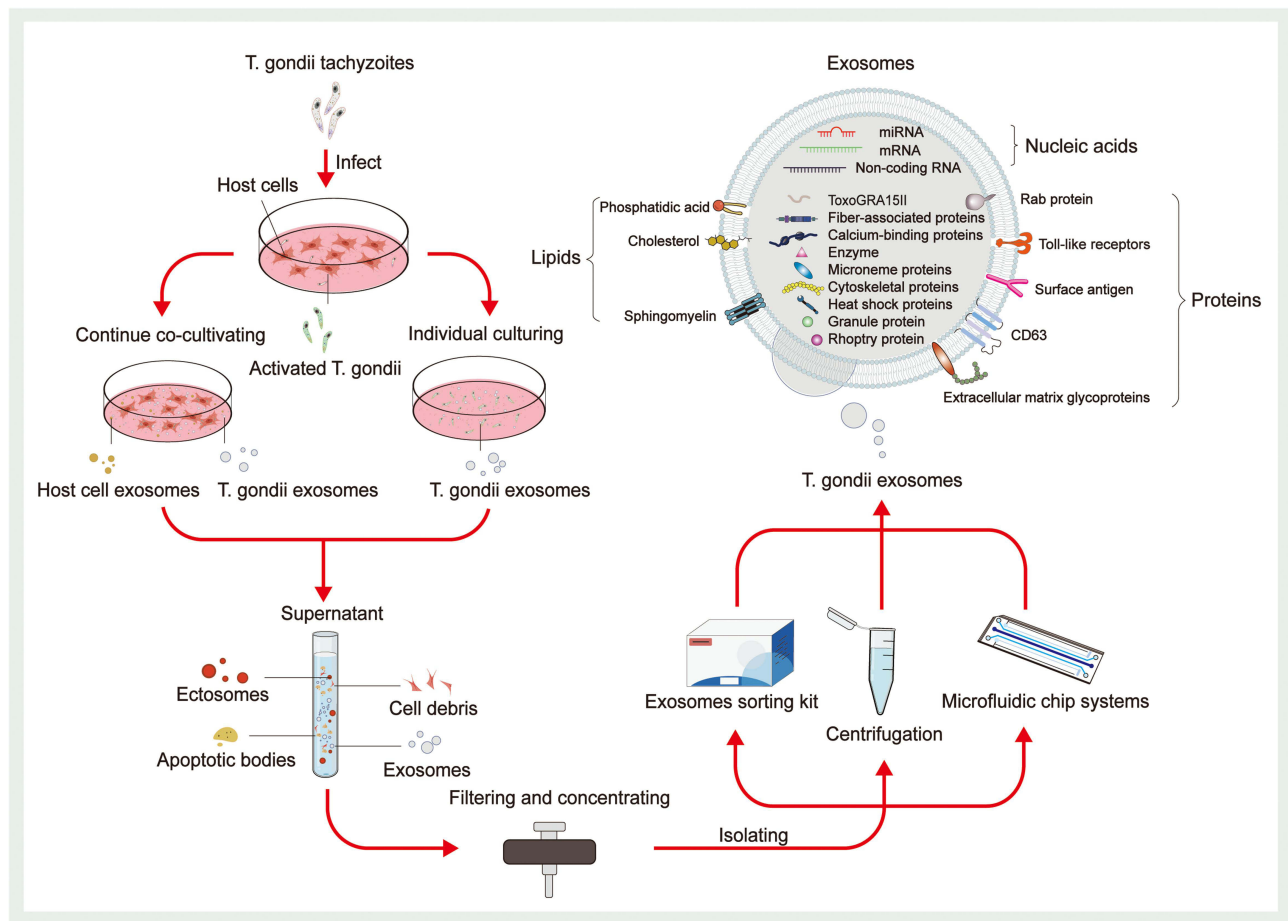


Figure 3 Isolation and composition of *T. gondii*-derived exosomes. Depending on the required purity and yield of *T. gondii*-derived exosomes, two common *T. gondii* culture techniques are used (continuous coculture and individual culturing). The former continuously co-cultures *T. gondii* with host cells and collects supernatants. The latter cultures *T. gondii* alone. In addition to exosomes, the supernatants also contained a large number of ectosomes, cell debris, and apoptotic bodies. Such components need to be detached by appropriate isolation methods. After filtration and concentration, the supernatant can be used for isolation. The most used methods for the isolation of *T. gondii*-derived exosomes include centrifugation and exosome sorting kits. In recent years, microfluidics chip systems have provided new possibilities for exosome isolation and characterization. Upon characterization, the researchers found that *T. gondii*-derived exosomes contain various proteins (including Rab protein, Toll-like receptors, surface antigen, CD63, extracellular matrix glycoproteins, fiber-associated proteins, calcium-binding proteins, enzyme, microneme proteins, cytoskeletal proteins, heat shock proteins, granule protein, rhoptry protein, and ToxoGRA15II), lipids (including phosphatidic acid, cholesterol, and sphingomyelin), and nucleic acids (including RNA, DNA, and non-coding RNA).

Composition of *T. gondii*-Derived Exosomes

The immunomodulatory functions of *T. gondii*-derived exosomes rely on their contents of proteins, nucleic acids, and lipids. Omics analysis of *T. gondii*-derived exosomes will contribute to unraveling their biological functions. *T. gondii*-derived exosomes contain various protein components, including ROP proteins, GRA proteins, microneme proteins (MIC), surface antigen (SAG), cytoskeletal proteins, heat shock proteins (HSP), and Rab proteins.^{37,82} These proteins widely participate in several physiological processes, such as invading host cells and regulating host immunity. Some researchers analyzed proteins of exosomes derived from *T. gondii*, *T. gondii*-infected human foreskin fibroblasts (HFFs), and *T. gondii*-uninfected HFFs utilizing NTA, qubit fluorometric quantification, and other proteomics-related techniques.⁸² Notably, 346, 69, and 15 proteins were successfully identified as specific to exosomes from the three sources, respectively, and 216 proteins were common. A comparison of the 216 common proteins with the EVpedia database revealed that 24.7% of proteins are among the top 100 proteins in the EVpedia database,¹⁰¹ including classical exosome protein markers such as extracellular matrix glycoproteins, calcium-binding proteins, fiber-associated proteins, membrane-bound protein family members, HSP70, and CD63. Later, another researcher successfully identified the protein composition of *T. gondii*-derived exosomes, which consisted of 3 MIC proteins, 11 GRA proteins, 16 ROP

