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Toxoplasma gondii adhesion and apoptosis of chicken erythrocytes

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Abstract

Toxoplasma gondii is thought to infect all nucleated cells in warm-blooded animals, including poultry, mammals, and humans. However, it is unclear whether *T. gondii* can infect chicken erythrocytes due to the nucleated nature of these cells. Due to the special role of chicken erythrocytes in innate immunity, we investigated the cell–cell interaction between *T. gondii* and erythrocytes to elucidate the role of chicken erythrocytes in *T. gondii* infection. Cellular apoptosis was analyzed by transwell assay and flow cytometry. An immunofluorescence method was used to examine the reorganization of vimentin during *T. gondii* infection in both Vero cells and chicken erythrocytes. The reorganization of actin was evaluated to further examine the invasion capacity of tachyzoites on chicken erythrocytes during infection. We discovered that *T. gondii* can adhere to but not invade chicken erythrocytes and eventually cause apoptosis in chicken erythrocytes. When tachyzoites were cocultured with chicken erythrocytes in vitro, the transcriptional levels of *T. gondii* MIC3, ROP16, and ROP18 were significantly decreased. In addition, the rearrangement of host cell vimentin, a type III cytoskeleton protein regulated by *T. gondii* infection, was not observed. Similarly, the parasite-induced ring-shaped actin structure was not formed in the host-parasite junction. *T. gondii* (RH strain) tachyzoites preferentially invaded Vero cells and replicated in chicken blood monocytes, but they were not found in chicken erythrocytes. These findings showed that although *T. gondii* could attach to the surface of chicken erythrocytes, but couldn't invade successfully. Interestingly, we found that the *T. gondii* secretome, lysates, and intact tachyzoites could cause apoptosis of chicken erythrocytes, which suggested a complex mechanism involved in the apoptosis of chicken erythrocytes induced by *T. gondii*. This study elucidated that *T. gondii* could not infect nucleated chicken erythrocytes and enriched our understanding of the transmission mechanism of *T. gondii* among avian species.

Keywords *Toxoplasma gondii*, Chicken erythrocytes, Invasion, Apoptosis

Introduction

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate intracellular parasite that can infect virtually all nucleated cells of vertebrates, including warm-blooded animals and humans (Sibley2003). Toxoplasmosis is a zoonotic parasitic disease, which is related to the health of humans and animals, therefore it cannot be ignored (Fu and Chen 2021; Cui et al. 2022). Similar to *Plasmodium*, *T. gondii* invasion is a multistep, complex process involving attachment and penetration (Shen and Sibley2012) that is essential for parasite proliferation in different host cells. *T. gondii*

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invasion efficiency differs greatly in different host species (Mukhopadhyay et al. 2020). Toxoplasmosis has a spectrum of manifestations, ranging from asymptomatic, such as that in avian species (Dubey et al. 2010), to lethal, such as that in immunocompromised patients (e.g., HIV and transplant patients) (Elmore et al. 2010).

Unlike *plasmodium* spp., where the invasion is more confined to human erythrocytes, *T. gondii* appears to invade all nucleated cells other than erythrocytes. In humans, *T. gondii* infects dendritic cells that act as a "Trojan horse" to disguise parasites that pass through the human blood–brain barrier without being detected by the host immune system (Lambert et al. 2009). Furthermore, macrophages were critical for host protection from *T. gondii* when mice were intraperitoneally injected with *T. gondii* tachyzoites (Jensen et al. 2011). Apart from human and murine models, experimental contamination with *T. gondii* oocysts via free-range chickens has shown that chicken, an avian species, has a high potential for parasite infection (Dubey et al. 2010). *T. gondii* has been found to replicate in chicken macrophages, suggesting its importance in activating host immune responses during infection (Quéré et al. 2013; Malkwitz et al. 2013). Furthermore, erythrocytes from various host species have been confirmed to be infected by tachyzoites (Tanabe et al. 1980; Schupp et al. 1978); therefore, *T. gondii* may have a high possibility of invading nucleated cell populations in chicken erythrocytes, promoting its dissemination in avian hosts. It is well known that chicken erythrocytes express Toll-like receptors and cytokines in response to pathogen infection. Compared to research on *T. gondii* invasion of leukocytes and phagocytes, which is reasonably well known, *T. gondii* invasion of chicken erythrocytes has remained elusive. Although it has been thought that *T. gondii* cannot invade nucleated chicken erythrocytes, the interaction between *T. gondii* and chicken erythrocytes has not been explored in depth.

This study aimed to explore the intricate cell–cell interaction to investigate the role of nucleated chicken erythrocytes in the dissemination of *T. gondii* infection. We performed experiments to study the interaction between chicken erythrocytes and *T. gondii*. This study found that *T. gondii* only invaded Vero cells but adhered to chicken erythrocytes to cause apoptosis when *Toxoplasma* was cocultured with Vero cells and chicken erythrocytes. Vimentin and F-actin, two main components of the cell cytoskeleton, were not reorganized when *T. gondii* was cocultured with chicken erythrocytes, proving the inability of *T. gondii* to invade chicken erythrocytes. In vivo, *T. gondii* was unable to invade chicken erythrocytes but could invade and replicate in chicken monocytes for further dissemination. Our findings indicated that *T. gondii* could not invade and replicate in chicken erythrocytes but could adhere to them and cause apoptosis.

