# Infection With *Trypanosoma Lewisi* or *Trypanosoma Musculi* May Promote The Spread of *Toxoplasma Gondii*

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**Research**

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# Abstract

**Background:** *Toxoplasma gondii* can infect almost all warm-blooded vertebrates with pathogensis being largely influenced by the host immune status. As important epidemiological hosts, rodents are globally distributed and are also commonly found infected with haemoflagellates, such as those in the genus *Trypanosoma*. We here address whether and how co-infection with trypanosomes can influence *T. gondii* infection in laboratory models.

**Results:** Rats of five strains, co-infected with *T. lewisi*, and mice of four strains, co-infected with *T. musculi*, were found to be more or less susceptible to *T. gondii* infection, respectively, with corresponding increased or decreased brain cyst burdens. Down-regulation of iNOS expression and decreased NO production or reverse were observed in the peritoneal macrophages of rats or mice, infected with trypanosomes, respectively.

**Conclusions:** *Trypanosoma lewisi* and *T. musculi* can modulate host immune responses, either by enhancement or suppression, and influence the outcome of *Toxoplasma* infection.


# Background

It isn't uncommon that a host is infected with two or more species of pathogens, including viruses, bacteria and parasites. A globally distributed parasite, *Toxoplasma gondii*, is one of the most successful parasites and can infect and propagate in almost all nucleated cells found within warm-blooded animals including humans [1, 2]. As a typical opportunistic pathogen, *T. gondii* usually establish long-term latent infections, which are largely asymptomatic in immune competent individuals. However, in fetus, newborns and immunocompromised patients, *T. gondii* infection will develop into potentially life-threatening toxoplasmosis [3], which reflects the nature of toxoplasmosis that its outcome is largely influenced by host immune status.

Extensive studies have been carried out, using animal models to discover the progression of *Toxoplasma* infection and factors influencing the host-parasite relationship. A key driver that controls *T. gondii* infection in laboratory rodents is the balance of expression of the enzymes iNOS and Arginase-1 [4–7]. High iNOS expression and low arginase expression is associated with resistance to *T. gondii* infection while the reverse expression levels are associated with susceptibility. However, there are significant differences between animal models in the laboratory and natural infections found in wild animals [8] including differences in environment and in immune status. Parasitism in wild animals is comment and rodents in the wild are highly likely to be infected with multiple pathogens. Thus, understanding the immune responses in relation to co-infection of *T. gondii* with other pathogens, has great potential to be both interesting and relevant in nature [9, 10].

It is widely documented that members of the genus *Trypanosoma* can induce severe depression of the host immune system [9, 11]. For example, *Trypanosoma brucei* induced suppression of responses to *Trichinella spiralis* in vaccinated NIH mice [9]. Chagas' disease at acute stage by *Trypanosoma cruzi*

infection is linked to immunosuppression in both patients and laboratory animals which facilitates the multiplication and dissemination of parasites in the mammalian host [9]. These raise questions as to whether and how other *Trypanosoma* species influences host immunity.

In the case of pathogens of rodents, *Trypanosoma musculi* and *Trypanosoma lewisi* are two globally distributed parasites of mice and rats, respectively. *Trypanosoma lewisi* can have prevalences of up to 56% rats [12–14] and has also been considered as pathogenic to humans [15–18]. Many previous studies have suggested that *T. lewisi* infection could induce an immunosuppressive effect on its hosts, resulting in a greater susceptibility to infection by other pathogens, including *Salmonella typhimurium, Cryptococcus neoformans* and *Toxoplasma gondii* [10, 19–21]. *Trypanosoma musculi* has similar biological characteristics to *T. lewisi* but restricted to mice and can also induce an effect on experimental infection of mice with *Toxoplasma* [22, 23].

Herein, our study aims to investigate the effect of trypanosome (*T. lewisi* and *T. musculi*) infection in rats and mice on infection with *T. gondii*, and to uncover the role of iNOS and NO in the trypanosome-induced immunoregulation.

# Materials And Methods

### Animals

Brown Norway (BN), Fischer 344 (F344) and Lewis (LEW) rats were purchased from the Vital River Experimental Animal Centre, Beijing, China. Sprague-Dawley (SD) and Wistar (WST) rats and four strains of mice (Swiss Webster, BALB/c, NIH and C57BL/6) were purchased from the Experimental Animal Centre, Guangdong Province, China. All rats used in this study weighed 180-220 g and mice weighed 20-25 g. Animals were kept in a specific pathogen free environment with free access to food and water. The protocols were approved by the Institutional Review Board of Animal Care at Sun Yat-Sen University under license of 3167227.

### Parasites

Tachyzoites of *T. gondii* RH-GFP strain [24] and cysts of *T. gondii* Prugniaud strain were obtained as below. Swiss Webster mice were intraperitoneally inoculated with $1\times10^5$ tachyzoites of RH-GFP. Peritoneal cavities of mice was harvested by injection with ice-cold D-Hanks 3 days post infection (dpi).Viable tachyzoites were harvested with differential centrifugation (50 × g 8 min at 4°C and 1500 × g for 10 min at 4°C) and resuspended in RPMI 1640 medium with 10% FBS. Swiss Webster mice were orally infected with *T. gondii* Prugniaud strain and tissue cysts were obtained from brains as previously described [25, 26]. Briefly, mice were anaesthetized by $CO_2$ at 60 dpi and the brain was removed and homogenized in 1 ml PBS (pH 7.2). Cysts within 10 µl samples were carefully quantified by microscopy using a cover slip (22 × 22 mm) at 100x magnification. Cyst in infected rats were determined similarity after homogenized in 2 ml PBS.

Two kinds of haemoflagellates, including *T. lewisi* (strain CPO02, isolated from wild rats, *Rattus norvegicus,* in Guangzhou, China) and *T. musculi* (provided by Dr Philippe Vincendeau from the Université de Bordeaux II), were used in this study [27, 28]. SD rats were intraperitoneally inoculated with $1\times10^6$ trypomastigotes of *T. lewisi*, while Swiss Webster mice were intraperitoneally inoculated with $1\times10^5$ trypomastigotes of *T. musculi*. Parasitemia in infected animals was monitored daily. Usually at 7-9 dpi, animals were killed by $CO_2$ treatment and the blood was drawn from the heart and diluted to designed concentration with PBS.