proteins, 12 SAG proteins, 3 hSP proteins, 5 Rab proteins, 10 kinases, 3 phosphatases, 7 proteases, and 215 other proteins (Figure 3).¹⁰²

T. gondii-derived exosomes also contain abundant nucleic acid components (Figure 3), including miRNAs, but their types, quantities, and functions remain unclear.^{37,103} Current research focuses on exosomes derived from host immune cells infected with *T. gondii*. *T. gondii* stimulates host dendritic cells to produce exosomes, which contain various mRNAs and miRNAs. After exosomes are internalized by recipient cells, these RNAs further influence biological processes such as immune responses in the organism by regulating gene expression. Several researchers have extracted and purified exosomal RNA from *T. gondii* (PRU strain) tachyzoites that infected HFFs using ultracentrifugation and an exosome sorting kit and analyzed it with microarrays. A total of 222 differentially expressed RNAs were found, and miR-23b was highly expressed. miR-23b can regulate the immune response in host cells by inhibiting the expression of the cytokine IL-17.¹⁰⁴ Some researchers have observed that exosomes derived from rat myoblast L6 cells infected with *T. gondii* (RH strain) tachyzoites contain 64 differentially expressed miRNAs. Eleven of these miRNAs are related to the cell cycle and proliferation and regulate various cell cycle-related genes, such as cytochalasin D2.¹⁰⁵ In recent years, another researcher performed a high-throughput analysis of exosomes derived from DC cells infected with tachyzoites of *T. gondii* (RH strain) and found 3434 more miRNAs than exosomes from DC cells not infected with *T. gondii*. Further bioinformatics analyses revealed that 12 of these stably enriched miRNAs were extensively involved in host innate immune regulatory processes.²⁶

Studies on the lipid composition of exosomes are limited. According to the ExoCarta database, exosome membranes contain 1116 lipids, including cholesterol, sphingomyelin, and phosphatidic acid (PA), in varying quantities.¹⁰⁶ The total amounts and components of exosomal lipids vary among different subtypes and are closely related to their original cells.¹⁰⁷ A study revealed that exosomes from sheep erythrocyte culture supernatants contained high levels of sphingomyelin, which is specific to the plasma membranes of sheep erythrocytes.¹⁰⁸ Exosomes secreted by B cells contain lipid components such as lysophosphatidic acid (LPA) and cholesterol (Figure 3).^{109,110} Studies on the lipid composition of *T. gondii*-derived exosomes are lacking, but researchers have hypothesized that *T. gondii*-derived exosomes contain specific lipid fractions from *T. gondii*. However, related issues remain to be explored.