Results

T. gondii adheres to chicken erythrocytes and induces cell apoptosis

The erythrocytes were inoculated with *T. gondii* tachyzoites and incubated for 12 h to investigate the ability of *T. gondii* to invade chicken erythrocytes. *T. gondii* adhered to chicken erythrocytes but failed to invade the host cells at 4 h postinfection (Fig. 1a), and the adhesion rate is about 40%. As the infection progressed, the erythrocytes began to undergo apoptosis. The erythrocytes with *T. gondii* infection displayed significant apoptosis at 12 h postinfection compared to the uninfected erythrocytes used as controls (Fig. 1b). This result suggested that *T. gondii* attachments or secretions may play a critical role in mediating erythrocyte apoptosis.

Invasion and replication of *T. gondii* in chicken blood cells in vivo

Freshly released 1×10^9 tachyzoites were intravenously injected into chickens to analyze the invasion and infection of various cells in chicken blood cells by *T. gondii* in vivo. Experimental results showed that *T. gondii* invaded and replicated in monocytes after tachyzoite injection but failed to invade erythrocytes (Fig. 2). The results further indicated the tropism of *T. gondii* for nucleated cells except for erythrocytes in vivo.

Rearrangement of vimentin in Vero cells but not in chicken erythrocytes

Previous studies have shown that *T. gondii* invasion into host cells depends on the rearrangement of vimentin, a key element of the host cell cytoskeleton that is highly conserved in all vertebrates (He et al. 2017; Ivaska et al. 2007). An indirect fluorescence assay was used to assess the expression of cell vimentin during infection in both chicken erythrocytes and Vero cells, where host cell vimentin was detected by a specific monoclonal antibody. The vimentin protein was distributed around the nucleus of the Vero cells and eventually extended and overcoated the PV membrane (Fig. 3a). However, vimentin protein was not reorganized in chicken erythrocytes during coincubation with *T. gondii* (Fig. 3b). To further evaluate the changes in chicken erythrocytes during coincubation with *T. gondii*, we examined the reorganization of the F-actin structure in an IFA assay. The expression of F-actin was detected during infection. The reorganization of F-actin is an indicator of host cell invasion and is formed in the moving junction structure (Gonzalez et al. 2009). As a result, reorganization of the F-actin

