High resistance to *Toxoplasma gondii* infection in inducible nitric oxide synthase knockout rats

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**Highlights**

- iNOS<sup>-/-</sup>-SD rats show strong resistance to *Toxoplasma gondii* infection
- iNOS<sup>-/-</sup>-SD rat PMs resist *T. gondii* infection through ROS upregulation
- The *T. gondii* infection results in PM pyroptosis in iNOS<sup>-/-</sup>-SD rats
- GRAs play a key role in the activation of resistance in iNOS<sup>-/-</sup>-SD rat PMs
Nitric oxide (NO) is an important immune molecule that acts against extracellular and intracellular pathogens in most hosts. However, after the knockout of inducible nitric oxide synthase (iNOS−/−) in Sprague Dawley (SD) rats, these iNOS−/− rats were found to be completely resistant to Toxoplasma gondii infection. Once the iNOS−/− rat peritoneal macrophages (PMs) were infected with T. gondii, they produced high levels of reactive oxygen species (ROS) triggered by GRA43 secreted by T. gondii, which damaged the parasitophorous vacuole membrane and PM mitochondrial membranes within a few hours post-infection. Further evidence indicated that the high levels of ROS caused mitochondrial superoxide dismutase 2 depletion and induced PM pyroptosis and cell death. This discovery of complete resistance to T. gondii infection, in the iNOS−/−-SD rat, demonstrates a strong link between NO and ROS in immunity to T. gondii infection and showcases a potentially novel and effective backup innate immunity system.

INTRODUCTION

Toxoplasma gondii is a globally distributed obligatory intracellular protozoan parasite that can infect a large number of warm-blooded vertebrates, including humans. It was estimated that one-third of the world’s human population is infected with T. gondii (Dubey and Frenkel, 1998). Once infected, this opportunistic pathogen is able to invade and propagate in virtually all nucleated host cells of warm-blooded animals with the exception of birds’ red blood cells (Dubey and Frenkel, 1998; Werk, 1985). Although chronic asymptomatic infection is found in most cases, it can cause severe toxoplasmosis in immunocompromised individuals including those who are undergoing chemotherapy, such as patients with AIDS, patients with cancer, and organ transplantation patients. It also causes abortion both in humans and domestic animals (Dupont et al., 2012; Flegr et al., 2014). It has long been known that there are huge differences in the outcome among different hosts infected with T. gondii, and the mechanisms of susceptibility and resistance are still hot topics to be investigated.

The mechanisms of host or host cell resistance to T. gondii infection have been extensively analyzed in mouse models. In these models, the resistance to T. gondii infection relies on a potent Th1 axis driven by IL-12-induced IFN-γ production (Dupont et al., 2012; Gazzinelli et al., 2014). This cytokine primes innate immune cells to express proinflammatory mediators, which favor the development of Th1 immune response against pathogens (James, 1995). In addition, the IFN-γ also upregulates the activity of inducible nitric oxide synthase (iNOS), which produces large amounts of nitric oxide (NO), a critical effector molecule that can restrict T. gondii growth in mice (Hunter and Sibley, 2012). NO is an essential signaling molecule in animals and is produced by a family of nitric oxide synthases (NOSs), including neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3), and particularly by inducible NO synthase (iNOS or NOS2), through the catalysis of L-arginine, the substrate of these enzymes (Alderton et al., 2001; Griffith and Stuehr, 1995; Martens et al., 2005; Masters et al., 1996; Nathan and Xie, 1994). NO, mainly produced by iNOS, is a primary killing factor of intracellular pathogens and plays an important role in the activity of macrophages (Bogdan, 2001; Schlüter et al., 1999). A large number of studies have demonstrated that NO is a microbicidal substance and is cytotoxic against many pathogens including viruses, bacteria, fungi, protozoan, and helminth parasites (Chen, 2016; Croen, 1993; Karupiah et al., 1993; Nathan and Hibbs, 1991; Shen et al., 2017a, b). The importance of NO in the mouse has been discussed extensively for many decades. For example, following the knockout of the iNOS gene, mice were found to be more susceptible to the
intracellular pathogens, Leishmania, T. gondii, and many others (Behnke et al., 2016; Croen, 1993; Yarovinsky, 2014).

The rat is another animal model system that has drawn recent attention in disease studies, particularly in the field of toxoplasmosis (Freyre et al., 2003a). Rats are highly resistant to T. gondii infection in comparison with mice, although the survival rate and tissue cyst numbers vary greatly among different rat strains and T. gondii strains (Freyre et al., 2003a, 2003b, 2004; Gao et al., 2015; Zenner et al., 1993). For example, when adults rats were orally infected with 200 T. gondii Prugniaud cysts, the Fischer 344 rat strain developed a large cyst burden (1231 ± 165.6, ranging from 820 to 1800), whereas four other strains of rat, including Brown Norway (BN), Sprague Dawley (SD), Wistar, and Lewis (LEW), developed no cysts (Gao et al., 2015). The subclinical patholgy of toxoplasmosis in rats is similar to that found in humans, which may make it a better model for human toxoplasmosis than mice (Dubey and Frenkel, 1998). Many studies have demonstrated that NO might play an important role in the control of T. gondii infection in rats, and mechanisms for this resistance have been proposed (Cavailles et al., 2014; Gao et al., 2015; Witola et al., 2017). Specifically, the higher expression levels of iNOS in rats, compared with the lower expression of this gene found in mice, which are more susceptible, correlates with a greater resistance to T. gondii infection in rats (Li et al., 2012; Zhao et al., 2013). However, these studies only provided indirect evidence of the role of NO owing to the limitations of the genetic tools available in rats until recently. Recently, iNOS knockout (iNOS−/−) SD rats were generated and used, in our laboratory, to directly reveal the role of NO against Leishmania and Schistosoma infections (Chen, 2016; Shen et al., 2017a, b). As NO plays different roles in mice and humans, we wished to understand the role of NO (or iNOS) in innate immunity against T. gondii infection in rats.

By using the iNOS−/−-SD rat, we measured the resistance of this animal to T. gondii infection. Surprisingly, both results from in vitro and in vivo studies clearly demonstrated that the individuals and the macrophages from the iNOS−/−-SD rats were completely resistant to the parasite infection. These results are unexpected and are contradictory to the results found for the mice model and may indicate a potential new or backup innate immunity system to control infections in rats.

RESULTS

iNOS−/−-SD rats are resistant to Toxoplasma gondii infection

In earlier stages of this work, we thought that after the deletion of the iNOS gene the rat would become more susceptible to infection by T. gondii and other intracellular pathogens in a similar manner to that found in mice and for other parasites. Our results showed that about 40% wild-type (WT)-SD rats died within two weeks from infection with the T. gondii strains RH or Tgctsd1 (a virulent T. gondii strain isolated from a cat in Shandong Province, China) (Figure 1A). However, to our surprise, iNOS−/−-SD rats did not display susceptibility but showed resistance to the infection when using different strains of T. gondii (Figure 1B), suggesting a significant burst of resistance after the knockout of iNOS. A brain cyst count in surviving rats revealed a relatively high burden in all examined WT-SD rats infected with strain Tgctsd1, but a lack of cysts in the 6 examined iNOS−/−-SD rats (Figure 1C). A similar phenomenon was observed in infections with the T. gondii Prugniaud (Pru) strain, although a lower cyst burden was found.