During the invasion of host cells by *T. gondii*, the various components mentioned above are loaded into exosomes and then involved in host cell adhesion, invasion, and intracellular localization. Examples include proteins such as MIC2 and MIC4, which are involved in the adhesion process;^{111,112} ROP and GRA proteins, which collectively participate in the formation of intracellular parasitophorous vacuoles;¹¹³ and RON proteins and AMA1 proteins, which form kinetic linkages during *T. gondii* exosome internalization and serve to exclude host plasma membranes.¹¹⁴

Immunomodulatory Activity of *T. gondii*-Derived Exosomes

A few studies have investigated the immunomodulatory function of *T. gondii*-derived exosomes. Some researchers successfully isolated and identified *T. gondii*-derived exosomes using TEM, NTA, and WB techniques and cocultured high concentrations of *T. gondii*-derived exosomes with a mouse macrophage line (RAW264.7). Finally, the extraction and analysis of the supernatants revealed that macrophages secreted significantly more IL-12, TNF- α , and IFN- γ but secreted significantly less IL-10. Researchers also immunized BALB/c mice with *T. gondii* RH strain tachyzoite exosomes (Exo-R) and *T. gondii* Me49 strain tachyzoite exosomes (Exo-M). High levels of IgG2 α were detected in serum from both groups of mice, and the levels of IgG2 α were significantly higher than those of IgG1, suggesting that *T. gondii*-derived exosomes induced the onset of a Th1-type cellular immune response in mice. Moreover, the percentages of CD8⁺ T cells in the spleens of exosome-treated mice and the concentrations of IFN- γ and IL-12 in the supernatants of splenic lymphocyte cultures were significantly higher than those in the controls, which supports the view that *T. gondii*-derived exosomes stimulate humoral and cellular immunity in mice (Figure 4).⁸³ Subsequent experiments on the function of *T. gondii*-derived exosomes in macrophages using similar methods revealed a significant increase in the concentrations of IL-12, IFN- γ , and TNF- α secreted by macrophages under the effect of *T. gondii*-derived exosomes, which was shown to be the result of exosomes triggering innate immunity by activating the c-Jun N-terminal kinase (JNK) signaling pathway.⁹⁷ In recent years, some researchers have also immunized mice with purified *T. gondii*-derived exosomes and found that IFN- γ and TNF- α levels were significantly increased in brain and spleen cells, and the mortality

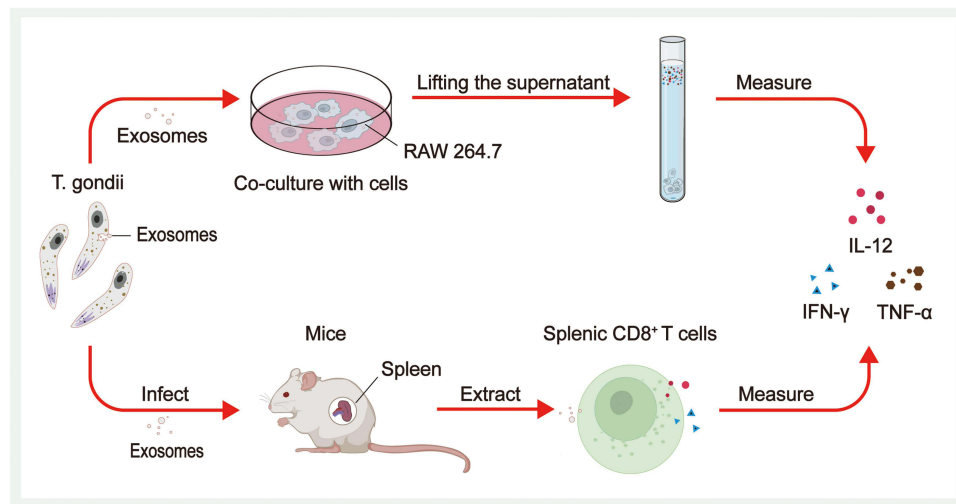


Figure 4 Immunomodulatory effect of *T. gondii*-derived exosomes. Both cellular and animal experiments revealed that high concentrations of *T. gondii*-derived exosomes stimulated increased secretion of IL-12, TNF- α , and IFN- γ from immune cells. Coculture of *T. gondii*-derived exosomes with RAW264.7 and an analysis of the final extracted supernatant revealed a significant increase in the secretion of IL-12, TNF- α , and IFN- γ from RAW264.7. The percentage of CD8⁺ T cells and the concentrations of IFN- γ and IL-12 produced by CD8⁺ T cells were significantly higher in the spleen cells of mice treated with exosomes.