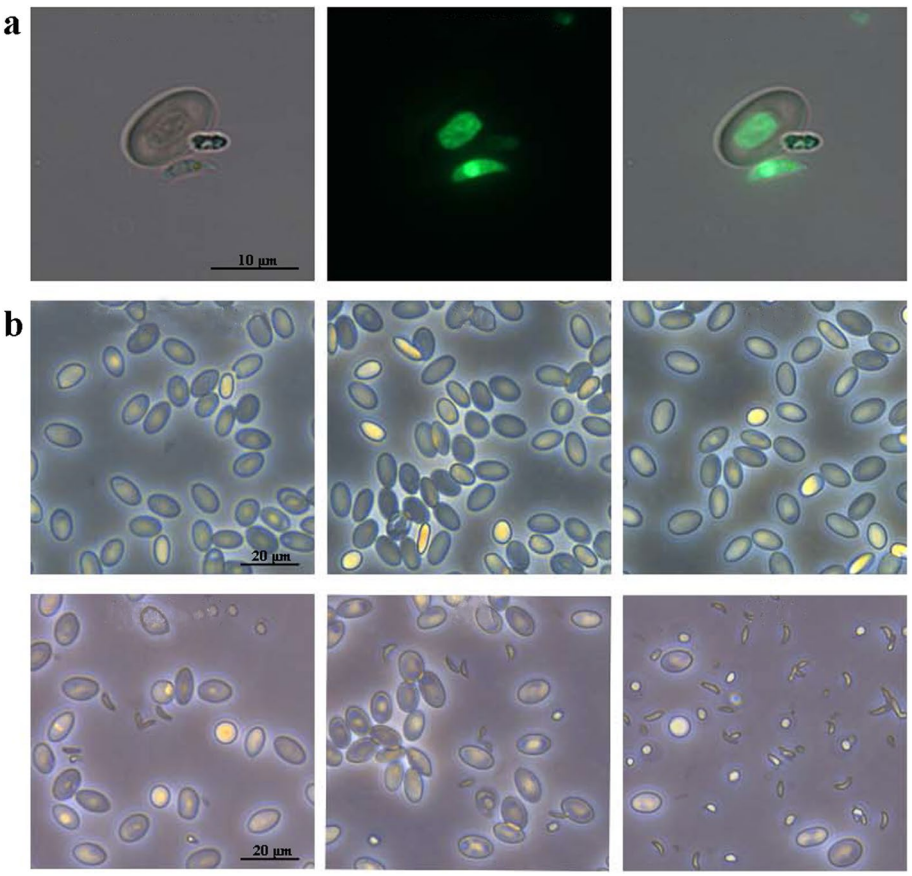

Fig. 1 Coincubation of *T. gondii* and chicken erythrocytes. **a** The initial adhesion between *T. gondii* and chicken erythrocytes at 4 h. The chicken erythrocytes were cocultured with tachyzoites. The erythrocytes and parasites were stained with acridine orange (scale bar: 10 μm). **b***T. gondii* was incapable of invading chicken erythrocytes. The coincubation of *T. gondii* with erythrocytes caused cellular apoptosis at 12 h postinfection (POI). The white arrow indicates the tachyzoites; the red arrow indicates the apoptotic cells (scale bar: 20 μm)

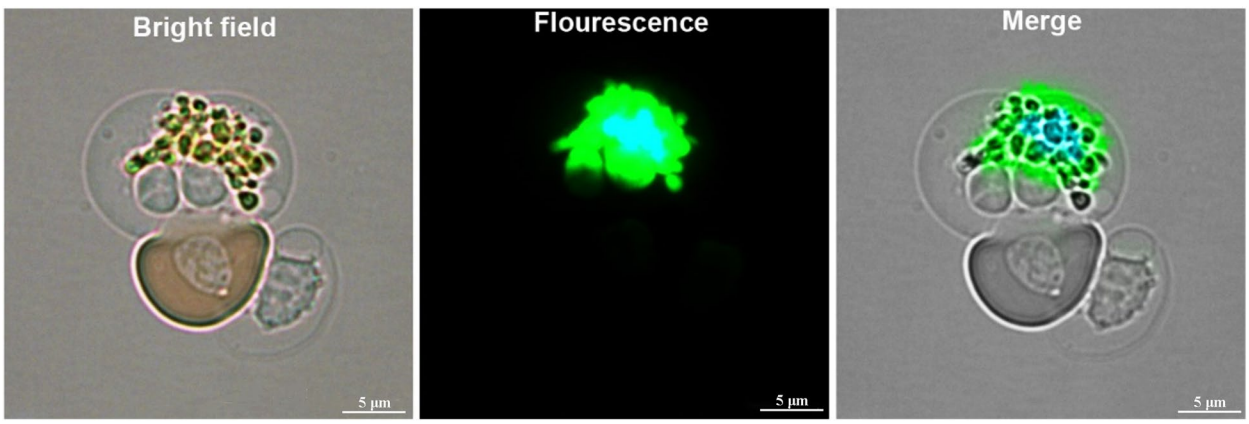

Fig. 2 Invasion and replication of *T. gondii* in chicken blood. *T. gondii* RH tachyzoites were intravenously injected into chickens. After the injection, blood cells were collected and stained with acridine orange. Scale bar: 5 μm

structure was not observed when *T. gondii* was coincubated with chicken erythrocytes (Fig. 3c), suggesting that *T. gondii* had an invasion defect when cocultured with chicken erythrocytes.

Analysis of cell apoptosis by transwell chamber and flow cytometry

Chicken erythrocytes were divided and incubated in the upper part of the Transwell chamber, and the contents of the lower chamber affected erythrocytes through the polycarbonate membrane. *T. gondii* secretions, lysates, and intact tachyzoites were coincubated with chicken erythrocytes in a transwell chamber for 24 h to determine if the observed cell apoptosis was caused by *T. gondii* secretory proteins (Fig. 4a). Erythrocyte apoptosis and necrosis were assessed using flow cytometry. When coincubated with *T. gondii* tachyzoites, 80.2% of live erythrocytes and 11% cell apoptosis were observed (Fig. 4b). After incubation with *T. gondii* lysates, there was a much higher proportion of live erythrocytes (85.9%) in the population (Fig. 4b). Furthermore, the proportion of live cells reached 92% when cocultured with *T. gondii* secretions (Fig. 4b). These findings show that *T. gondii* could cause apoptosis in chicken erythrocytes. The secretome and lysates of *T. gondii* partially account for *the* induced apoptosis. The mechanism by which *T. gondii* triggers the apoptosis of chicken erythrocytes is rather complicated.

Transcriptional levels of MIC3, ROP16 and ROP18 decreased significantly in vitro when *T. gondii* was cocultured with chicken erythrocytes

To explore the mechanisms of apoptosis, we examined the secretion of *T. gondii* virulence factors. *T. gondii* tachyzoites were coincubated with chicken erythrocytes. *T. gondii* tachyzoites cultured in Vero cells (in vitro) or collected from mice (in vivo) were used as controls. The transcriptional expression of the MIC3, ROP16 and ROP18 genes of *T. gondii* was analyzed by real-time PCR. The results showed that the transcriptional expression levels of MIC3, ROP18 and ROP16 of *T. gondii* coincubated with chicken erythrocytes for 24 h significantly decreased compared with the tachyzoites collected from mice or cultured in Vero cells in vitro (Fig. 5). The data suggested that the inability to invade chicken erythrocytes is far more likely because these virulence factors are not highly expressed.