## Methodology of animal experiments

*In vivo* experiments were conducted with four groups of rats (6 each) and five groups of mice (10-13 each). In rats, "*Tl*" group was inoculated with $1\times10^6$ *T. lewisi* trypomastigotes, while "*Tg*" group was inoculated with 200 cysts of the *T. gondii* Pru strain. "*Tl,Tg*" and "*Tg,Tl*" groups were firstly inoculated with former and then 4-6 days later inoculated with latter, respectively. In mice, "*Tm*" group was inoculated with $1\times10^5$ *T. musculi* trypomastigotes, "*Tg*" group was inoculated with 5 cysts of the *T. gondii* Pru strain. "*Tm,Tg*" and "*Tg,Tm*" groups were separately inoculated with indicated parasites with interval of 5 days, while "*Tg+Tm*" group was inoculated both parasites on the same day. Infected animals were monitored for 50 (rats) or 30 (mice) days until sacrificed.

*In vitro* experiments were carried out with pre-infected five SD rats ($1\times10^6$ *T. lewisi* trypomastigotes) and six Swiss Webster mice ($1\times10^5$ *T. musculi*), Rat and mouse peritoneal macrophages were isolated at 6 dpi and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) at 37 °C with 5% $CO_2$. Another five SD rats and six Swiss Webster mice were intraperitoneally inoculated with a saline solution as a control.

## Peritoneal macrophage isolation and cultivation

The method for isolation of macrophages, described by El-Mahmoudy [29], was used in this study. Animals, sacrificed by $CO_2$ treatment, were injected intraperitoneally with 5 ml (mouse) or 15 ml (rat) ice cold D-Hank's solution and then peritoneal cells were harvested and separated by centrifugation at 250 × g for 10 min at 4 °C. The cells were then washed by D-Hank's solution, and suspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and penicillin (100 U/ml), and then left to adhere for 2 hrs at 37 °C in an incubator with 5% $CO_2$. Non-adherent cells were removed after 4 hours..

## Determination of *T. gondii* intracellular multiplication

Rat or mouse macrophages were challenged with *T. gondii* RH strain tachyzoites at a multiplicity of infection (moi) 1:1. Extracellular *T. gondii* were then washed out after 1 hr incubation together, at which the time point was defined as 1 hr for the start of the experiment. Thereafter, the cells were observed with an inverted fluorescence microscope at the desired time. The numbers of *T. gondii* were counted in 100 host macrophages and an average determined.

## Measurement of iNOS and arginase activity

Nitrite content as a reflection of NO production was determined by the Griess reaction as described [30]. Briefly, 100 µl supernatant or standard solution ($NaNO_2$) was incubated in triplicate with 100µl of Griess reagent (0.5% sulfanilamide, 0.05% naphthyldiaminedihydrochloride in 5% $H_3PO_4$) for 10 min. The plates were read at 550 nm in an ELISA reader (Multiskan MK3, Thermo Labsystems, Finland).

Arginase activity of purified macrophages was measured by a colorimetric method as described [31]. Briefly, 10 mM $MnCl_2$ and 0.5 M L-arginine were successively added to macrophage lysates for 1 hr at 37 °C. The reaction was stopped by addition of an acid solution ($H_2SO_4$: $H_3PO_4$: $H_2O$ = 1:3:7), and the urea generated by arginase was analyzed by addition of a-isonitrosopropiophenone at 100 °C for 45 min. The colored product was quantified by absorption at 550 nm in an ELISA reader. Arginase activity was determined as the amount of urea produced from total protein of peritoneal macrophages.

## Measurement of gene expression by mRNA analysis

Total RNA from treated and non-treated macrophages was extracted using Trizol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Total RNA was converted to cDNA using a set of oligo (dT) primer and SuperScript TM III First-Strand Synthesis System (Invitrogen, Carlsbad, USA). cDNA (1µg) was used as a template for amplifying iNOS, arginase-1 and GAPDH (internal standard) genes by PCR using the following primers: arginase-1, 5'-AAG AAA AGG CCG ATT CAC CT-3' and 5' -CAC CTC CTC TGC TGT CTT CC-3', 201 bp; rat-iNOS, 5'-CTA CCT ACC TGG GGA ACA CCT GGG-3' and 5' -GGA GGA GCT GAT GGA GTA GTA GCG G-3', 442 bp; mouse-iNOS, 5'-GCC TCG CTC TGG AAA GA-3' and 5' -TCC ATG CAG ACA ACC TT-3', 499 bp; GADPH, 5' -AAT GCK TCC TGY ACC ACC AAC TGC-3' and 5' -TTA GCC AWA TTC RTT GTC RTA CCA GG-3', 513 bp. For semiquantitative PCR, the cycling protocol was: 94 °C for 1 min, 60 °C for 1.5 min, and 72 °C for 1.8 min. For arginase-1, rat-iNOS, mouse-iNOS, 27 cycles were used, but for GAPDH, only 20 cycles were used. Amplified DNA products were separated on 1% agarose gel and photographed using an electronic documentation system (Biostep, Germany) after staining with ethidium bromide.

## Measurement of gene expression using western Blotting

Cells were lysed in SDS loading buffer, fractionated in SDS-PAGE and transferred onto immunoblot polyvinylidene difluoride membrane (Pall, USA). The membrane was probed using rabbit polyclonal iNOS antibody (Thermo, USA) and rabbit polyclonal arginase-1 antibody (Santa Cruz, USA). β-tubulin was stained with antibody (NOVUS, USA) as a control. Horseradish peroxidase-labeled secondary antibodies (Cell Signaling, USA) and DAB (3,3',5,5'-tetramethylbenzidine) Detection Kit (Tiangen, China) were used for antibody detection. Relative expression of iNOS and Arginase was calculated by normalizing with the β-tubulin control.