rate of *T. gondii*-infected mice was also significantly decreased.¹¹⁵ Another study revealed that *T. gondii* secretes exosomes after host invasion, and the exosomes present *T. gondii* antigens to T cells either directly or via antigen-presenting cells, which activate T cells and immune responses.³⁹

Immunomodulatory Effect of *T. gondii*-Infected Cell-Derived Exosomes

Other studies on the immunomodulatory effect of *T. gondii*-associated exosomes have focused on exosomes derived from infected host cells.^{60,116} After invading the host, *T. gondii* can stimulate the production of exosomes by dendritic cells, macrophages, monocytes, and even tumor cells. In 2004, researchers stimulated host dendritic cells to produce exosomes using soluble *T. gondii* antigens and attempted to prepare cell-free vaccines based on exosomes. After injecting exosomes into mice, a significant increase in IL-2 and IFN- γ levels in mouse splenocytes was observed, suggesting the generation of vigorous Th1-type cellular immune responses and a favorable anti-infective effect.¹¹⁷ After phagocytosis of *T. gondii*, macrophages can release exosomes with *T. gondii* antigens, and these exosomes activate other macrophages to release cytokines, such as TNF- α , and destroy pathogens.¹¹⁸ The *T. gondii*-infected human monocyte cell line THP-1 also releases exosomes that stimulate uninfected THP-1 cells to produce TNF- α and other proinflammatory mediators to protect against infection.¹¹⁸ Exosomes derived from infected dendritic cells of pregnant maternal mice protect murine fetuses from vertically transmitted infections, and cystic loads in the brains of pups were found to be significantly reduced. This effect was accompanied by strong immune responses and changes in the expression levels of numerous cytokines in the serum of mice.¹¹⁹ In recent years, several studies have attempted to validate the therapeutic effect of exosomes secreted by dendritic cells infected with the *T. gondii* Me49 strain (Me49-DC-Exos) in mouse colorectal cancer models. Significant inhibition of tumor growth was observed, and subsequent experiments verified that Me49-DC-Exos achieved tumor inhibition by regulating the proportion of myeloid-derived suppressor cells through the suppression of signal transducer activator of transcription 3 (STAT3).¹¹⁶ Researchers followed up with experiments comparing the effects of uninfected dendritic cell exosomes (DC-Exos) with those of Me49-DC-Exos in colon cancer-bearing mice. Tumor growth was significantly inhibited in the Me49-DC-Exo group compared to the DC-Exo group, and the proportion of M2 macrophages in mouse blood decreased markedly. In subsequent cellular experiments, this outcome resulted from exosomes regulating SOCS1 gene expression, and an increase in SOCS1 gene expression increased the proportion of M1 macrophages.⁶⁰ Recently, several researchers have extracted exosomes from *T. gondii*-infected human hepatoblastoma cells, treated them with alum adjuvant, and performed immune induction experiments on cerebral cyst-loaded mice. The expression of IFN- γ and IL-4 and the numbers of CD4⁺ and CD8⁺ T lymphocytes were significantly elevated in mice,

suggesting the successful induction of humoral immunity and mixed Th 1/Th 2 cell immunity and a marked reduction in the brain cyst load by 75% in mice.¹²⁰ These findings provide new insights into tumor immunotherapy. The ability of *T. gondii*-derived exosomes to modulate host immune responses confers their potential as tumor immunotherapy vaccines (Figure 5). In addition, *T. gondii*-derived exosomes may also affect the host cell cycle and proliferation. Researchers have found that L6 cells infected with *T. gondii* RH strains have a decreased proliferative capacity and an increased proportion of cells in S phase or G2/M phase.¹⁰⁵