Discussion

The invasion capacity of *T. gondii* into chicken erythrocytes was explored in our study. When compared with tissue cell lines (Vero and HFF cells) and immune cells (DCs and RAW264.7 cells), we found that *T. gondii* binds to chicken erythrocytes but fails to invade and replicate in them. This conclusion was in line with a previous study in which nucleated erythrocytes were unsuitable host cells for *T. gondii* (Malkwitz et al. 2017). Meanwhile, chicken erythrocytes displayed a high proportion of cellular apoptosis. *T. gondii* relies on the integrity of the host cell and a continuous supply of essential metabolites to maintain its life cycle. However, inducing apoptosis in certain immune cell populations might be required to downregulate parasite-specific immune responses, contributing to parasite survival (Lüder et al. 2001). Chicken erythrocytes are an intrinsic part of the innate immune system, so apoptosis could result from the host immune response triggered by *T. gondii*. Some proteins secreted by parasite secretory organelles are deeply involved in apoptosis modulation, such as the molecules secreted by rhoptries, Rop16 and Rop18, GRA15 and GRA16 secreted by dense granules (Mammari et al. 2019). Therefore, we hypothesized that parasite secretions may have contributed to the observed cellular necrosis. However, coincubation of chicken erythrocytes with *T. gondii* tachyzoite secretions did not result in a substantial increase in cell necrosis (Fig. 2), suggesting that *T. gondii* secretory proteins were not the primary contributors to cellular apoptosis. There might be a more complex process involved in the apoptosis of chicken erythrocytes caused by *T. gondii*.

Invasion-related proteins secreted by parasites, such as MICs and ROPs, play a critical role in host cell invasion during infection (Wang et al. 2020). MIC3, a parasite-derived molecule that promotes *T. gondii* adhesion and invasion of host cells, has been shown to play a role in *T. gondii* virulence in mice (Cérède et al. 2005; El Hajj et al. 2008). ROP16 and ROP18 are two important *Toxoplasma* kinases belonging to the ROP2 clade (Bradley and Sibley2007), which modulate parasite virulence by interacting with host cell proteins such as ATF6 and STAT (Saeij et al. 2006; Jensen et al. 2013; Yamamoto et al. 2011; Fentress et al. 2010), facilitating *T. gondii* infection and proliferation. ROP18 was identified as a major factor contributing to strain-specific differences in virulence

(See figure on next page.)

Fig. 3 Vimentin and actin rearrangement in cells infected with *T. gondii* at different times. **a**, **b** Vero cells (**a**) and chicken erythrocytes (**b**) were infected with *T. gondii* RH strain tachyzoites, and the rearrangement of cells was evaluated at 0 h (uninfected), 8 h, and 20 h postinoculation using a specific anti-vimentin antibody. The red signal indicates vimentin expression in host cells during *T. gondii* invasion. Scale bar: 20 μm. **c** The F-actin structure was not observed during *T. gondii* invasion. The reorganization of actin was assessed at different time points of 0 h (uninfected cells) and 8 h postinoculation. The red signal indicates actin expression in host cells during invasion. Scale bar: 10 μm