## Statistical analysis

Cyst counts from each group are given as a mean ± standard error of mean (SEM), and presented using GraphPad Prism version 5. Other results are expressed as mean ± standard deviation (SD). Odds ratios were calculated with 0.5 added to counts to avoid dividing with zero [32] and preformed two-tailed $\chi^2$-test with Yates's correction. The student´s *t*-test was used to compare differences between two unpaired samples and statistical differences were designated by */# ($p \leq 0.05$), **/## ($0.001 < p \leq 0.01$) and ***/### ($p \leq 0.001$).

# Results

### Survival in animals co-infected with *T. gondii* and *T. musculi/ T. lewisi* is associated with splenomegaly

As shown in supplementary table 1, rats are high resistant to *T. gondii* infection, as all showed zero mortality. However, infection of *T. lewisi* may kill rats of SD, BN, LEW and WST strains, except F344. In generally, additional *T. gondii* infection may not change the mortality caused by *T. lewisi*, only *Tl,Tg* group of BN rats showed significant high mortality. Considering the parasitemia, *T. lewisi* could survive in the blood for more than 40 days in four strains of rats (BN, F344, SD, WST) (Supplementary figure 1), the LEW strain rats were so resistant to *T. lewisi* that no parasitemia was observed. In WST groups, all rats developed similar levels of parasitemia. In F344 and SD groups, lower parasitemia peaks of *T. lewisi* were found in those that were co-infected with *T. gondii* than in singly infection groups (*Tl* vs *Tl,Tg/Tg,Tl*: F344, $p = 0.0079/0.0005$; SD, $p < 0.0001/0.0001$), while BN missed many data due to animal deaths. Spleen indexes [spleen weight (mg)/body weight (g)] in rats on 60 dpi (Table 1) showes *T. lewisi* infection resulted significant splenomegaly, which *T. gondii* resulted splenomegaly F344 rats but reverse effect in other rats. In case of co-infection, all rats had higher spleen indexes than the *T. gondii* groups.

As shown in Table 2 and supplementary figure 2, *T. musculi* is nonlethal to mice with 100% survival. However, mice are highly susceptible to *T. gondii* infection (even with the avirulent Pru strain) and showed various levels of mortality, as C57BL/6, the most susceptible mice, survived only 15% (2/13) and BALB/c, the most resistant mice, all alive. In co-infected groups, there was generally a decreased survivability compared to both singly infected controls, but only significant for the "*Tm,Tg*" group of BALB/C mice ($p = 0.025$).

During the infection of *T. musculi* (30 days) only the parasitemia found in "*Tm,Tg*" group of BALB/C mice was significantly higher than the "*Tm*" group at their peaks between days 9 or 12 ($p = 0.0418$), while other groups were not (Supplementary figure 2). Importantly, most C57BL/6 mice died before showing high parasitemia, indicating the death may due to immune-storm of co-infection.

Spleen indexes in surviving mice on 60 dpi (Table 3) showes either *T. gondii* or *T. musculi* infection resulted significant splenomegaly, whereas co-infection generally enhanced the splenomegaly, compared with the *T. gondii* groups. Data for C57BL/6 mice were limited due to few was survived.

### *Toxoplasma gondii* cyst burden in the brains of co-infected animals

We investigated the variance in susceptibility to *T. gondii* in mice and rats when co-infected with *T. musculi* (mice) and *T. lewisi* (rats). A surprising result was that mice of Swiss Webster and NIH improved their resistance, to a greater or lesser degree, to the *T. gondii* PRU strain when co-infected with *T. musculi* (Figure 1). Heavy cyst burdens were detected in the brains of mice infected with *T. gondii* only (Mean±SEM of group "*Tg*": BALB/c, 355±71.81, n=13; NIH, 3629±626.9, n=10; Swiss Webster, 23198±3472, n=3), while lower numbers of brain cysts were detected in the brains of *T. musculi* pre-infected groups (Mean±SEM of group "*Tm, Tg*": BALB/c, 206±96.36 , n=6; NIH, 1353±508.4, n=8; Swiss Webster, 124.3±15.67, n=3) with the difference being significant in the NIH ($p = 0.015$) and Swiss Webster strains ($p < 0.001$) (Figure 1). However, when *T. musculi* was coinfected with *T. gondii* at the same time (*Tm*+*Tg*) or subsequently (*Tg*, *Tm*) there was little influence on cyst burden, in the mice strains except in the case of Swiss Webster where a significant drop in cyst number occurred when *T. gondii* infected mice were post-infected with *T. musculi.* Cyst burden in C57BL/6 mice could not be determined due to high lethality (more than 85%) after infection with *T. gondii*.

Interestingly, in contrast to the mice, we found (Figure 2) that rat strains F344 and BN pre-infected with *T. lewisi* showed a greater susceptibility to *T. gondii* than the *T. gondii* singly infected rats (Mean±SEM of group "*Tl, Tg*": F344, 2103±234.6, n=5; BN, 936.0±259.5, n=5; SD, 90.0±60.0, n=5; vs group "*Tg*": F344, 216.0±47.6, n=5; BN, 0±0, n=5; SD, 0±0, n=5). However, there was no significant difference between the other coinfected groups (*Tg*, *Tl* i.e. post-infection with *T. lewisi*) and the *T. gondii* infected groups (*Tg*). In the case of the remaining rat strains, WST and LEW, these are very resistant to *T. gondii* infection and no *Toxoplasma* cysts were found at all (by microscopic examination and *T. gondii*-PCR) even when coinfected with *T. lewisi* (data not shown).