To further confirm the resistance in iNOS−/−-SD rats against T. gondii infection, homogenized brains and other organs from infected iNOS−/− and WT-SD rats were intraperitoneally (i.p.) injected into mice. None of the organ samples collected from the iNOS−/−-SD rats were found to be positive after 10 days of infection with the T. gondii RH strain, while 17 brains, 2 spleens, and 13 lungs were found to be positive in the WT-SD groups (Table 1). This demonstrated that T. gondii could not survive in the iNOS−/−-SD rats beyond, at least, 10 days post-infection (dpi). Similar results were obtained, on day 60, in the infection with other strains of T. gondii, PLK/RED, and Tgctsd1 (Table 1). In addition, the amount of IFN-γ in sera collected from both the iNOS−/− and WT-SD rats, 3–6 days after infection with T. gondii, was significantly increased (Figure S1).

To clarify the involvement of cells in the resistance to T. gondii in iNOS−/−-SD rats, rat neonatal muscle fibroblast cells (NMFCs) were first tested. Both the WT and iNOS−/−-SD rats’ NMFCs were significantly resistant to the T. gondii RH/GFP strain when IFN-γ was supplemented in the medium (Figures 1E–1G). In addition, lipopolysaccharide (LPS) was also found to slightly enhance the resistance, and a synergistic effect was observed with IFN-γ. Similarities in the mode of resistance were observed when the T. gondii
PLK/RED strain was used (Figures S3A–S3C). The behaviors of rat NMFCs, with respect to resistance to T. gondii infection, were almost identical in both iNOS/C0/C0 and WT-SD rats. This suggested that the specific resistance in iNOS/C0/C0-SD rats against T. gondii infection might be independent of muscle cells.

After excluding the possibility that muscle fibroblasts were playing a major role in defense against T. gondii, we then asked the question of how T. gondii could be completely killed in the iNOS/C0/C0-SD rats? To our knowledge, this might not be a traditional immunological pathway that has previously been described that could account for this. It is well known that macrophages are primarily and frequently attacked by T. gondii and many other pathogens. We, therefore, focused on macrophages. Our results demonstrated that, although NO was not detected in the freshly obtained iNOS/C0/C0-SD rat PMs (Figure S2),...
surprisingly the knockout rat PMs showed a strong resistance to \textit{T. gondii} infection even without IFN-γ stimulation (Figures 2A and 2B). In contrast, the \textit{T. gondii} grew well in freshly harvested WT-SD rat PMs, unless rat PMs were pre-treated with IFN-γ, LPS, or sodium nitroprusside (SNP) (Figures 2A and 2C). These findings were confirmed by experiments with the \textit{T. gondii} PLK/RED strain in rat PMs (Figures S3D–S3F) and bone marrow-derived macrophage cells (Figures S3G and S3H). When the NO content in medium supernatants was assayed, the NO level in the WT-SD rat PMs group was positively correlated with \textit{T. gondii} tachyzoites within 1 h post-infection (hpi) with \textit{T. gondii}, which was even higher than the naturally higher levels of ROS found in the, naturally resistant, LEW rat PMs. Meanwhile, an increase was not detected in the WT-SD rat PMs (Figures S3D–S3F) and bone marrow-derived macrophage cells (Figures S3G and S3H). When the NO content in medium supernatants was assayed, the NO level in the WT-SD rat PMs group was positively correlated with \textit{T. gondii} growth inhibition, which could be enhanced by the presence of an NO donor compound SNP (Figures 2A and 2C), while results from the \textit{iNOS}−/−-SD groups still showed a strong resistance to \textit{T. gondii} infection no matter whether NO was present or not (Figure S2).

\textit{iNOS}−/−-SD rat PMs infected with \textit{T. gondii} infection caused ROS upregulation and superoxide dismutase 2 depletion

NO, representing reactive nitrogen species (RNS) can synergistically work with reactive oxygen species (ROS) to create a variety of intermediates (Nathan and Shiloh, 2000). Therefore, we wondered about the status of ROS in the macrophages prepared freshly from the \textit{iNOS}−/−-SD rats. Our results clearly showed a significant increase of ROS levels in the \textit{iNOS}−/−-SD rat PMs within 1 h post-infection (hpi) with \textit{T. gondii} which was even higher than the naturally higher levels of ROS found in the, naturally \textit{Toxoplasma} resistant, LEW rat PMs. Meanwhile, an increase was not detected in the WT-SD rat PMs (Figures 2D and 2E). Importantly, reduced glutathione (GSH), an intracellular antioxidant (also referred to as a cellular ROS scavenger), was also found to be significantly increased in parallel with the levels of ROS (Figure 2F), indicating an oxygen stress response in the infected cells.

Table 1. Bioassay of organs from WT and \textit{iNOS}−/−-SD rats infected with different strains of \textit{T. gondii} tachyzoites

| Rats + \textit{T. g} (dpi) | Blood | Brain | Heart | Liver | Spleen | Lung | Kidney |
|---------------------------|-------|-------|-------|-------|--------|------|--------|
| WT-SD rats + RH/GFP (10 d) | 0/10 1 | 9/10 <0.001 | 0/10 1 | 0/10 1 | 2/10 0.500 | 8/10 0.007 | 10/10 1 |
| \textit{iNOS}−/−-SD rats + RH/GFP (10 d) | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| WT-SD rats + RH/GFP (20 d) | 0/10 1 | 6/10 0.034 | 0/10 1 | 0/10 1 | 0/10 1 | 5/10 0.093 | 10/10 1 |
| \textit{iNOS}−/−-SD rats + RH/GFP (20 d) | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| WT-SD rats + RH/GFP (30 d) | 0/10 1 | 2/10 0.500 | 0/10 1 | 0/10 1 | 0/10 1 | 0/10 1 | 10/10 1 |
| \textit{iNOS}−/−-SD rats + RH/GFP (30 d) | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| WT-SD rats + PLK/RED (60 d) | 0/9 1 | 4/9 0.082 | 0/9 1 | 0/9 1 | 0/9 1 | 0/9 1 | 0/9 1 |
| \textit{iNOS}−/−-SD rats + PLK/RED (60 d) | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 |
| WT-SD rats + Tgctsd1 (60 d) | 0/9 1 | 3/9 0.206 | 0/9 1 | 0/9 1 | 0/9 1 | 0/9 1 | 0/9 1 |
| \textit{iNOS}−/−-SD rats + Tgctsd1 (60 d) | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 |
| WT-SD rats + PBS (30 d) | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |

The number of \textit{T. gondii} positive/tested rats is shown. Half a gram tissue from each rat was removed, homogenized, and then intraperitoneally injected into a Swiss Webster mouse. Data were analyzed using the Fisher’s exact test. Significance was accepted at <0.05, No., number of positive organs detected.

Effect of \textit{T. gondii} infection on the parasitophorous vacuole membrane and mitochondria of \textit{iNOS}−/−-SD rat PMs

The parasitophorous vacuole membrane (PVM) is a key barrier required for parasite survival within the host cell (Molestina and Sinai, 2005; Sinai and Joiner, 1997; Suss-Toby et al., 1996). ROS are very important...
Figure 2. Resistance of iNOS<sup>−/−</sup>-SD rats to T. gondii is dependent on PMs and ROS bursts

(A) The status of T. gondii RH/GFP strain tachyzoites in the iNOS<sup>−/−</sup>-SD and WT-SD rat PMs treated with different compounds 48 hours post-infection (hpi), scale bars equal 50 μm. LPS, lipopolysaccharide; IFN-γ, interferon-gamma; SNP, sodium nitroprusside.