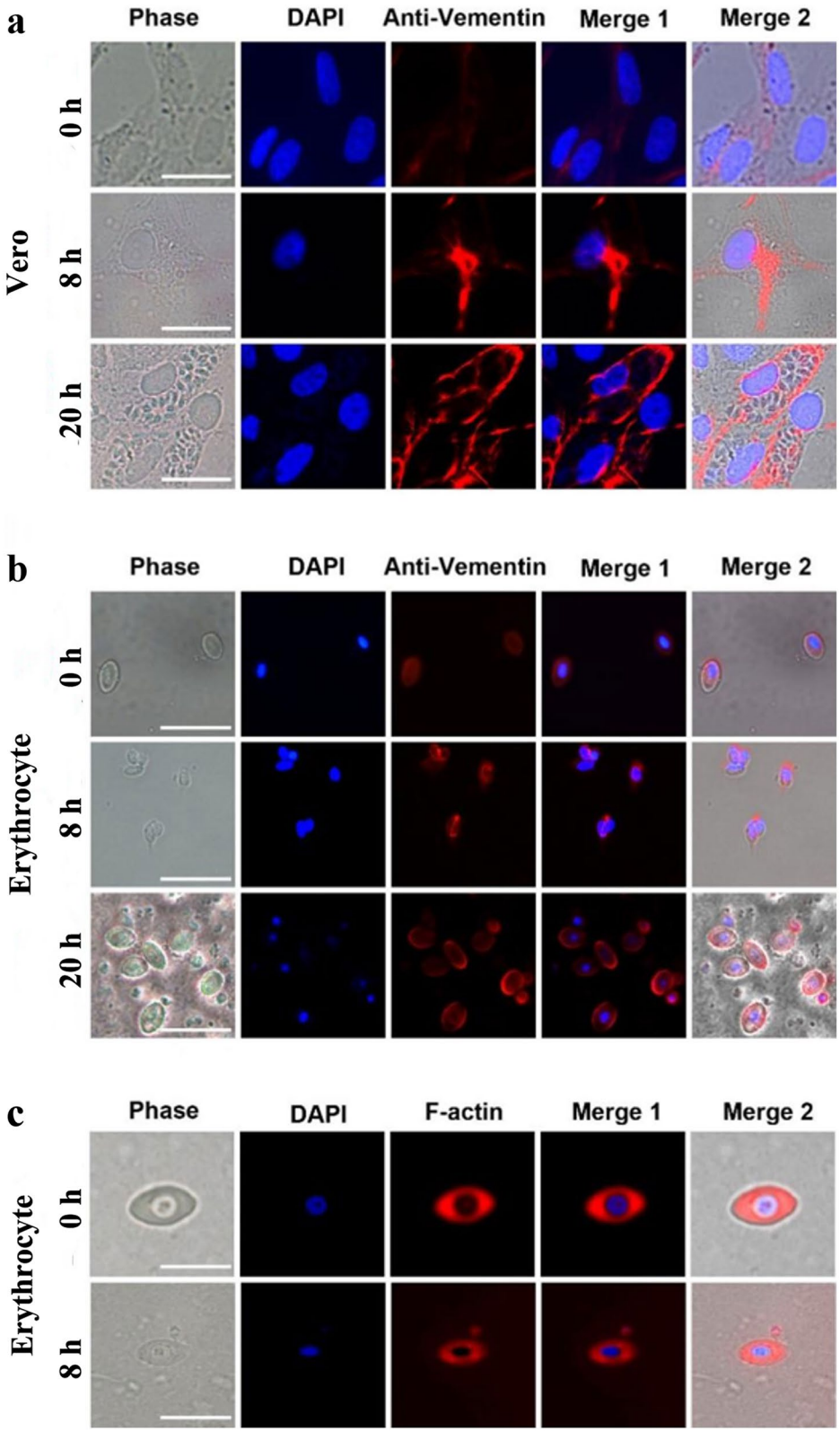

Fig. 3 (See legend on previous page.)