## Variation in NO/urea production and iNOS/arginase expression in the peritoneal macrophages of mice or rats is related to the proliferation status of *T. gondii*

It has been previously demonstrated that there is competition for the substrate arginine between the enzymes iNOS and arginase [5]. Therefore, we analyzed the level of NO/urea production and iNOS/arginase expression in peritoneal macrophages isolated from Sprague- Dawley rats (SD) and Swiss Webster mice which were pre-infected with *T. lewisi* and *T. musculi*, respectively. Increased amounts of NO production was found in primary cultured peritoneal macrophages taken from mice pre-infected with *T. musculi* (25.82±1.88 μM from *T. musculi* pre-infected mice after culture for 24 hrs compared with 4.44±2.80 μM from control mice at 24 hrs, $p = 0.0004$) (Figure 3A). In addition, a higher level of iNOS mRNA expression (reference gene, GADPH) was observed in peritoneal macrophages from mice pre-infected with *T. musculi* whereas iNOS mRNA expression could not be detected in control mouse peritoneal macrophages (Figure 3C). Western blot analysis (reference protein, β-actin) demonstrated a slightly higher expression of iNOS protein in *T. musculi* pre-infected mouse than normal mouse peritoneal macrophages. Measurement of arginase mRNA expression and urea production, in primary cultured peritoneal macrophages from mice, showed higher levels in the control group (0.34±0.04 mM from control mice after 24 hrs culture and 0.23±0.05 mM from *T. musculi* pre-infected mice at 24 hrs, $p = 0.043$) (Figure 3B). However, there is no distinct difference in arginase expression between control and *T.*

*musculi* pre-infected mice by western blot analysis (Figure 3D). This suggest that the drop in urea production in the pretreated mice was probably due to competition, by increased iNOS expression. for the arginase/iNOS substrate arginine.

Unlike the effect of *T. musculi* on mouse, NO production was significantly lower in *T. lewisi* pre-infected rats (15.26±2.03 μM) than control rats (35.34±3.34 μM, $p < 0.0001$), and after only 12 hrs of culture this difference already existed ($p < 0.0001$) (Figure 4A). In addition, it can be seen that the expression of iNOS mRNA (reference gene, GADPH) and protein (reference protein, β-actin) were clearly decreased in SD rats after *T. lewisi* infection (Figure 4C and 4D). However, the urea production and mRNA/protein expression of arginase were significantly higher in *T. lewisi* pre-infected rats (Figure 4B, 4C and 4D).

It is known that NO is an important effector molecule involved in the resistance of rat/mouse peritoneal macrophages to *T. gondii* infection. In addition, it has been confirmed that a lower level of NO production was detected in the peritoneal macrophages of *T. lewisi* infected SD rats. Therefore, we expected that there would be a proliferation of *T. gondii* in macrophages from rats pre-infected with *T. lewisi*. Indeed, the proliferation of *T. gondii* tachyzoites (RH strain) dramatically increased in peritoneal macrophages from *T. lewisi* pre-infected SD rats (Figure 5) and this was associated with the observed lowered NO production. The reproductive rate of *T. gondii* tachyzoites is clearly inhibited in the peritoneal macrophages from the *T. lewisi* uninfected control SD rats.

In contrast in mice, it seems that the increase in NO production after infection with *T. musculi* confers no obvious resistance to proliferation of *T. gondii* tachyzoites in the peritoneal macrophages of mice (data not shown). However, in vivo, the infection of mice with *T. musculi* was observed to extend the survival time of hosts infected with *T. gondii* tachyzoites (RH strain). This was especially notable when the host was infected *T. musculi* before *T. gondii* (Supplementary figure 3).

# Discussion

*Toxoplasma gondii*, as a successful parasite, can infect almost all warm-blooded vertebrates and therefore has the potential to interact with a wide range of other pathogens that infect its potential hosts. Rodents potentially play a key role in *T. gondii* transmission and this raises the broad question as to whether co-infection with typical rodent trypanosomes, such as *T. lewisi* and *T. musculi*, has any effect on the transmission of *T. gondii*?

Previous research has shown that *Trypanosoma lewisi* and *T. musculi*, like other species of trypanosome, can suppress the immunocompetence of hosts and increase susceptibility to opportunistic infections such as *Toxoplasma gondii* [19]. It has also been demonstrated that *T. lewisi* and *T. musculi* can reduce immunity by influencing the function of macrophages [19]. However, the mechanism of these processes of immunosuppression is still unclear. It is well known that peritoneal macrophages of the rat are naturally resistant to *T. gondii* infection [33] and recent studies have demonstrated that the mechanism of such resistance is strongly linked to higher expression of inducible nitric oxide synthase (iNOS) and lower expression of arginase in the rat peritoneal macrophage [5]. In this study, we propose that *T. lewisi* and *T.*

*musculi* may modulate NO production and iNOS expression in peritoneal macrophages to affect the immunity status of their hosts and that this, in turn, influences infection with *T. gondii*.

Consistent with previous studies, this study demonstrated that *T. lewisi* could increase the susceptibility of immunocompetent rats to *T. gondii*. More importantly, for the first time, it has demonstrated that the host immunosuppression, caused by *T. lewisi*, acts through inhibiting the expression of iNOS. This consequently lowers the production of NO and, at the same time, increases the expression of arginase and, consequently, urea production. This increase is clearly related to the progression of infection of *T. lewisi* as it is not found in the delayed co-infection group. The formation of cysts in the brains in different strains of rats, following infection with the *T. gondii* Prugniaud (cyst-forming) strain, showed rat strain-specific variation. We found that the cyst burden in the brains of F344 rats was significantly worse than in the BN or SD rats in the "*Tl*, *Tg*" groups. A previous study has shown that the encystment of *Toxoplasma* in brains of rats that were pre-infected with *T. lewisi* is dependent on rat strain but not the inoculation dose of *Toxoplasma* or the age of the rats [34]. Similarly, our previous study [4] demonstrated that different strains of adult and newborn rats (LEW, WST, SD, BN and F344) exhibited remarkable differences in susceptibility to cyst formation with the *T. gondii* Prugniaud strain and that this was related to the inherent ratio of iNOS/Arginase expression in peritoneal macrophages. For example, that study showed that F344 rats had a higher susceptibility than BN or SD rats in both adult and newborn stages. However, even newborn rats of the LEW or WST strains exhibited a high degree of resistance to cyst formation with either a lower number or absence of cysts in the brain. These results are consistent with the results confirmed by Li and colleagues that showed that lower iNOS expression and NO production were found in F344 and BN rats while LEW and WST rats express higher levels of iNOS with increased NO production [5]. Since these inbred lines of rats represent a tiny sample of the phenotypic variability in global rat populations, it is likely, in nature, that there will be a high degree of individual variability in the iNOS/Arginase expression balance found in wild populations of rats. Based on our data, it is likely that, in nature, pre-infection with *T. lewisi* is detrimental to survivability following infection with *T. gondii*. We would predict that, in areas of high prevalence of *T. lewisi* infections, natural selection would drive rat populations towards higher frequencies of rats with inherent high resistance to *T. gondii* and inherently high iNOS/Arginase expression ratios (as observed in strains like LEW and WST).