Potential of *T. gondii*-Derived Exosomes in Tumor Immunotherapy

Although few relevant studies are available, the potential of *T. gondii*-derived exosomes in tumor immunotherapy remains noteworthy. First, numerous studies have reported the role of *T. gondii* in immune modulation and tumor therapy. As mentioned above, direct use of attenuated *T. gondii* strains or *T. gondii* extracts such as TLA, GRA, and ROP can stimulate host immune cells, including dendritic cells, macrophages, CD8⁺ T cells, and NK cells, and induce significantly increased expression of cytokines and other factors, such as IL-12, IFN- γ , and TNF- α ; such strong immune responses can simultaneously inhibit tumor progression.^{24,67} Essentially, this effect stems from *T. gondii*'s Profilin-like proteins, ToxoGRA15II, RNA, and other immunogenic contents.^{48,70} These components, when recognized by host immune cells, activate a series of immune responses, such as subsequent antigen processing and antigen presentation, which in turn play an anti-tumor role. The composition and function of exosomes are closely related to their original cells. During the formation of exosomes, associated molecules in the cytoplasm, such as nucleic acids, lipids, and proteins, are endocytosed and transferred to early endosomes. Early endosomes mature and differentiate into late MVBs or endosomes, which are degraded after binding with lysosomes or fusing with plasma membranes and are subsequently released to extracellular sites to form exosomes.^{121,122} In summary, exosomes contain specific substances, such as proteins from the cells of origin, which could provide *T. gondii*-derived exosomes with immunomodulatory and anti-tumor effects similar to those of living *T. gondii* strains.

As an anti-tumor tool, *T. gondii*-derived exosomes possess numerous advantages over live *T. gondii* strains. For example, as exosome extraction and identification techniques continuously develop, obtaining high-precision and high-purity target exosomes becomes simpler.¹²³ Compared to live *T. gondii* strains, exosomes can be collected and preserved

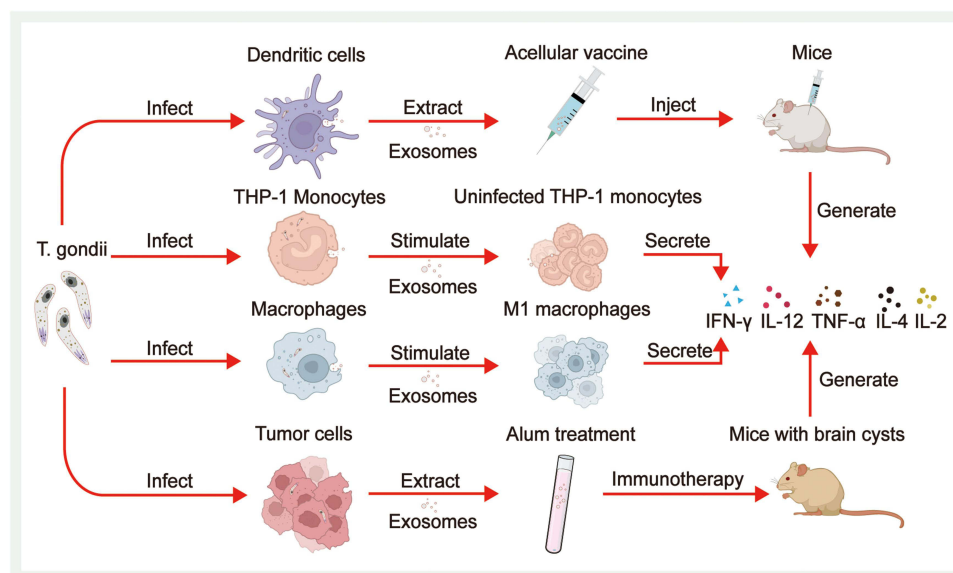


Figure 5 Immunomodulatory effects of *T. gondii*-infected cell-derived exosomes. After invading the host, (*T. gondii*) stimulates dendritic cells, monocytes, macrophages, and even tumor cells to produce exosomes. After injecting the acellular vaccine made from exosomes produced by dendritic cells into mice, a significant increase in cytokine levels in splenocytes is observed. *T. gondii*-infected human monocyte cell line THP-1 also releases exosomes that stimulate uninfected THP-1 monocytes to secrete TNF- α and other proinflammatory mediators. Macrophages phagocytose *T. gondii* and release exosomes containing *T. gondii* antigens, which stimulate other macrophages to polarise toward the M1 type and secrete cytokines. Exosomes extracted from *T. gondii*-infected tumor cells, alum processed, and subjected to immune induction experiments in mice with brain cysts also showed a significant increase in cytokine expression.