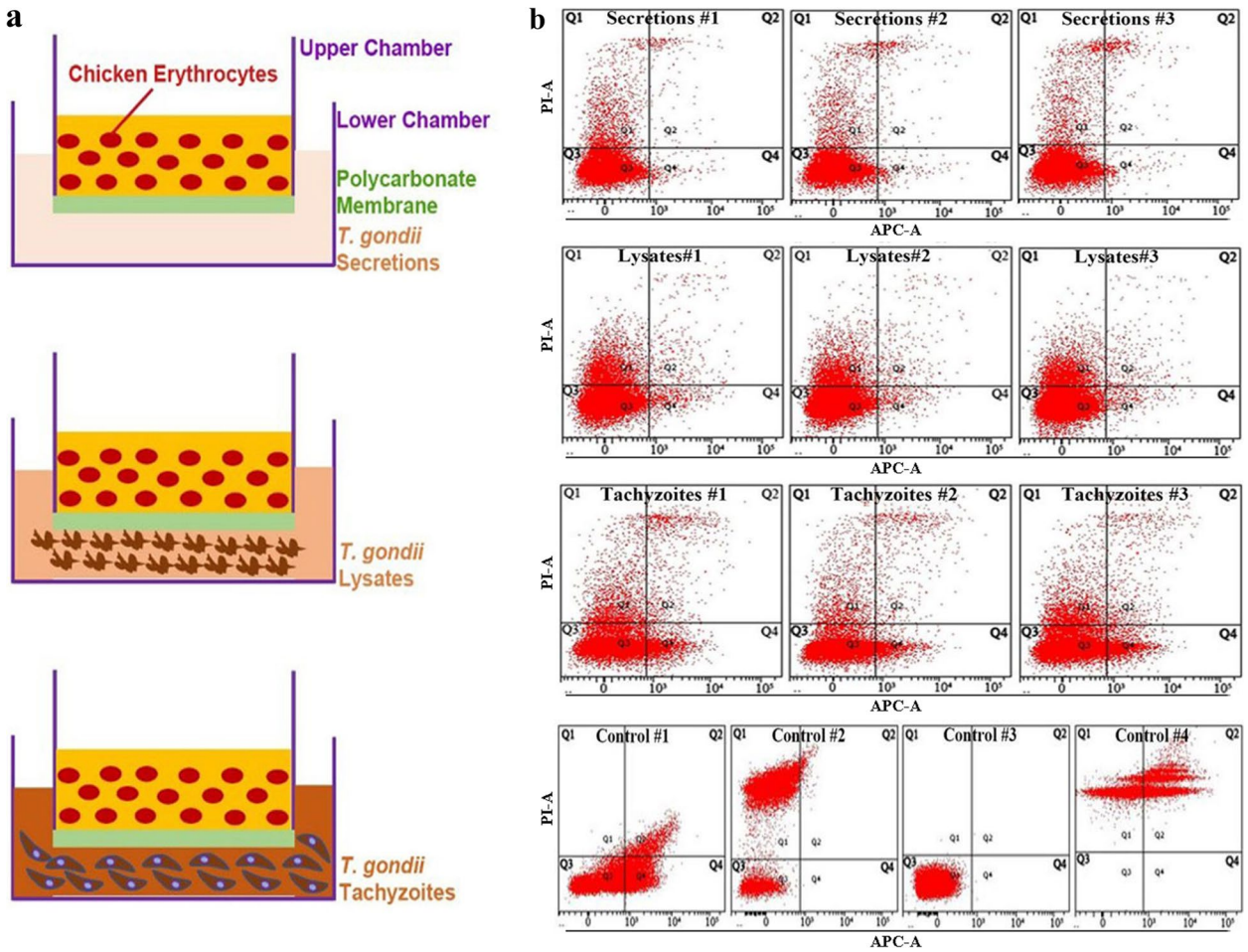

Fig. 4 Analysis of cellular apoptosis during *T. gondii* infection. **a** Schematic of transwell chamber analysis. Chicken erythrocytes were placed in the upper chamber, and *T. gondii* RH tachyzoites, secretions, and lysates were placed separately in the lower chamber. **b** Cellular apoptosis was analyzed by flow cytometry. After incubation of chicken erythrocytes with tachyzoites, secretions, and lysates for 24 h, the proportion of live cells was evaluated using flow cytometry. Flow cytometry showed necrotic cells at the upper left and normal cells at the lower left. Late apoptotic cells are shown on the upper right; late apoptotic cells are shown on the lower right. Experiment without dead cells was used as Control 1, experiment without apoptotic cells was used as Control 2, experiment with only living cells was used as Control 3, experiment without living cells was used as Control 4. The relative fluorescence intensity is represented by an axis

(Saeij et al. 2006; Taylor et al. 2006), and the upregulation of ROP18 expression could enhance *T. gondii* virulence (Hunter and Sibley2012). When chicken erythrocytes were incubated with *T. gondii* tachyzoites, MIC3, ROP16, and ROP18 transcription levels decreased. This could be due to the failure to invade chicken erythrocytes.

Host cell entry is an active process involved in gliding motility powered by an actin-myosin motor (Sibley2010), which requires the reorganization of the host cell cytoskeleton (Sweeney et al. 2010). Many invading pathogens have been shown to need host cell vimentin, which is dynamically expressed and undergoes a complicated phosphorylation pattern during pathogen infection (Sihag et al. 2007). It has been reported that host cell vimentin can inhibit *T. gondii* invasion. When vimentin was knocked out, the efficiency of *T. gondii* invasion increased significantly. Vimentin reorganization can facilitate *T. gondii* invasion (He et al. 2017). In addition, *T. gondii* infection was previously shown to control vimentin expression in different host cells (He et al. 2017), with cell vimentin expression transferred and reorganized across the PVs within 1 h after infection (Halonen and Weidner 1994). In this study, Vimentin was not rearranged in erythrocytes because there was no parasite invasion.

Since ROP18 has been shown to affect the phosphorylation pattern of cell vimentin (He et al. 2017), we investigated vimentin rearrangement in the presence