In this study, we found that infection with *T. musculi* could improve the immunocompetence of mice infected with the *Toxoplasma* cyst forming strain (Prugniaud). In the "*Tm*, *Tg*" group, where pre-infection with *T. musculi* was performed, there was also a considerable improvement in mouse survivability following infection with *T. gondii* tachyzoites (RH strain). This improvement was related to the increase in NO production and iNOS expression in mouse peritoneal macrophages found during *T. musculi* infection. In nature, therefore, this mechanism will drive natural selection to increase the frequency of *T. musculi* and *T. gondii* co-infected mice due to improved survivability. We would predicit that this will increase the opportunities for the spread of *T. gondii* to other kinds of hosts in areas where co-infection is high. However, this prediction seems contrary to the previous study by Piccolo-Johanning et al [23], who proposed that *T. musculi* could inhibit the immunocompetence of Swiss Webster mice by producing increased cyst burdens and a shortened lifespan. These differences may be attributed to the use of

different *T. gondii* strains - their use of TFC, TLW and TBT stains may behave differently to the PRU strain we used. Clearly further studies are required to clarify the interactions between different parasite strains with different host strains.

Rats, infected with *T. gondii*, play a vital role in epidemiology of toxoplasmosis because they can serve as reservoirs of infection for cats, dogs, and possibly for pigs [33]. Therefore, we speculate that the immunosuppressive effect of *T. lewisi* on rats could, though natural selection, help to drive the distribution of co-infected animals, in nature, and that could have a strong influence on the transmission and maintenance of the life cycle of *T. gondii.* In addition, *T. lewisi* has been suggested as a potential human pathogen, as it shows resistance to lysis by normal human serum and apolipoprotein L-1, and due to a number of reported cases of human infection [14, 17, 18]. In the rare cases of human infection, it is possible that *T. lewisi* infection may hasten the onset of toxoplasmosis and lead to more severe symptoms.

In conclusion, we propose that the coinfection of two or more pathogens may be beneficial to the spread and/or maintenance of some parasites, but on the other hand may also be harmful to the health of the hosts.

# Abbreviations

BN: Brown Norway; F344: Fischer 344; LEW: Lewis; SD: Sprague-Dawley; WST: Wistar; iNOS: Inducible Nitric Oxide Synthase.

# Declarations


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### Authors' contributions

JM Gao, SQ Yi, QQ Geng and ZR Lun designed the study. JM Gao, ZS Xu and DH Lai drafted the manuscript and undertook data extraction and screening. G Hide, ZR Lun and DH Lai critically reviewed the paper. All authors contributed to the acquisition, analysis, or interpretation of data for the work. All authors approved the final version and agree to be accountable for all aspects of the work.


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### Availability of data and materials

Data supporting the conclusions of this article are included within the article.

### Ethics approval and consent to participate

The animal ethical approval of sample collection was obtained from the Institutional Review Board of Animal Care at Sun Yat-Sen University (permit no. 3167227).

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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# Tables

Table 1
Summary of the spleen index of rats infected with *T. gondii* and/or *T. lewisi.*

| Rat strains | Spleen index | | | | |
|---|---|---|---|---|---|
| | ***Tg, Tl*** | ***Tl,Tg*** | ***Tg*** | ***Tl*** | **Control** |
| BN | 6.98 ± 0.26*** | 5.22 ± 0.13*** | 1.67 ± 0.04### | 5.85 ± 0.41## | 2.39 ± 0.16 |
| F344 | 4.59 ± 0.09*** | 5.63 ± 0.22*** | 3.1 ± 0.13# | 4.95 ± 0.25### | 2.58 ± 0.03 |
| SD | 2.96 ± 0.17*** | 4.30 ± 0.05*** | 1.92 ± 0.05## | 4.76 ± 0.43## | 2.32 ± 0.09 |
| WST | 6.26 ± 0.31*** | 5.19 ± 0.61*** | 2.28 ± 0.07## | 5.06 ± 0.10### | 2.83 ± 0.14 |

Spleen index = spleen weight (mg)/body weight (g). n = 6. */***, vs *Tg* group, $p < 0.05$ or $p < 0.001$, respectively; #/##/###, vs control group, $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively.

Table 2
Summary of survivability of mice infected with *T. gondii* and/or *T. musculi*.

| **Mouse strain** | **% Survival (No. survivors/No. infected)**<br>**Crude Odds Ratio (95% CI)** | | | | | |
|---|---|---|---|---|---|---|
| | ***Tm*** | ***Tm, Tg*** | ***Tm+ Tg*** | ***Tg, Tm*** | **Sum of co-infections** | ***Tg*** |
| BALB/c | 100.0% (13/13)<br>- | 46% (6/13)<br>0.032(0.002–0.652)[a] | 69% (9/13)<br>0.078(0.004–1.63)[b] | 100% (13/13)<br>1.00(0.018–54.2) | 72% (28/39)<br>0.134(0.007–2.422)[c] | 100% (13/13)<br>ref |
| C57BL/6 | 100.0% (6/6)<br>- | 8% (1/13)<br>0.552(0.063–4.87) | 0%(0/13)<br>0.170(0.007–3.92) | 0%(0/13)<br>0.170(0.007–3.92) | 3% (1/39)<br>0.179(0.021–1.506)[d] | 15% (2/13)<br>ref |
| NIH | 100.0% (6/6)<br>- | 62% (8/13)<br>0.515(0.102–2.59) | 83% (10/12)<br>1.4(0.224–8.77) | 58% (7/12)<br>0.455(0.089–2.33) | 68% (25/37)<br>0.680(0.170–2.719) | 77% (10/13)<br>ref |
| Swiss Webster | 100.0% (12/12)<br>- | 58% (7/12)<br>0.722(0.148–3.53) | 60% (6/10)<br>0.765(0.146–4.02) | 50% (6/12)<br>0.529(0.109–2.56) | 56% (19/34)<br>0.666(0.177-2.50) | 67% (8/12)<br>ref |