(B and C) The mean parasite loads, judged by fluorescence intensity, in iNOS<sup>−/−</sup>-SD (B) and WT-SD (C) rat PMs; MOI = 2, cells were transferred into 24-well plates for further experimentation, 5 x 10<sup>5</sup> cells per well, in triplicate, n = 3.

(D) Detection of T. gondii tachyzoites and ROS in rat PMs infected T. gondii. Rat PMs were transferred to confocal dishes, 10<sup>6</sup> per dish with or without T. gondii PLK/RED strain tachyzoites at an MOI = 2 for 1 h and then washed with 1 x PBS 3 times, incubated in RPMI-1640 for 1 hour, followed by another 30-min incubation with DCFH-DA (2',7'-dichlorofluorescin diacetate) at 10 μM. The resulting oxidized form (DCF) was observed by confocal microscopy, bar = 50 μm.

(E) The oxidized DCF in the rat PMs was detected by flow cytometry. Rats were i.p. infected with 10<sup>7</sup> T. gondii PLK/RED strain tachyzoites and then 1 h later rat PMs were collected following i.p. injection with 0.5 mL of PBS and subjected to flow cytometry (n = 3, >10,000 counts).
Figure 2. Continued
(F) The GSH level in the rat PMs. Rat PMs were harvested as above but at a later time point (3 hpi); n = 3.
(G) The protein level of SOD2 was measured by Western blotting. Rat PMs were harvested at 3 hpi as above; β-tubulin was performed on the same blot and used to normalize the signal.
(H, I and J) The infection rate of T. gondii RH/GFP strain tachyzoites in the WT-SD (H), iNOS−/−-SD (I), and LEW (J) rat PMs cultured with or without MTBAP and GSH. Cells were transferred into 24-well plates for subsequent investigation, 5 × 10^5 per well; MOI = 2; in triplicate, n = 3. All data are presented as mean ± SD and are representative of three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns: no significant difference. See also Figures S2, S3, S5, and S6.

Effectors in the host cellular immune response to intracellular pathogens, which need to cross to the PVM to be able to damage T. gondii. When we monitored the structure of the PVM in the infected cells 3 hpi by transmission electron microscopy, the resulting images clearly revealed that the structure of the PVM in iNOS−/−-SD rat PMs was blurred and damaged (Figures 3C and 3D, red arrows). However, the PVMs of WT-SD rat PMs were structurally clear (Figures 3A and 3B, white arrows). ROS is considered a double-edged molecule; it is not only toxic to parasites but also damage host cells (Zhang et al., 2016). The mitochondrial membranes in the iNOS−/−-SD rat PMs infected with T. gondii were found to be scattered, be distorted, and lacked intact internal structures (Figure 3G, red arrows). In contrast, the uninfected iNOS−/−-SD rat PMs’ and infected/uninfected WT-SD rat PMs’ mitochondria appeared normal (Figures 3E, 3F, and 3H, black arrows). The damage in the infected iNOS−/−-SD rat PMs might be the consequences of specific ROS bursts in the infected cells. These results suggested that the burst of ROS might be the key reason for PVM and host mitochondrion disruption in the iNOS−/−-SD rat PMs infected with T. gondii.

Rapid cell death and pyroptosis in the iNOS−/−-SD rat PMs infected with T. gondii
As revealed by propidium iodide (PI) and Hoechst staining, T. gondii infection was found to induce cell death in all freshly collected rat PMs tested from different strains of rats, especially in the iNOS−/−-SD rat PMs (which increased up to 22%–31%) (Figures 4A and 4B). Data from flow cytometry analysis also confirmed these findings. This result further supported the observations that the major proportion of cell death was necrosis both in the LEW and iNOS−/−-SD rat PMs (Figure 4C). We then investigated the expression of pyroptosis-specific markers including caspase-1, IL-1β, and IL-18 by Western blotting. The results showed that pro-caspase-1, the precursor of caspase-1, remained unchanged in the rat PMs post-infection, while the mature form caspase-1 and the downstream cytokines IL-1β and IL-18 were found to be increased in the iNOS−/−-SD and LEW rat PMs when infected with T. gondii (Figures 4D and 4E). These findings were further confirmed by using an inhibitor (VX765) of caspase-1 which protected T. gondii from damage and resulted in a higher infection ratio (Figure S4). In contrast, however, the caspase-3-p12, a specific signal for apoptosis, was not detected (Figure 4D). Furthermore, treatment to inhibit the activity of caspase-3-p12 by affecting a rat-specific upstream protein NLRP3 using the inhibitor MCC950 resulted in no change in infection ratio compared with the control in the iNOS−/−-SD and LEW rat PMs (Figure S4). Taken together, these results clearly support the cell fate as pyroptosis but not apoptosis in the iNOS−/−-SD rat PMs infected with T. gondii. Thus, the explanation for the complete resistance to T. gondii infection found in the iNOS−/−-SD rat is related to cell pyroptosis as a mechanism. This raises a further critical question as to how T. gondii infection can cause pyroptosis in the PMs of the iNOS−/−-SD rat.

Effect of GRA43 on the induction of pyroptosis in iNOS−/−-SD rat PMs
Previous studies suggested that GRAs such as GRA43 are linked to pyroptosis in LEW rat macrophages (Wang et al., 2019a). To test if these proteins were the main cause of pyroptosis in the PMs of iNOS−/−-SD rat, we generated a GRA43 knockout T. gondii strain (RhΔgra43) which was then used to infect the rat PMs from different rat strains. Results clearly indicated that RHΔgra43 strain indeed produced a significantly higher infection ratio in the PMs of iNOS−/−-SD and LEW rats than those found in the parental RH strain (Figures S5B and S5C). Furthermore, our results also demonstrated that the RHΔgra43 strain caused much less ROS in the PMs of iNOS−/−-SD and LEW rats (Figures S5D and S5E). However, this effect was not observed in the iNOS−/−-SD rat PMs infected with the RHΔrop18 strain (Figure S6). These results indicate that T. gondii GRA43 is a key trigger for causing the pyroptosis in the iNOS−/−-SD rat PMs.

DISCUSSION
The biological functions of NO have been extensively investigated, and the effect of this molecule against pathogens is also well understood. A large number of early studies based on mouse and rat models have
shown that NO produced by iNOS is a critical anti-
T. gondii host factor (Alexander et al., 1997; Li et al., 2012; Zhao et al., 2013). In addition, our earlier studies in rats also indicated that NO played an important role against Leishmania and Schistosoma infections (Chen, 2016; Shen et al., 2017a, b).