in large quantities upfront without longer-term live cell maintenance cultures to maintain their activity. In addition, *T. gondii*-derived exosomes are significantly superior to live *T. gondii* strains in terms of biosafety. *T. gondii* contains other unidentified components, and even after attenuation, direct use in patients may still have unforeseen adverse effects. Exosomes are composed of phospholipid bilayers, which can be easily accessed by receptor cells through ligand–receptor binding or direct membrane fusion, endocytosis, and even phagocytosis.^{124,125} They can also cross biological barriers such as the BBB, intestinal barrier, and placental barrier to reach specific sites and therefore could be used in various drug delivery routes.¹²⁶ Exosomes, as natural carriers of intercellular information, possess high stability, low toxicity, and low immunogenicity in the circulation, as well as excellent modifiability; thus, they can be used for the loading and transportation of therapeutic drugs as novel drug delivery platforms and have high potential as immunomodulatory and anti-tumor agents.^{127,128} In summary, the significant potential of *T. gondii*-derived exosomes in the field of tumor immunotherapy merits attention.

Prospects of *T. gondii*-Derived Exosomes in Tumor Therapy

Immunotherapy or chemotherapy drugs need to be delivered precisely to target cells to achieve therapeutic efficacy and avoid the adverse effects associated with whole-body drug administration. However, deep tumors remain difficult to treat pharmacologically due to the presence of biological barriers such as the BBB. In addition, the nutritional blood vessels of tumors are mostly distributed more densely around the periphery of tumors, making it difficult to transport drugs through the circulation to the center of tumors, which affects drug efficacy.¹²⁹ Some researchers have found that some chemotherapy drugs are poorly targeted when conducting tumor chemotherapy, leading to decreased anti-tumor effects and an increased incidence of various adverse reactions, such as neurotoxicity¹³⁰ and cardiovascular toxicity.¹³¹ Research on drug delivery systems (DDSs) to improve drug efficacy is emerging. DDSs are designed to efficiently deliver drug molecules to targets and reduce the incidence of adverse reactions. Popularly utilized DDSs include nanomaterials, liposomes,¹³² and hydrogels.¹³³

In recent years, studies have consistently documented the potential of engineered exosomes with appropriate modifications as drug carriers in various oncology therapies.^{127,134} Engineered exosomes, as novel drug carriers for tumor therapy, offer long half-lives, better biocompatibility, low toxicity, greater tissue targeting ability, and high modifiability compared to current well-established DDSs, such as liposomes and polymer nanoparticles,^{135,136} and have relatively fewer adverse effects.¹³⁷ The introduction of anti-tumor drugs into exosomes by electroporation and other techniques to obtain nanoplateforms, the so-called Trojan horse, which can cross biological barriers and penetrate deeply into tumor tissues, dramatically improves the therapeutic effect of these drugs.¹³⁸ Biomodification of exosomes is also possible using targeting peptides¹³⁹ or aptamers,¹⁴⁰ which can endow exosomes with significantly higher tumor affinity and specific targeting capabilities, further enhancing their efficacy and preventing adverse reactions. Accordingly, researchers have developed exosomes expressing characteristic exosomal membrane proteins and loaded them with drugs such as doxorubicin (Dox) by electroporation. In experiments in which tumor-loaded mice were treated, these exosomes exhibited significant tumor growth inhibition without remarkable toxicity.^{141,142}

T. gondii-derived exosomes possess multiple advantages from exosomes, as they inherit the characteristics, components, and ability of *T. gondii* to modulate the host immune response, which can overcome various shortcomings of existing DDSs and has promising applications in tumor therapy (Figure 6). *T. gondii*-derived exosomes can activate host antigen presentation, stimulate host immune cell secretion, weaken the immunosuppressive microenvironment, and greatly increase the expression of cytokines such as IL-12, TNF- α , and IFN- γ . When applied for tumor immunotherapy, engineered *T. gondii*-derived exosomes can accurately and efficiently deliver drugs such as PD-1 monoclonal antibody, Dox, and RNA to target cells. In addition, these exosomes weaken the immunosuppressive microenvironment induced by tumor cells, restore the anti-tumor effects of T cells and other immune cells, and transform tumors from “cold” to “hot”, resulting in efficient anti-tumor effects on deep tumors. However, there are still challenges that need to be overcome on the path to clinical translation of *T. gondii*-derived exosomes. Firstly, both existing *T. gondii* culture techniques are flawed (Figure 3). Co-culture with host cells results in exosomes from host cells being mixed in the collected supernatant. Individual culturing of *T. gondii* reduces the activity of *T. gondii*, leading to a reduction in the quality and quantity of exosomes secreted by *T. gondii*. Secondly, *T. gondii* can be divided into various subtypes; therefore, it is necessary to