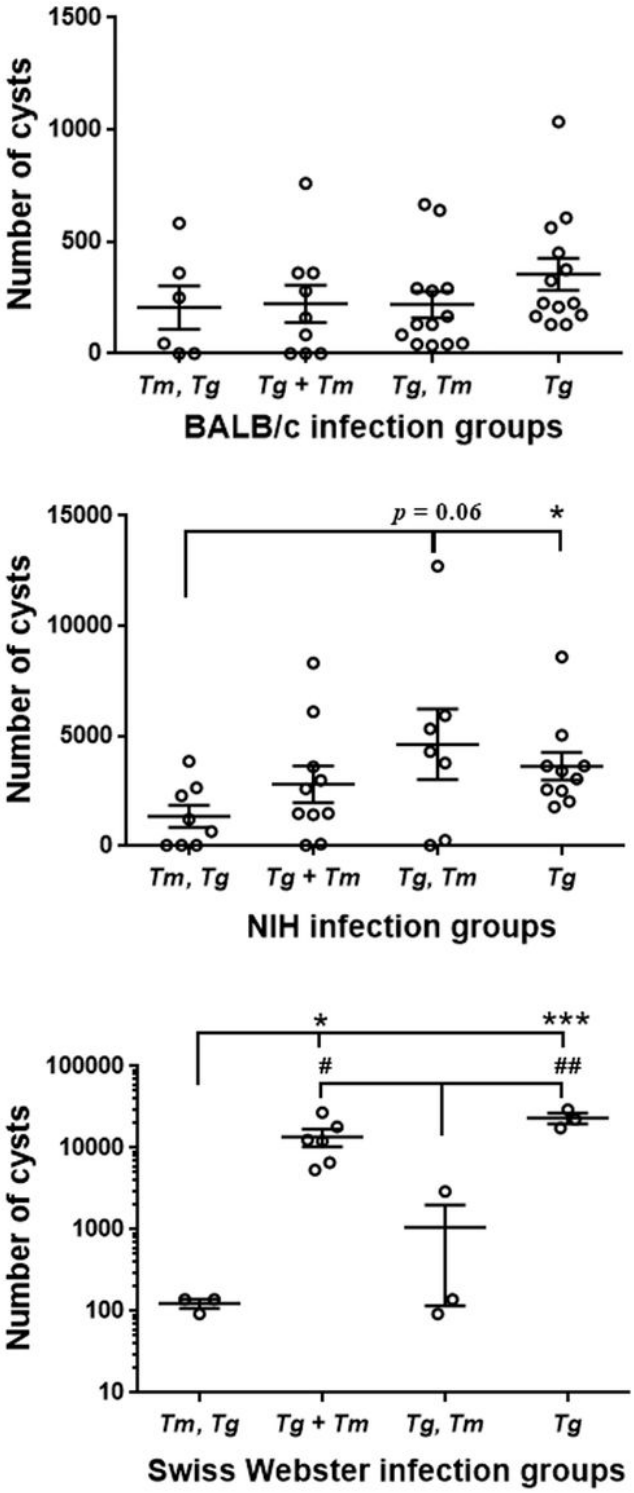
The columns refer to the following: *Tm* (infected singly with *T. musculi*); *Tg* (infected singly with *T. gondii*); *Tm,Tg or Tg,Tm* (infection with *T. musculi* first followed by infection with *T. gondii* five days later or *vice-versa*); *Tm+ Tg* (both parasites infected simultaneously). The term “ref” refers to the reference for the odds ratio. a, $p = 0.013$; b, $p = 0.050$; c, $p = 0.054$; d, $p = 0.057$.

Table 3
Summary of the spleen index of mice infected with *T. gondii* and/or *T. musculi*.

| Mice strains | Spleen index | | | | | |
|---|---|---|---|---|---|---|
| | *Tm,Tg* | *Tg,Tm* | *Tm+ Tg* | *Tg* | *Tm* | Control |
| BALB/c | 12.19 ± 0.38*** | 12.86 ± 0.70*** | 13.48 ± 1.65*** | 7.42 ± 0.45### | 16.25 ± 1.41### | 4.14 ± 0.15 |
| NIH | 11.62 ± 1.63 | 12.63 ± 1.07 | 14.86 ± 2.49 | 10.01 ± 1.61## | 15.99 ± 0.95### | 4.35 ± 0.17 |
| Swiss Webster | 7.15 ± 0.84* | 18.03 ± 0.86*** | 7.75 ± 1.59 | 4.09 ± 0.57 | 11.92 ± 0.85### | 4.35 ± 0.40 |
| C57BL/6 | - | - | - | - | 14.38 ± 1.06### | 3.55 ± 0.13 |

Spleen index = spleen weight (mg)/body weight (g). n = 6. */***, vs *Tg* group, $p < 0.05$ or $p < 0.001$, respectively; ##/###, vs control group, $p < 0.01$ or $p < 0.001$, respectively.