To our surprise, however, the iNOS−/−-SD rat was found to be extremely resistant to T. gondii infection resulting in failure to form cysts and to cause active infection. Traditionally, mice macrophages were found to be more susceptible to T. gondii infection if the iNOS gene was knocked out or inhibited (Alderton et al., 2001; Scharton-Kersten et al., 1997). This demonstrated that NO or NOS played an essential role in protecting mice from toxoplasmosis (Alexander et al., 1997). Unlike mice, rats are relatively resistant to T. gondii infection with different rat strains showing different rates of survival and tissue cyst formation (Freyre et al., 2003a, 2003b, 2004; Gao et al., 2015; Zenner et al., 1993). Once infected, despite strain variation, most rats remain asymptomatic and usually develop only a chronic infection with the production of cysts (Gao et al., 2015). This has been attributed to the high levels of expression of iNOS and high concentrations of NO in rats (Li et al., 2012). For example, with a lower iNOS expression and lower NO level, BN rats are more susceptible to T. gondii infection than the high iNOS expression and high NO levels found in LEW rats (Li et al., 2012). Our results, however, demonstrated that the iNOS−/−-SD rat showed a resistance to T. gondii infection that was dramatically high. This result was unexpected and surprising to us. Further characterization showed a significant increase of ROS in the iNOS−/−-SD rats infected with T. gondii and suggested that ROS was linked to this resistance. Previous studies indicated that the macrophages from BN rats exhibited much lower levels of ROS than LEW rat PMs (Witola et al., 2017). Interestingly, although the expression of the iNOS gene and the levels of NO were lower in the SD rat PMs than those found in the LEW rat (Gao et al., 2015), our results show that the ROS levels in both SD and LEW rat PMs are similar. Therefore, it seems that the different expression levels of iNOS or the amount of NO are linked to the differences in susceptibility of the SD and LEW rats against T. gondii infection and are therefore the key determinants of innate susceptibility/resistance. However, our results from the iNOS−/−-SD rats contradicted this because both iNOS−/−-SD and LEW rats showed similar resistance to T. gondii infection. These observations implied that there is the existence or activation of a hidden and aggressive immune mechanism in SD rats, active against T. gondii infection, when iNOS was absent.

Figure 3. The ultrastructure of the parasitophorous vacuole membrane (PVM) and host mitochondrion in the WT and iNOS−/−-SD rat PMs infected with T. gondii
(A–D) The PVM structures of WT-SD rat PMs infected with T. gondii are normally clearly seen with a double membrane (A, B, white arrows), while blurred membranes in PVMs were observed in the iNOS−/−-SD rat PMs infected with this parasite (C, D, red arrows).
(E–H) The host mitochondrial morphology in PMs from WT (E, F) and iNOS−/−-SD (G, H) with or without T. gondii infection. Intact membranes with clear outer double membranes and internal ridges were observed in WT SD PMs infected with or without T. gondii (E,F, black arrows) and in iNOS−/−-SD PMs without T. gondii infection (H, black arrows), while blurred inner membranes were found in the iNOS−/−-SD PMs infected with T. gondii (G, red arrows). Rat PMs were obtained at 3 hpi, rats aged 4 weeks had 10^7 T. gondii PLK/RED strain tachyzoites i.p. injected. HC, host cell; HM, host cell mitochondrion; HN, host cell nucleus; N, T. gondii nucleus; PG, polysaccharide granules; M, T. gondii mitochondrion; bar = 500 nm.
Owing to the significant increase of ROS found in freshly collected iNOS−/−-SD PMs, infected with T. gondii, we thought that ROS might play a role in this resistance in the iNOS−/−-SD rat. It is well known that ROS, produced by oxidases and nonenzymatic sources, are highly reactive metabolites of molecular oxygen and comprise hydrogen peroxide, superoxide radicals, and hydroxyl radicals (Andreyev et al., 2005). These molecules have wide biological functions in different animals from invertebrates to humans, and one important role is in host defense against intracellular pathogens ranging from viruses to parasites. The generation of ROS was found to limit the survival and proliferation of T. gondii in infected mammalian cells (Aline et al., 2002; Buzoni-Gatel and Werts, 2006; Miller et al., 2006; Murray et al., 1985). However, ROS molecules are a double-edged sword. They can kill pathogens and also damage host cell lipids, DNA, RNA, and proteins, resulting in cellular damage and even cell death (Aicardo et al., 2016). Although the effect of ROS against T. gondii had been reported in the LEW rat (Cavailles et al., 2014; Witola et al., 2017), in the early stages of this work it was hard for us to understand the nature of the mechanism (or reason) for this increased ROS in the infected iNOS−/−-SD rat macrophages. From electron microscopy and cell culture
observations, we found damaged PVMs and disruption of host cell mitochondria as well as increased cell death in the infected iNOS<sup>−/−</sup>-SD rat PMs. These observations were linked to the increase of ROS in the infected cells because similar results were not observed in the PMs isolated from infected WT-SD rats. Clearly, ROS are the main factor for the death of cells and the parasite in the infected PMs. They not only are toxic to the PVM of *T. gondii* tachyzoites but can also damage the host cell mitochondrion and subsequently cause cell death. Several studies have demonstrated that serious mitochondrial damage always accompanies cell death (Fan et al., 2017; Wang et al., 2019b). Other studies have shown that removal of oxidants by MnTBAP(III) in LEW macrophages leads to a significant increase in cells containing tachyzoites and is accompanied by a decreased resistance to *T. gondii* infection (Witola et al., 2017). Our results also demonstrated the presence of more parasites and less cell death in infected iNOS<sup>−/−</sup>-SD rat PMs when treated with MnTBAP(III) or GSH. Therefore, the innate response, by rapid killing of *T. gondii* tachyzoites, in the iNOS<sup>−/−</sup>-SD rat PMs is dependent on the highly active ROS but is countered by the high cost of host cell death. This suggests that a two-level defense system may exist in rats: an iNOS-based system that drives the normal response against pathogens but causes minimal host damage and a much more self-destructive, ROS-based, system that kicks in if iNOS is absent or perhaps fails to cope with the infection.

This raises the question as to how ROS is upregulated in the macrophages of infected iNOS<sup>−/−</sup>-SD rats. Interestingly, other studies showed that the resistance of the LEW rat was linked to inherently high transcript levels of Cytp2d3, Cytp25, and Cybrd1 (Witola et al., 2017), whose gene products promote mitochondrial electron transfer, the Fenton reaction, and generate large amounts of ROS (Hrycay and Bandiera, 2015). However, high levels of these corresponding enzymes were not found in the iNOS<sup>−/−</sup>-SD rat PMs infected with *T. gondii* (NCBI number: PRJNA642722), which suggests that other unknown pathways related to the upregulation of ROS must exist in this iNOS<sup>−/−</sup>-SD rat. Based on the comparison of metabolic pathways between WT and iNOS<sup>−/−</sup>-SD rats, no significant differences were found (data not shown). We thought, therefore, that perhaps other factors, derived from *T. gondii*, might cause the upregulation of ROS in iNOS<sup>−/−</sup>-SD rats. It has been reported that some PRRs and GRAs play an extremely important function in the invasion and development of *T. gondii* in the host cell (Gold et al., 2015). Among them, some have been investigated well. GRA43 is a dense granule protein colocalized with GRA7 and can influence the correct localization of other GRAs to the PVM, a key requirement for the development of *T. gondii* within the host cell (Wang et al., 2019a). GRA7 is a transmembrane protein secreted from *T. gondii* that was considered to interact with ROP complexes in the host cytosol and play a critical role in MyD88-dependent TRAF6 activation and ROS production in macrophages (Alaganan et al., 2014; Yang et al., 2016). By comparison with the freshly collected WT-SD rat PMs infected with *T. gondii*, our data showed that the iNOS<sup>−/−</sup>-SD rat PMs infected with *T. gondii* presented higher transcript levels of MyD88 and TRAF6 (Table S1), followed by a burst of ROS and the killing of the parasite in the iNOS<sup>−/−</sup>-SD rat PMs. However, after the knockout of GRA43, the *T. gondii-gra43* tachyzoites failed to induce the upregulation of ROS in iNOS<sup>−/−</sup>-SD rat PMs, while the *T. gondii-gra43* strain could proliferate intracellularly. These results demonstrated that the GRA43 might interfere with the extension of GRA7 into the host cytosol resulting in a failure to induce the host ROS burst in iNOS<sup>−/−</sup>-SD rat PMs. Moreover, the accumulation of ROS in macrophages is considered to be closely related to the amount of NO content in cells. Studies indicated that NO produced by iNOS could promote the accumulation of SOD2, one of the major mitochondrial antioxidants that can significantly suppress the ROS (Kaneko et al., 2012). Therefore, iNOS or NO depletion may eliminate the suppression and uplift the ROS, followed by the ROS depletion by SOD2 in a manner similar to our observations in the iNOS<sup>−/−</sup>-SD rat PMs infected with *T. gondii*. However, the supply of NO to the iNOS<sup>−/−</sup>-SD rat PMs in vitro did not block the ROS anti-*T. gondii* pathway, which excluded the direct role of NO in ROS suppression. Indeed, it was shown that the iNOS monomer could be localized to the peroxisome which, in turn, was believed to alleviate damaging nitrogen or oxygen radicals (Loughran et al., 2005; Stolz et al., 2002). This may explain why TRAF6 and MyD88 were upregulated in WT-SD rat PMs infected with *T. gondii*, compared with the uninfected WT-SD rat PMs, but that the ROS did not increase significantly. Therefore, we conclude that the absence of iNOS and the normal secretion of *T. gondii* GRA7 jointly promoted the ROS burst and that the burst of ROS not only was toxic to the parasite but also damaged the host cell mitochondrion and resulted in the depletion of SOD2. In addition, we also observed the depletion of SOD2 in the iNOS<sup>−/−</sup>-SD rat PMs. SOD2 can banish superoxides (O<sub>2</sub><sup>−</sup>) and reduce the mitochondrial membrane oxidation pressure (Beccana et al., 1986). In other words, the depletion of SOD2 may act in a parallel way to uplift ROS.