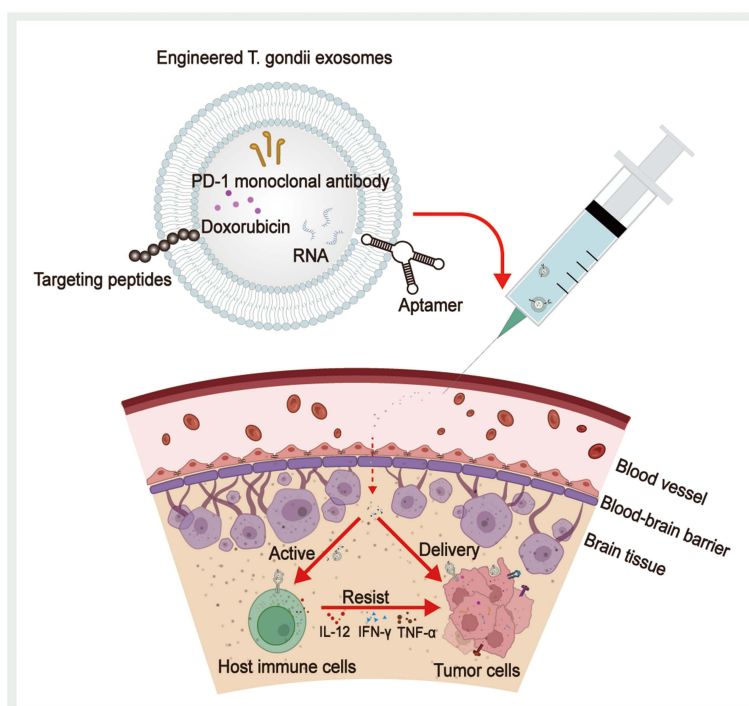


Figure 6 Prospects of *T. gondii*-derived exosomes in tumor therapy. Immunotherapeutic or chemotherapeutic drugs need to be delivered precisely to the target tissues to achieve therapeutic efficacy and avoid the adverse effects associated with systemic drug administration. Biomodification of *T. gondii*-derived exosomes using targeting peptides or aptamers can endow *T. gondii*-derived exosomes with significantly higher tumor affinity and specific targeting capabilities. These engineered *T. gondii*-derived exosomes can be loaded with doxorubicin, PD-1 monoclonal antibody, and RNA to cross biological barriers such as the blood-brain barrier to reach deep-seated tumors and can also proactively activate host immune cells to increase the expression of cytokines such as IL-12, IFN- γ , and TNF- α , or directly deliver loaded drugs to the tumor cells to achieve anti-tumor effects.

investigate the distinct host immunomodulatory functions of exosomes from different subtypes of *T. gondii* for the sake of safer clinical application. In addition, because *T. gondii*-derived exosomes contain a variety of antigenic components from *T. gondii*, they acquire the ability to modulate host immunity while inevitably potentially causing adverse reactions in the host. Therefore, more research is necessary to investigate the specific *T. gondii* antigenic components in *T. gondii*-derived exosomes and their mechanisms of host immunomodulatory effects. Pursuing the goal of achieving precise sorting of *T. gondii*-derived exosomes, and even attenuating or removing specific antigenic components. In addition, with further research on *T. gondii*-derived exosomes and advances in related production technologies, the use of laboratory-produced DDSs containing *T. gondii* components may be able to activate the host immune response while offering a better biosafety profile. Finally, because therapy with *T. gondii*-derived exosomes relies on an intact immune system, it may be significantly less effective when used in tumor patients with severe immunocompromise. To date, research on the application of *T. gondii*-derived exosomes remains in the exploratory stage. *T. gondii*-derived exosomes may become an excellent tool for tumor therapy in the future.