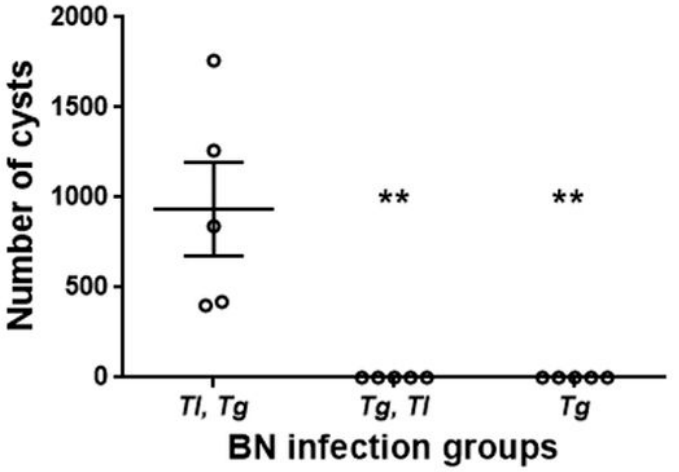
# Figures

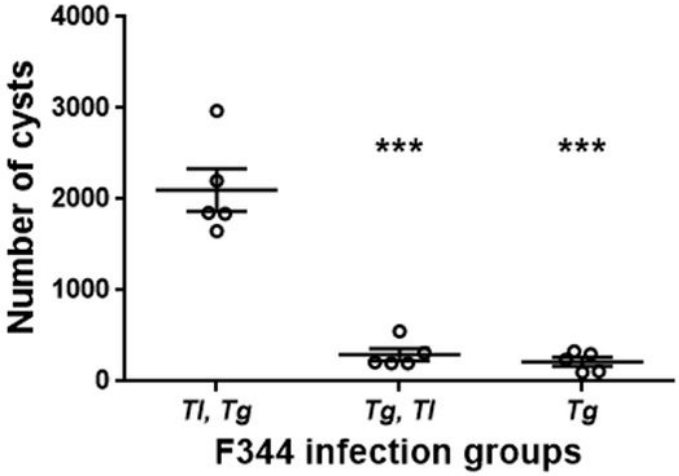


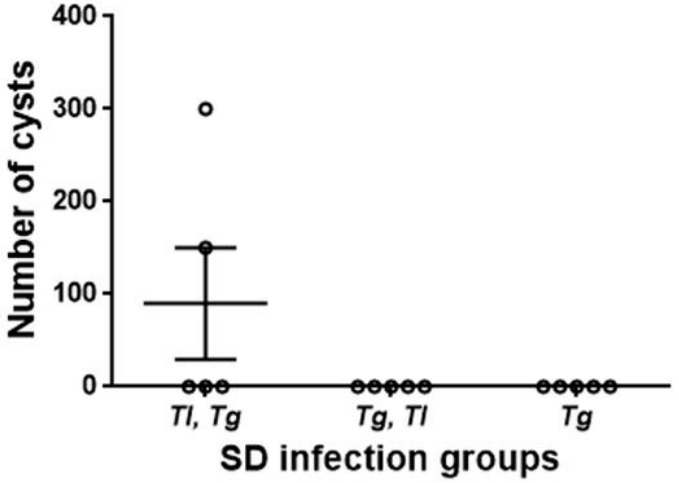

## Figure 1

Differences in Toxoplasma cyst numbers in the brains of four strains of mice. Each strain of mice was divided into four groups, which were coinfected with 5 cysts from the T. gondii Pru strain and 105 trypomastigotes of T. musculi or singly infected with T. gondii. Tg (infected singly with T. gondii); Tm,Tg or Tg,Tm (infection with T. musculi first followed by infection with T. gondii five days later or vice-versa); Tm + Tg (both parasites infected simultaneously). The cyst numbers in the brains of all inoculated mice

were detected 60 days later. The mean and standard error of the mean of each group was indicated. Significant differences are indicated */***, vs Tg group, $p < 0.05$ or $p < 0.001$, respectively; #/##, vs Tg,Tm group, $p < 0.05$ or $p < 0.01$, respectively.