ROS production in macrophages is always related to inflammasome activation, which may directly cause cellular damage, oxidative stress, and pyroptotic processes (Shen et al., 2020; Wang et al., 2019b).
Pyroptosis in pathogen restriction is a critical innate immune response to prevent intracellular infection (Boucher et al., 2015). It is emerging as an important mechanism of microbial clearance, during which the activation of caspase-1 leads to the production of IL-1β and IL-18 (Boucher et al., 2015; Xia et al., 2019). In LEW rat macrophages, T. gondii infection could activate the NLRP1 inflammasome, secretion of mature IL-1β, resulting in pyroptosis, and inhibition of parasite replication (Cavailles et al., 2014; Cirelli et al., 2014; Ewald et al., 2014). Subsequent research has confirmed that three dense granule proteins (GRAs) promote parasite-stimulated pyroptosis in LEW rat macrophages (Wang et al., 2019a). Our results directly show that the iNOS−/−-SD rat PMs infected with T. gondii could induce cell pyroptosis; however, when the GRA43 protein is absent, the resistance to T. gondiiΔgra43 was decreased in iNOS−/−-SD rat PMs. Pyroptosis in iNOS−/−-SD rat PMs may only be limited to T. gondii, or other apicomplexans, which carry out active invasion and secrete GRA proteins but not to those that adopt passive invasion like Leishmania amazonensis (LV78) (Chen, 2016) by phagocytosis. Another well-known virulence factor in T. gondii, rhoptry protein ROP18, did not seem to be involved in the activation of ROS/NLRP1 resistance as found previously in the LEW rat (Cavailles et al., 2014).

In summary, our studies have potentially uncovered a novel, non-traditional, innate immune pathway in the iNOS knockout SD rat which, in the absence of iNOS, enables complete resistance to T. gondii infection (Figure S7). This resistance mechanism is mainly dependent on the upregulation of ROS and subsequent pyroptosis in rat PMs. The activation of the ROS/NLRP1 pathway requires the secretion of the T. gondii GRA43 protein. Because WT-SD rats are incapable of activating the ROS/NLRP1 pathway, iNOS is considered to be an essential negative factor that modulates the pathway into a pre-activated state. Hence, in the SD rat, there exists a dormant powerful, but suicidal pathway, of pyroptosis that is specific to T. gondii and is locked down by iNOS. This finding may inspire some future novel therapeutic strategies for the prevention of domestic livestock or human toxoplasmosis and, possibly, other infectious diseases.

Limitations of the study

Owing to the limitations on the usage of animals, the detection of the parasite at earlier time points, during the acute stage of infection within the first 10 days, was not performed. As a result, we were unable to compare parasite burdens between WT and iNOS−/−-SD rats in the very early stages of infection, nor were we able to directly demonstrate the anti-tachyzoite capability of the peritoneal macrophages obtained during the acute stage.

For in vitro experiments, a high level of IFN-γ (100 ng/mL) was used in the positive controls. We recognize that this does not directly reflect the physiological levels measured in in vivo sera (averaging 200–400 pg/mL) (Figure S1). However, we used these higher levels, in the in vitro functional assays, to show the proof of concept that WT rat macrophages can eliminate T. gondii in an IFN-γ-dependent manner. By contrast, in the knockout mutant, rat macrophages could eliminate the parasite even in the absence of IFN-γ. We recognize that it would be desirable to match the IFN-γ concentrations in in vitro and in vivo experiments. However, the range of physiological in vivo values of IFN-γ concentration can vary widely in different hosts, such as mice, rats, and humans, and is dependent on immunological status. Even in our own study just on rats alone (Figure S1), the ranges of IFN-γ concentrations varied between 50 and 600 pg/mL in the WT rats, depending on the immunological status. Furthermore, in vivo concentrations of IFN-γ are often only measured as average values, while in reality, there will be localized variations in the microenvironments of individual macrophages (which, of course, produce IFN-γ themselves). While we recognize the desirability of closely matching IFN-γ concentrations in in vitro and in vivo experiments, we believe that this does not have an impact on the overall conclusions of our study.

Throughout the study, we used peritoneal macrophages that were freshly collected before being exposed to a variety of experiments. These freshly isolated macrophages are in a similar physiological condition and show most of the physiological functions of in vivo macrophages. We believe that this accurately represents what is happening to these macrophages in vivo; however, we do recognize the possibility that these cells may have altered behavior once harvested. We recognize that further work may be needed to fully establish the link between the in vitro and in vivo experiments.

While we have demonstrated that GRA43 triggered the T. gondii resistance pathway in iNOS−/−-SD rat PMs, we recognize that some other GRAs and secreted proteins may also contribute to the initiation of
T. gondii resistance. Therefore, further studies are needed to better understand the full story with respect to the detailed identities of these resistance triggers.

STAR METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103280.

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AUTHOR CONTRIBUTIONS
Conceptualization: ZRL and DHL; methodology: ZRL, DHL, and ZJW; formal analysis: ZJW, DHL, GH, and MY; Investigation: ZJW, SMY, JMG, and PZ; visualization: ZJW and DHL; project administration: ZRL and DHL; funding acquisition: ZRL, DHL, and GH; Writing – original draft: ZJW, DHL, and ZRL; Writing – review & editing: ZJW, GH, MY, DHL, and ZRL.