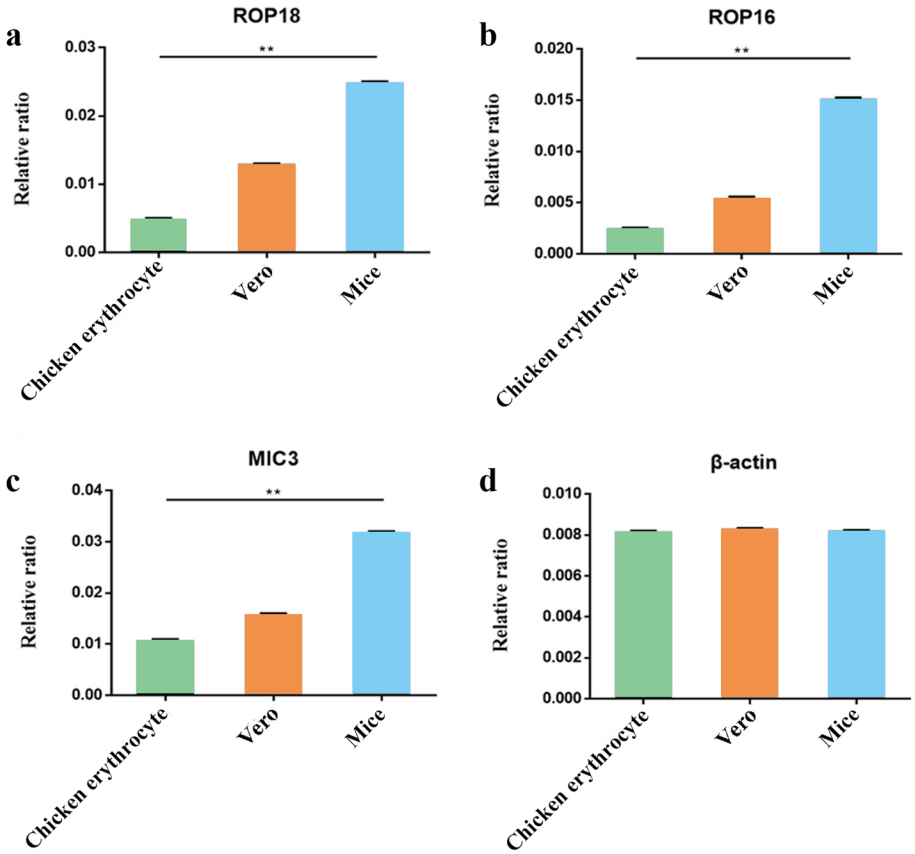

Fig. 5 Evaluation of transcriptional expression of virulence factors in *T. gondii*. *T. gondii* tachyzoites were incubated with erythrocytes in vitro. *T. gondii* cultured in Vero cells or isolated from mice in vivo were used as controls. The transcriptional levels of ROP18 (**a**), ROP16 (**b**), and MIC3 (**c**) were decreased significantly in *T. gondii* coincubated with chicken erythrocytes, while actin showed no significant difference (**d**). Error bars represent the mean ± SD ($n = 3$). $^{**}p < 0.01$

of *T. gondii* with Vero cells and chicken erythrocytes. A substantial arrangement of vimentin was present in Vero cells infected by *T. gondii* tachyzoites for different periods (8, 20 and 24 h post-infection). In contrast, cell cytoskeletal reorganization was not observed in chicken erythrocytes, confirming the failure to invade chicken erythrocytes. The decreased ROP18 level might be because parasites cannot invade chicken erythrocytes, which inhibits the rearrangement of host vimentin. Additionally, the reorganization of actin was also examined during *T. gondii* infection. It was previously reported that the F-actin structure was formed and localized at MJ throughout the entire process of *T. gondii* invasion (Gonzalez et al. 2009). In the present study, the ring-shaped F-actin structure was not formed when tachyzoites were incubated with chicken erythrocytes, indicating that *T. gondii* had a defect in invading chicken erythrocytes. The invasion tropism of *T. gondii* was also explored in our study, and we found that *T. gondii* preferentially invaded Vero cells when coincubated with chicken erythrocytes. Moreover, the invasion capability of *T. gondii* on chicken erythrocytes in vivo was further investigated by endovascularly injecting *T. gondii* tachyzoites into chickens. *T. gondii* replicated in chicken monocytes but failed to invade erythrocytes. These data support the conclusion that *T. gondii* cannot invade chicken erythrocytes but can infect other cell types, including monocytes, for dissemination (Malkwitz et al. 2017).

Conclusion

This study revealed that *T. gondii* cannot infect nucleated chicken erythrocytes and increased our knowledge of the transmission mechanism of *T. gondii* across avian species.

Methods

Ethics statement

The Ethics Committee of Animal Experimentation of Shenyang Agricultural University's Laboratory Animal Center approved the experiments using five chickens purchased from Liaoning Changsheng Biotechnology Company in China.

Chicken erythrocyte culture

Chicken erythrocytes were harvested as previously described (Malkwitz et al. 2017). Phosphate buffered saline (PBS) was used to dilute chicken blood collected from the wing vein. The erythrocytes were suspended in RPMI-1640 medium mixed with 10% chicken serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and amphotericin B (0.0025 mg/mL) after centrifugation at 1500 rpm for 10 min. The erythrocytes were cultured and incubated at 42 °C with 5% CO_2.

T. gondii culture

Tachyzoites of the virulent *T. gondii* RH strain (Type I) were cultured in Vero cell monolayers using DMEM containing 2% FCS, penicillin (200 U/mL) and streptomycin (0.25 mg/mL) and incubated at 37 °C with 5% CO_2. To harvest parasites, the freshly released tachyzoites were centrifuged at 2500 × *g* for 5 min, and the pellet was resuspended and purified using a 5 μm filter (Millipore, USA).

T. gondii infection assay

To assess *T. gondii* infection in chicken erythrocytes, freshly released *T. gondii* tachyzoites were inoculated into cultivated chicken erythrocytes. In both infected and uninfected groups, cell-based morphology and parasite replication were tracked daily following inoculation to measure the invasion efficiencies of *T. gondii.*

Chicken infection analysis

The freshly released 1×10^9 tachyzoites were intravenously injected into five chickens to assess *T. gondii* invasion potential in vivo. PBS solution was injected into a chicken wing vein as a control. Following injection, chicken blood was collected for acridine orange staining using a fluorescence microscope (Leica DM4B, Wetzlar, Germany).