Conclusion

The emergence of tumor immunotherapy has brought a new pathway for tumor treatment but also confronts obstacles such as low response rates and adverse effects. The ability of *T. gondii* to modulate host immunity and combat tumors has been demonstrated in some studies, making it a possible tool for adjuvant tumor immunotherapy. However, *T. gondii* cultures are technically sensitive and suffer from biosafety concerns, limiting the availability of applications in tumor immunotherapy. In order to address these problems, finding new ways to utilize *T. gondii* has become critical. Evidence provided herein suggests that *T. gondii* can also secrete exosomes and that they may be a potent tool to assist in tumor immunotherapy. Firstly, *T. gondii* exosomes are characterized by low toxicity and immunogenicity. Secondly, it can be collected in large quantities in advance without long-term live cell culture. Finally, *T. gondii* exosomes contain fewer

unidentified components than live *T. gondii* strains, making their biosafety performance superior. Based on these, the modified engineered *T. gondii* exosomes platform has a longer half-life, better biocompatibility, and better targetability. Such platforms can load anti-tumor drugs (such as PD-1 monoclonal antibody, Dox, and RNA) across biological barriers (such as the BBB) to precisely reach the tumor sites. Meanwhile, it can also stimulate immune cells, increase the expression of various cytokines, relieve the tumor-induced immunosuppressive microenvironment, and ultimately enhance the anti-tumor efficacy. Further work is required to bring engineered *T. gondii* exosomes into clinical application. Firstly, researchers are needed to examine and minimize the cytotoxicity and immunogenicity of *T. gondii*-derived exosomes. Secondly, existing exosome isolation and extraction techniques have been applied most frequently to various cells and relatively few to *T. gondii*. Thus, to increase the purity and yield of the exosomes obtained from *T. gondii*, it is necessary to further test the ability of *T. gondii* to secrete exosomes, as well as to develop the isolation and extraction techniques suitable for *T. gondii*. Thirdly, more animal experiments should then be carried out to clarify the specific mechanism of the immunomodulatory function of engineered *T. gondii*-derived exosomes on organisms, to assess their anti-tumor and biosafety properties, and to study their stability within the blood circulation, pharmacokinetics, and pharmacodynamics. Finally, the criteria for approval for marketing can only be met after a series of rigorously designed clinical trials with the cooperation of clinicians. In conclusion, continued understanding, experimentation, and application of the immune response regulation mechanisms and potential as carriers of anti-tumor drugs of *T. gondii* exosomes can provide new perspectives in tumor immunotherapy.

Abbreviations

BBB, Blood-brain barrier; CCR3, Chemokine receptor 3; CAR-T, Chimeric antigen receptor T-cell; JNK C-Jun N-terminal kinase; CTL, Cytotoxic T lymphocyte; DC-Exos, Dendritic cell exosomes; GRA, Dense granule protein; Dox, Doxorubicin; DDSs, Drug delivery systems; Me49-DC-Exos, Exosomes secreted by dendritic cells infected with the *T. gondii* Me49 strain; HSP, Heat shock proteins; HFFs, Human foreskin fibroblasts; ICB, Immune checkpoint blockade; ILCs1, Innate lymphoid cells Group 1; IFN- γ , Interferon- γ ; IL-12 Interleukin 12; LPA, Lysophosphatidic acid; MMP-9, Matrix metalloproteinase-9; MIC, Microneme proteins; MVBs, Multivesicular bodies; NTA, Nanoparticle tracking analysis; NK, Natural killer; PAMPs, Pathogen-associated molecular patterns; PA, Phosphatidic acid; CD63 Recombinant lysosomal associated membrane protein 3; ROP, Rhoptry protein; SEM, Scanning electron microscopy; SEC, Size-exclusion chromatography; SAG, Surface antigen; SAG1, Surface antigen 1 (also named P30); SPR, Surface plasmon resonance; TLA, *T. gondii* lysate antigen; Exo-M, *T. gondii* Me49 strain tachyzoite exosomes; CPS *T. gondii* nonreplicating avirulent uracil auxotroph vaccine strain; Exo-R *T. gondii* RH strain tachyzoite exosomes; ToxoGRA15II *T. gondii* virulence-associated dense granule protein; TLRs, Toll-like receptors; *T. gondii*, *Toxoplasma gondii*; STAT3, Transcription 3; TEM, Transmission electron microscopy; TNF- α , Tumor necrosis factor- α ; UMP, Uridine-5'-monophosphate; WB, Western blotting.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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