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The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-SOD2 antibody produced in goat | Sigma-Aldrich | Cat#SAB2501676; RRID: AB_2893357; Lot#10972P1 |
| Anti-β-tubulin mouse monoclonal antibody | TransGen | Cat#HC101; RRID: AB_2893358; Lot#L20817 |
| Rabbit anti-rat caspase-1 antibody | Abcam | Cat#ab179515; RRID: AB_2884954; Lot#GR3232708 |
| Rabbit anti-rat caspase-3 antibody | Abcam | Cat#ab179517; RRID: AB_2893359; Lot#GR3271024-1 |
| Rabbit anti-rat IL-1β antibody | Abcam | Cat#ab9787; RRID: AB_308787; Lot#GR161754-57 |
| Rabbit anti-rat IL-18 antibody | Abcam | Cat#ab191860; RRID: AB_2750951; Lot#GR3243446-9 |
| Anti-GAPDH mouse monoclonal antibody | TransGen | Cat#HC301; RRID: AB_2629434; Lot#M10814 |
| HRP-labeled Goat Anti-Rabbit IgG (H+L) | Beyotime | Cat#A0208; RRID: AB_2892644; Lot#01151818022 |
| Rabbit anti-goat IgG HRP-conjugated antibody | Gentex | Cat# GTX228416-01; RRID: AB_2887582; Lot#41715 |
| Goat anti-mouse IgG (H+L) secondary antibody, HRP | Invitrogen | Cat#31430; RRID: AB_228307; Lot#UB278606 |
| Toxoplasma gondii SAG1 monoclonal antibody | Invitrogen | Cat#MAS-18268; RRID:AB_2539642; Lot#TJ2659778 |
| Goat anti-mouse IgG (H+L) cross-adsorbed ready probes™ secondary antibody, alexa flour 594 | Invitrogen | Cat#R37121; RRID: AB_2556549; Lot#2014703 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Lipopolysaccharide | Sigma-Aldrich | Cas: SMB00610 |
| Interferon-gamma | Sigma-Aldrich | Cas: I3275 |
| Sodium nitroprusside | Sigma-Aldrich | Cas: 13755-38-9 |
| Mn(III) tetrakis (4-benzoic acid) porphyrin | Merck | Cas: 55266-18-7 |
| 2’·7’-dichlorofluorescin diacetate | Beyotime | S0033M |
| VX765 | Meilunbio | JYY-S81449-Smg; Cas: 273404-37-8 |
| MCC950 | MedchemExpress | Cas: HY-12815A |
| **Critical commercial assays** |        |            |
| Nitric oxide content assays kit | Beyotime | S0023 |
| Total Glutathione Assay Kit | Beyotime | S0052 |
| Hoechst 3342 and Propidium Iodide staining Kit | Beyotime | C1056 |
| Annexin V-FITC and Propidium Iodide staining Kit | Beyotime | C1062M |
| **Deposited data** |        |            |
| Raw and analyzed data | This paper | SRA: PRJNA642722 |
| Experimental models: Cell lines |        |            |
| Neonates rat muscle fibroblast | This paper | Cat#CL-0137 |
| L929 | Procell life Science&technology Co., Ltd | |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
- Further information and requests should be directed to and will be fulfilled by the corresponding author, Zhao-Rong Lun (lsslzr@mail.sysu.edu.cn).

**Materials availability**
- This study did not generate new unique reagents.

**Date and code availability**
- The transcriptome sequence data has been deposited at NCBI and are publicly available as of the date of publication. Accession number is listed in the key resources table. Original western blot images and microscopy data reported in this paper will be shared by the lead contact upon request.
- This paper does not report any original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

This work was conducted in accordance with protocols approved by the Laboratory Animal Use and Care Committee of Sun Yat-Sen University under the license no. 31772445. Sprague Dawley (SD) rats and Swiss Webster mice were purchased from the Laboratory Animal Center of Sun Yat-sen University, Lewis (LEW) rats were purchased from Vital River Laboratories (Beijing, China), while the source of *iNOS*−/− rats (SD background) was described previously (Shen et al., 2017b). All rats were 4–5 weeks old and weighed 90 ± 20 g, while all Swiss Webster mice weighed 25–30 g. All animals were allowed free access to water and food under specific pathogen-free conditions. *T. gondii* RH/GFP and PLK/RED strains were provided by Prof. Xue-Nan Xuan, Obihiro University of Agriculture and Veterinary medicine. *T. gondii* RH-Δrop18 strain was provided by Jian-Du, Anhui Medical University. RH and PLK strains of *T. gondii* were maintained in human foreskin fibroblasts (HFF), with DMEM supplemented with 10% fetal bovine serum (FBS), 100 μg/ml gentamicin, and 10 mM glutamine at 37°C, 5% CO₂, after thawing from the liquid nitrogen. Tgctsd1 was maintained in mice. In general, tachyzoites were harvested from the peritoneal cavity of infected Swiss Webster mice by inoculation of ice-cold PBS or from the cultures. Rat muscle fibroblast cells split from neonates’ rat leg, cultured in plates in an incubator with RPMI-1640 supplemented with 10% FBS, 100 μg/ml gentamicin, and 10 mM glutamine at 37°C, 5% CO₂. Rat PMs were isolated from adult rats peritoneal and incubated in RPMI-1640 supplemented with 10% FBS, 100 μg/ml gentamicin, and 10 mM glutamine.
glutamine at 37°C, 5% CO2. Bone marrow precursor cells were isolated from donor rats and differentiated into macrophages as described (Hoffmann et al., 1989; Pfannes et al., 2001). To generate T. gondii RH-Δgra43 strain, GRA43 locus-specific CRISPR plasmid, pSAG1-Cas9-sgUPRT was replaced by a UPRT targeting guide RNA (gRNA) with a GRA43 gRNA using site-directed mutagenesis as described (Shen et al., 2014, 2017a).

METHOD DETAILS

Animals and parasites
Sprague Dawley (SD) rats and Swiss Webster mice were purchased from the Laboratory Animal Center of Sun Yat-sen University, Lewis (LEW) rats were purchased from Vital River Laboratories (Beijing, China), while the source of iNOS−/− rats (SD background) was described previously (Shen et al., 2017b). All rats were 4–5 weeks old and weighed 90 ± 20 g, while all Swiss Webster mice weighed 25–30 g. All animals were allowed free access to water and food under specific pathogen-free conditions.

In order to clarify whether different strains of Toxoplasma gondii showed infection profile differences in WT and iNOS−/−-SD rats, T. gondii I strain RH/GFP, type II strain PLK/RED or PRU and a Chinese strain Tgctdsd1 (Chinese III) were used (Gao et al., 2017). RH and PLK strains of T. gondii were maintained in human foreskin fibroblasts (HFF), with DMEM supplemented with 10% fetal bovine serum (FBS), 100 μg/ml gentamicin, and 10 mM glutamine at 37°C, 5% CO2, after thawing from the liquid nitrogen. Tgctdsd1 was maintained in mice. In general, tachyzoites were harvested from the peritoneal cavity of infected Swiss Webster mice by inoculation of ice-cold PBS or from the cultures.

Chemicals
Key chemicals used in our experiments are listed as follows: Lipopolysaccharide (LPS) (Sigma, USA), interferon-gamma (IFN-γ) (Sigma, USA), sodium nitroprusside (SNP) (Sigma, USA), Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) (Merck, USA), VX765 (Meilunbio, China), MCC950 (MedChemExpress, USA).

Antibodies
Anti-SOD2 antibody produced in goat (1:1000; Sigma, USA), anti-β-tubulin mouse monoclonal antibody (1:1000; TransGen Biotech, China), rabbit anti-rat caspase-1 antibody (1:1000; Abcam, England), rabbit anti-rat caspase-3 antibody (1:1000; Abcam, England), rabbit anti-rat IL-1β antibody (1:1000; Abcam, England), rabbit anti-rat IL-1β antibody (1:1000; Abcam, England), anti-GAPDH mouse monoclonal antibody (1:1000; TransGen Biotech, China). Secondary antibody: HRP-labeled goat anti-rabbit IgG (H + L) (1:1000; Beyotime, China), goat anti-mouse IgG (H + L) secondary antibody, HRP (Invitrogen, USA), rabbit anti-goat IgG HRP-conjugated antibody (1:1000; Gentex, USA), Toxoplasma gondii SAG1 monoclonal antibody (1:20; Invitrogen, USA), goat anti-mouse IgG (H + L) cross-adsorbed ReadyProbes™ secondary antibody, and alexa fluor 594 (1:1000; Invitrogen, USA).