Immunofluorescence assay

An immunofluorescence method was used to check the expression of vimentin protein in both Vero cells and chicken erythrocytes to examine the reorganization of vimentin during *T. gondii* infection (He et al. 2017; Halonen and Weidner 1994). In a single-well chamber, 1×10^5 Vero cells and chicken red blood cells were cocultured for 12 h before 1×10^6 T. *gondii* tachyzoites were inoculated into the cell mixtures. After incubation, parasite-infected monolayers were fixed with 4% formaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 20 min at room temperature. Following three PBS washes, the slides were blocked for 30 min at 37 °C with 3% BSA. After three more washes with PBS, the samples were incubated overnight at 4 °C with an anti-Vimentin-specific monoclonal antibody. After five washes in PBS, the slides were incubated for 30 min at 37 °C with goat anti-mouse IgG secondary antibodies (Invitrogen, USA). The slides were then washed five times with PBS to develop DAPI nuclear staining, and the sample images were visualized and captured using fluorescence microscopy (Leica DM4B, Wetzlar, Germany). The reorganization of actin was evaluated to further examine the invasion capacity of tachyzoites on chicken erythrocytes during infection. Tachyzoites were incubated with chicken erythrocytes for 0, 2, 12 and 20 h before being placed in Immuno-Fluorescence Assay (IFA), and an Actin-Tracker Red-Rhodamine antibody (Beyotime, Beijing) was used to detect F-actin expression of the host and parasites during infection.

Cellular apoptosis analysis by transwell assay and flow cytometry

As previously described, the effect of *T. gondii* on chicken erythrocyte apoptosis was assessed using a polycarbonate membrane-0.4 M transwell chamber (Corning, Guangzhou, China) (Lüder et al. 2001). To obtain parasite secretions, freshly egressed tachyzoites were collected and centrifuged at 2500 rpm for 10 min. The parasites were then suspended in egress buffer (141.8 mM NaCl, 5.8 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, 25 mM HEPES, and 2% ethanol, pH 7.2) after being washed twice with intracellular buffer (5 mM NaCl, 142 mM KCl, 1 mM $MgCl_2$, 2 mM EGTA, 5.6 mM glucose, and 25 mM HEPES at a pH of 7.2), and the parasite suspension was incubated at 37 °C for 30 min. *T. gondii* secretions were obtained by centrifugation at 4 °C and 1000 × *g* for 5 min, followed by extraction and centrifugation at 4 °C and 2000 × *g* for 5 min. In addition, to obtain parasite lysates, freshly released tachyzoites were also collected and centrifuged at 2500 rpm for 10 min. The parasites were resuspended in 1 mL of PBS buffer and then transferred to an ultrasonic crushing apparatus for 15 min. The secretions of *T. gondii* were harvested and cultured in the lower part of the Transwell chamber. Meanwhile, tachyzoite lysates and intact tachyzoites were incubated separately in the lower part of the Transwell chamber. In parallel, chicken erythrocytes were incubated in the upper part of the Transwell chamber. The experiment was performed in triplicate. The cell cultures were labeled with FITC-labeled Annexin V after incubation, and the cell viability of each group was measured and evaluated separately using flow cytometry (BD Biosciences).

Real-time PCR analysis

The transcriptional levels of virulence factors ROP18, ROP16, and MIC3 were calculated using the real-time PCR method to determine whether *T. gondii* virulence was reduced when cocultured with chicken erythrocytes

Table 1 Primers used in real-time PCR analysis

Gene	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)
ROP16	GCAGCAGCATGACTTCTTT	AGTTGGATGTTCGGGATAA
ROP18	CAGCCAACTCTTCATTCGTCTC	CAAATCGGGCAGCCTCAA
MIC3	ATGCACGTCCCGTTGGGT	TCGTCGGCACTGGGTTGA

(Edvinsson et al. 2006). In this experiment, *T. gondii* tachyzoites were cultured with chicken erythrocytes and Vero cells in vitro. Transcription analysis of the three proteins was carried out using *T. gondii* that was cocultured with either chicken erythrocytes or Vero cells or *T. gondii* tachyzoites collected from mice. After inoculation, total RNA was individually extracted with TRIzol reagent (Invitrogen, USA). The cDNA was then synthesized using a reverse transcription reagent (Takara, Dalian, China) according to the manufacturer's instructions. Following reverse transcription, the cDNA was used as a template to amplify the ROP18, ROP16, and MIC3 genes using the real-time PCR method with specific primers (Table 1).

Abbreviations

ATF6	Recombinant activating transcription factor 6
GRA15	Dense granule protein 15
GRA16	Dense granule protein 15
MIC3	Microneme protein 3
MJ	Moving junction
PV	Parasitophorous vacuole
ROP2	Rhoptry protein 2
ROP16	Rhoptry protein 16
ROP18	Rhoptry protein 18
STAT	Signal transducer and activator of transcription
T. gondii	*Toxoplasma gondii*

Authors' contributions

YN designed the experiments. LC, SX and ZX performed formal analysis, data analysis and the experiments. LC, SX, and ZX wrote the original draft. SE, JT and YN reviewed and revised the paper. HYH and DY visualized the data. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate

All animal experimental procedures were approved by the Shenyang Agricultural University (SYAU) Institutional Animal Care and Use Committee (IACUC) and conformed to national, international and university guidelines for animal care. SYAU protocol Number: SYXK (Liao)2021-0010. The animals were handled in accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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