In vivo parasite infection and bioassay
To determine the survival ratio of SD rats infected with T. gondii tachyzoites, each SD rat was intraperitoneally (i.p.) inoculated with 107 tachyzoites (strains of RH/GFP, PLK/RED or Tgctdsd1) in 500 μL PBS. The sera were collected from the group of SD rats infected with T. gondii as above and the control group of rats were injected only with 500 μL PBS. Sera were collected from infected rats at 3, 6 and 9 dpi. IFN-γ concentration was determined using the rat IFN-γ ELISA kit (ExCell Biotech, China).

Furthermore, to test cyst forming ability, iNOS−/−-SD rats were i.p. infected with 106 tachyzoites of a cyst forming strain of T. gondii Tgctdsd1 as described previously (Gao et al., 2017), while Prugniaud (PRU) strain was used as a reference. T. gondii cyst numbers were counted in brain homogenates from the infected SD rats at 60 dpi.

For bioassay, rats infected with T. gondii RH strain were sacrificed at 3, 6, 10, 20 and 30 dpi, while rats infected with the PLK/RED and Tgctdsd1 strains were killed at 60 dpi. Heart, liver, spleen, lung, kidney and brain (3 and 6 dpi rats were not detected) were collected from each sacrificed rat and 0.5 g tissue from each organ was homogenized in 1 mL PBS (pH = 7.4) and was i.p. inoculated into a swiss mouse. The mice were monitored in a daily manner until they were sacrificed 30 days later.
Histological analysis of brain tissue sections
To monitor the brain cysts in rat, brain tissues were fixed with 4% paraformaldehyde solution for 24h, then dehydrated, embedded in paraffin and sectioned into slices of 4–6 μm. Slices were hydrated in xylene and a descending sequence of ethanol, following hematoxylin-eosin (H&E) stain.

Preparation of primary fibroblasts, peritoneal macrophages and bone marrow derived macrophages
Rat neonates were sterilized with 75% alcohol for 5 min and sacrificed. Muscle tissues from legs were collected, snipped into pieces and digested with 0.05% (w/v) trypsin/0.53 mM EDTA at 37°C for 5 min until termination with 10% FBS in RPMI-1640. Muscle cells were passed through a filter with 70.0-μm pore (Biologix, USA) to discard debris and were cultured in the plates in an incubator with 5% CO₂ at 37°C for 4 hours. Non-adherent cells were washed away and the remaining cells, termed SD rat neonatal muscle fibroblast cells (NMFCs), were continuously cultured for further experiments. Rat NMFCs were also subcultured and stored in liquid nitrogen for later use.

For the macrophage isolation, adult rats were sterilized and sacrificed as above. They were then i.p. inoculated with 15 ml ice cold PBS. Peritoneal fluid was collected, centrifuged and washed with FBS-free RPMI-1640 medium, and cells were transferred to a 24-well (5×10⁵ per well) plate. Non-adherent cells were washed away after 1h incubation and the remaining cells, the rat peritoneal macrophages (PMs) were used for further experiments.

Bone marrow precursor cells were isolated from donor rats and differentiated into macrophages as described (Hoffmann et al., 1989; Pfannes et al., 2001). Briefly, bone marrow cells were washed out with RPMI-1640 from the femurs and tibias isolated from SD rats aged 6 weeks. Macrophages were obtained by cultivation of the precursor cells for 5–7 days in a differentiation medium of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% L-cell-conditioned medium, 10% heat-inactivated FBS, 1% penicillin and streptomycin (Invitrogen, USA) at 37°C and 5% CO₂.

Treatments of rat cells and parasite infection
Rat NMFCs and rat PMs were pre-treated with the designated factor(s) of either 100 ng/ml lipopolysaccharide (LPS) or/and 100 ng/ml interferon-gamma (IFN-γ) for 12 hours, or with 25 μM sodium nitroprusside (SNP) for 12h, or with 50 μM VX765 or 10 μM MCC950 for 2 hours.

All T. gondii tachyzoites were collected and purified with a 5.0-μm pore filter (Millipore, USA). The filtrate was then centrifuged at 1000x g for 10 min at 4°C, resuspended in RPMI-1640 medium (GIBCO, USA) supplied with 10% FBS. For in vitro experiments, tachyzoites were added to the cells in RPMI-1640 medium at a multiplicity of infection (MOI) of 2 and were allowed to invade for 1 hour at 37°C in 5% CO₂. The free tachyzoites were washed away with PBS and the complete RPMI-1640 medium was pretreated with designated factor(s). This was recorded as 0 hpi (hours post infection).

Determination of nitric oxide (NO), reactive oxygen species (ROS) and glutathione (GSH)
To test the NO content, rat PMs were treated with LPS or/and IFN-γ as described above. Media from the preparations with or without T. gondii were collected at 24 hpi and centrifuged. Supernatants were used to determine NO content as previously described (Li et al., 2012).

ROS generated in RPMs was determined with 2’,7’-dichlorofluorescin diacetate (DCFH-DA) (Beyotime, China) using flow cytometry or confocal microscopy as described (Liu et al., 2018). For flow cytometry, rats were i.p. injected with 10⁵ tachyzoites, and RPMs were collected 1h later. The harvested cells were then incubated in FBS and phenol red free RPMIM-1640 with 10 μM DCFH-DA (Beyotime, China) at 37°C for 30 min. Subsequently, cells were washed with ice cold PBS three times and analyzed using a FACSCanto II flow cytometer (BD Biosciences, USA) at 495 nm excitation and 529 nm emission.

For confocal microscope analysis, the naive rat PMs were collected from uninfected rats, grown on the confocal dishes for adherence for 1h. After washing, PMs were infected with T. gondii for 1h and non-invaded parasites were washed away. After another hour of incubation, the DCFH-DA probe was added
as described above. Samples were then washed with ice cold PBS three times and imaged under the LSM880 confocal microscope (Zeiss, Germany).

To test the GSH content, RPMs were obtained from infected rats 3 hours after inoculation as mentioned above, cellular lysis was conducted in ice-cold buffer and the protocol was followed as reported (Fan et al., 2012). The GSH content in the cell lysis was measured following the manufacturer’s instructions for the Total Glutathione Assay Kit (Beyotime, China).

**Sample preparation for transmission electron microscopy**

Rat PMs were isolated from infected and noninfected rats 3 hpi with $10^7$ *T. gondii* PLK/RED strain tachyzoites as mentioned above, and were fixed, dehydrated, and embedded. Ultrathin sections were observed under the JEM1400 electron microscope (JEOL, Japan).

**Western-blotting**

Rat PMs were harvested as described above and were lysed by rapid repeated freezing in liquid nitrogen and thawing in a 37°C water bath (three times). Protein concentrations were determined using the BCA protein assay kit (Beyotime, China). Samples were denatured, resolved on 15% SDS-PAGE gels, transferred onto polyvinylidene fluoride (PVDF) membranes (Pall, USA), blocked with 5% nonfat milk in TBS (Tris-buffered saline) and subsequently probed with designated 1st and 2nd antibodies. Signals were detected using a Tanon™ High-sig ECL Western Blotting Detection Kit (Tanon, China) using Thomas’ methods (Hnasko and Hnasko, 2015).

**Determination of the effect of GSH and MnTBAP on the growth of *T. gondii* in SD and LEW RPMs**

Fresh rat PMs isolated from SD and LEW rats were transferred at a density of $5 \times 10^5$ per well onto the 24-well plates with 1 ml of supplemented RPMI-1640 medium mentioned as above. Triplicate wells were treated with the ROS scavenger Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) (Gauuan et al., 2002) (200 µM, Merck, USA) and antioxidants of GSH (Zeevalk et al., 2007) (2 mM, Beyotime, China), incubated for 6 h, and then were infected with tachyzoites of *T. gondii* RH/GFP strain at a MOI of 2, followed by incubation at 37°C with 5% CO₂ and humidity. After incubation at 12, 24 and 48h, the cultures were analyzed by fluorescence microscopy using the fluorescein isothiocyanate (FITC) channel to measure the parasite GFP fluorescence. After 24h time points of analysis, the medium was completely aspirated from each well and replaced with an equivalent volume (1 mL) of fresh medium containing fresh GSH or MnTBAP at the respective final concentration. To determine the *T. gondii* infection ratio in the *iNOS<sup>−/−</sup>*-SD, WT-SD and LEW rat PMs treated with or without GSH and MnTBAP, microscopic images of fields of view were captured for infection analysis. Fluorescent quantification was done using ImageJ software (version 1.37).

**Cell viability assay**

To assess cell viability after infection with *T. gondii* in the presence of the ROS burst, Hoechst 33342 and Propidium Iodide (PI) staining assays were performed (Chen et al., 2015). PMs were freshly isolated from *iNOS<sup>−/−</sup>*-SD, WT-SD and LEW rat strains. Cells were transferred into 24-well plates as described above with RPMI-1640 medium complemented with FBS (10%). Tachyzoites of the *T. gondii* RH/GFP strain were infected at a MOI of 2. PMs with or without *T. gondii* infection were incubated for 4 h at 37°C with 5% CO₂ and humidity. After 4h, the cells were washed twice with PBS and 1 mL of RPMI-1640 with 5 µL propidium iodide (PI) and 5 µL Hoechst (Beyotime, China) was added. Cells were incubated at 4°C for 20 min, then washed twice with PBS and pooled in 1 mL of PBS. Images were taken using a ZEISS microscope and the stained cells were counted. Confocal imaging was performed using a ZEISS 880 Confocal laser microscope. WT-SD rat PMs as the positive control were incubated with apoptosis inducers (Beyotime, China) at a dilution of 1:1000 for 4 h.

For cell death verification, fresh PMs isolated from *iNOS<sup>−/−</sup>*-SD, WT-SD and LEW rat were transferred to flow tubes (1 $\times 10^6$ per tube) with 2 mL of complete RPMI-1640 medium at 37°C with 5% CO₂ and humidity. Rat PMs with or without *T. gondii* infection at a MOI of 1 were incubated for 4h at 37°C with 5% CO₂ and humidity. Positive control WT-SD rat PMs were incubated as described above. PMs were then collected by centrifugation and assessed with Annexin V-FITC and PI staining (Beyotime, China) for 10 min as described.
(Wang et al., 2012). Data acquisition was performed by flow cytometry on a MoFlo-XDP (Beckman Coulter, USA) and analysis was conducted with the FlowJo software (v10.3).

GRA43 knock-out and cell infection

To generate the GRA43 locus-specific CRISPR plasmid, pSAG1-Cas9-sgUPRT was replaced by a UPRT targeting guide RNA (gRNA) with a GRA43 gRNA using site-directed mutagenesis as described (Shen et al., 2014, 2017a). To construct a homologous template for the GRA43 knockout, the 5’ and 3’ homology arms of GRA43 gene were amplified by PCR from the RH strain genome and the selection marker DHFR was amplified from pUPRT::DHFR-D. The 5’ and 3’ homology arms, as well as DHFR, were cloned into pHC19 using the ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China) (Figure S8). All primers used to construct plasmids are listed in Table S2. All plasmids were confirmed by DNA sequencing. To generate the GRA43 knockout RH strain (RH-Dgra43), an homology template was amplified from plasmid pGRA43::DHFR and co-transfected with pSAG1-Cas9-sgGRA43 into 10^7 RH tachyzoites which were suspended in 100 μL Cytomix using an Amaxa electroporation system and the X-001 program. Transfectants were selected with 2 μM pyrimethamine (Sigma Aldrich, USA), cloned by limiting dilution in 96-well plates and verified by diagnostic PCRs (primers listed in Table S2) (Figure S8).

Immunofluorescence assays (IFA)

To assess the ROP18 or GRA43 knock-out T. gondii infection ratio, the infected RPMs were subjected to immunofluorescence assays as reported (Du et al., 2014). Briefly, PMs cultured on the coverslips in 24-well plates were infected with RH, RH-Drop18 and RH-Dgra43 tachyzoites at a MOI of 2 respectively. At 24 or 48 hours after infection, cells were washed with PBS three times and fixed in 4% paraformaldehyde (PFA) for 15 min, then were quenched and permeabilized with 0.2% Triton X-100 and 0.2 M glycine in PBS for 20 min, and then blocking in 3% bovine serum albumin (BSA) in PBS for 30 min. A subsequent incubation with mouse monoclonal anti-SAG1 primary antibody (Invitrogen) was applied for 3 h at 37°C, followed by rabbit anti-mouse Alexa Fluor 594 (Thermo Fisher) for 1 h at 37°C. After washed with PBS three times, cells were stained with 4,6-Diamidino-2-phenylindole (DAPI) and examined under a microscope (Leica). Images were recorded for further use.

Determination of ROS in RPMs infected with the T. gondii RH-Dgra43

Rat PMs were collected and incubated in a 24-well plate as mentioned before. Cells were infected with T. gondii RH or RH-Dgra43 and non-infected cells were used as control. The DCFH-DA probe was added to the cells at 1 hpi and 3 hpi respectively, then they were incubated for 30 min and washed as mentioned above (Liu et al., 2018). Results were observed and recorded under a Zeiss microscope and were analyzed by ImageJ (Version 1.52a).

RNA extraction, library preparation and RNA sequencing

Rats were i.p. injected with 10^7 tachyzoites or equal volume of 1×PBS. After 3 h inoculated, 1×10^7 peritoneal cells were used for total RNA extraction for each rat. Total RNA of each sample from individual animals was extracted using TRIzol Reagent (Invitrogen). RNA samples were quantified and qualified by 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop (Thermo Fisher Scientific Inc.) and 1% agarose gel. 1 μg total RNA with RIN value above 7 was used for following library preparation. RNA library preparation and sequencing were performed at the GENEWIZ Inc High Throughput Sequencing according to the manufacturer’s protocol (NEBNext® UltraTM RNA Library Prep Kit for Illumina®). Libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to manufacturer’s instructions (Illumina, San Diego, CA, USA). Sequencing was carried out using a 2x150bp paired-end (PE) configuration; image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. The sequences were processed and analyzed by GENEWIZ Inc. All raw data were up-loaded to NCBI and the bioproject number is PRJNA642722.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed and illustrated using GraphPad Prism software (version 8.0). The data are given as the means ± standard deviation or means ± standard error of mean and are representative from at least three independent experiments. Statistical significance was accepted at p < 0.05, using the Student’s unpaired t test or and performed two-tailed χ2-test (Fisher’s exact test).