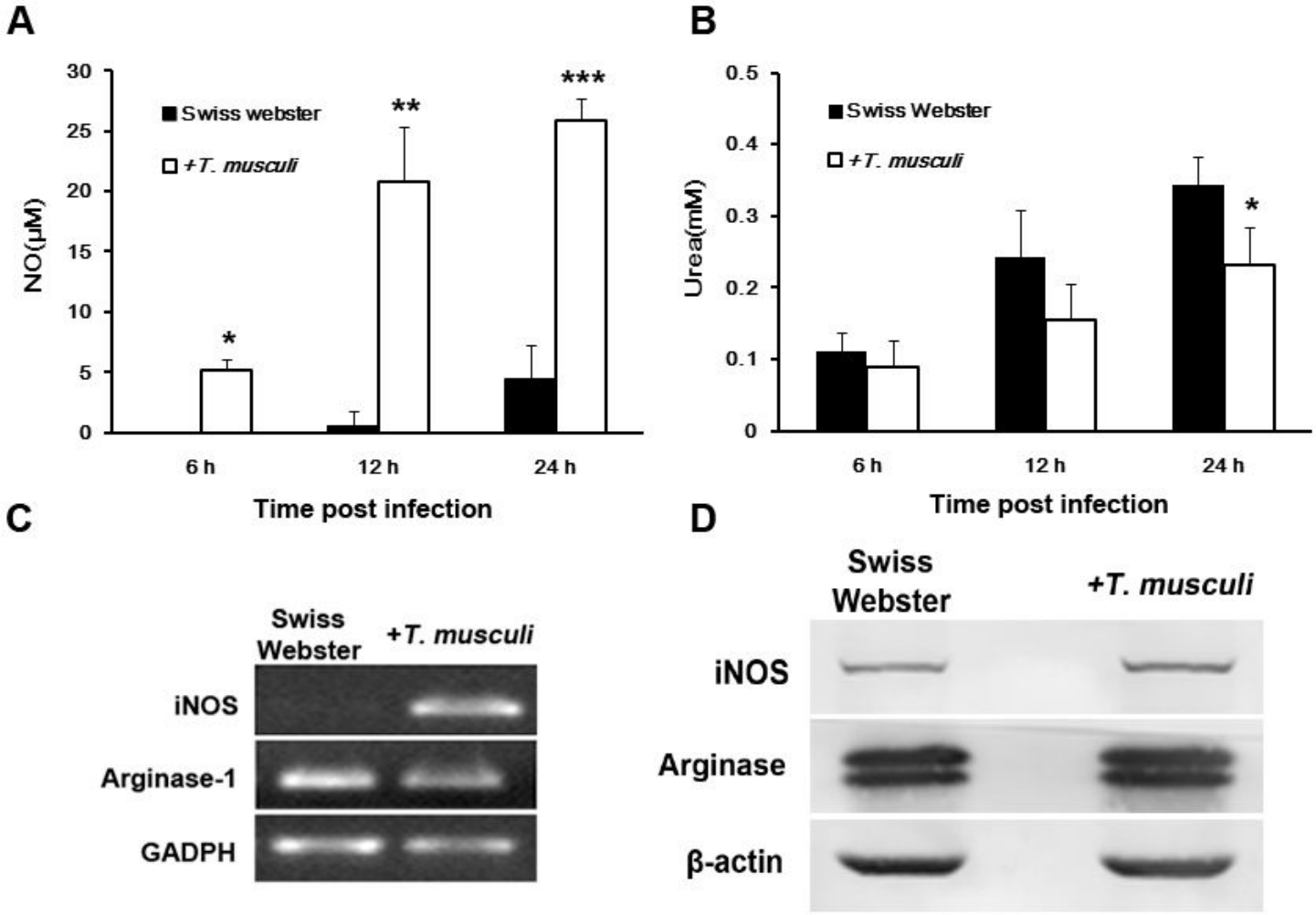




## Figure 2

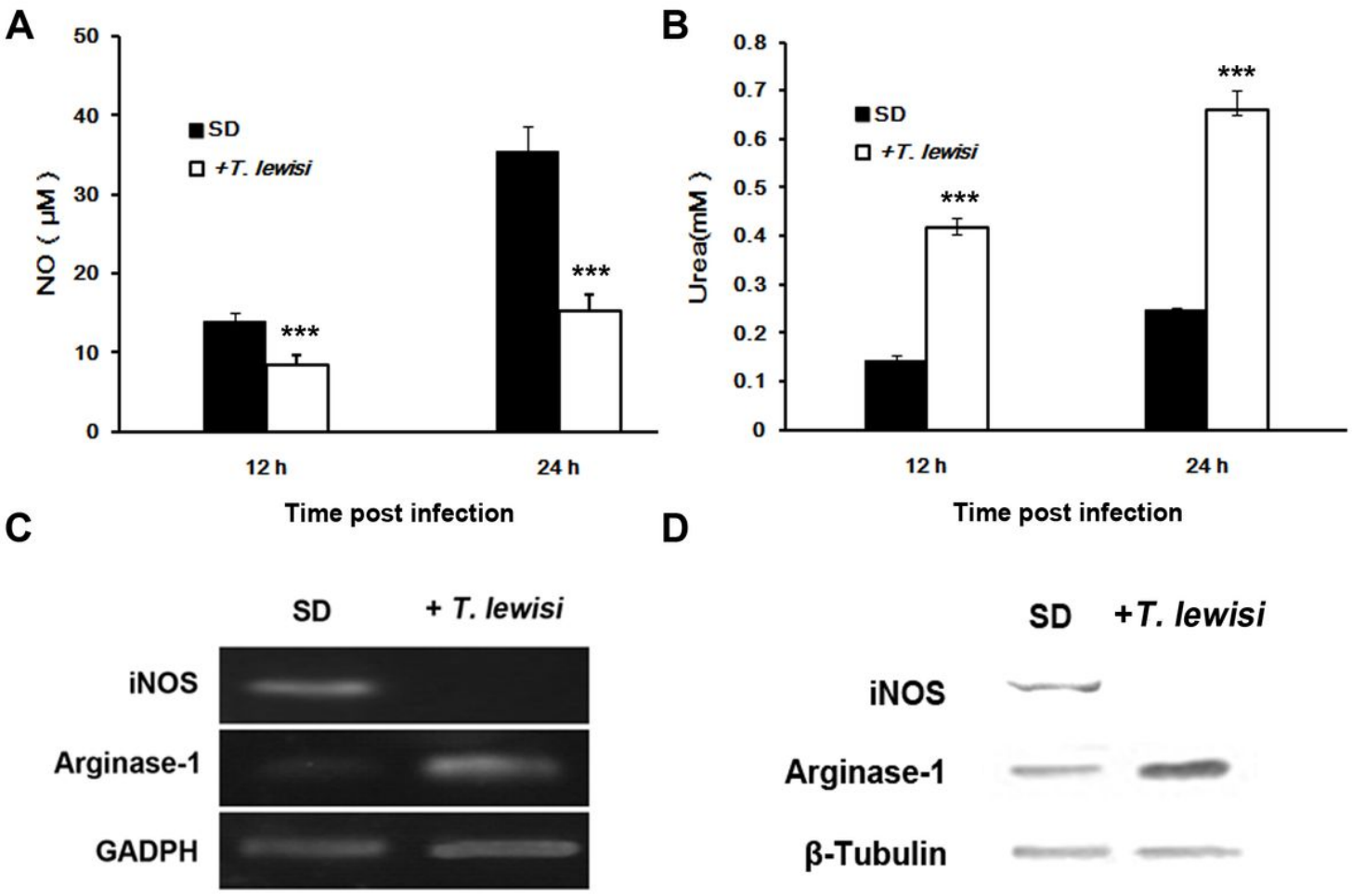
Differences in Toxoplasma cysts numbers in the brains of five strains of rats. Each strain of rats was divided into three groups, which were coinfected with 200 cysts of T. gondii Pru strain and 106

trypomstigotes of T. lewisi or only infected with T. gondii. Tg (infected singly with T. gondii); TL,Tg or Tg,TL (infection with T. lewisi first followed by infection with T. gondii five days later or vice-versa).The cyst numbers in the brains of all inoculated rats were detected 60 days later. The mean and standard error of the mean of each group was indicated. Significant differences are indicated **, $p < 0.01$; ***, $p < 0.001$



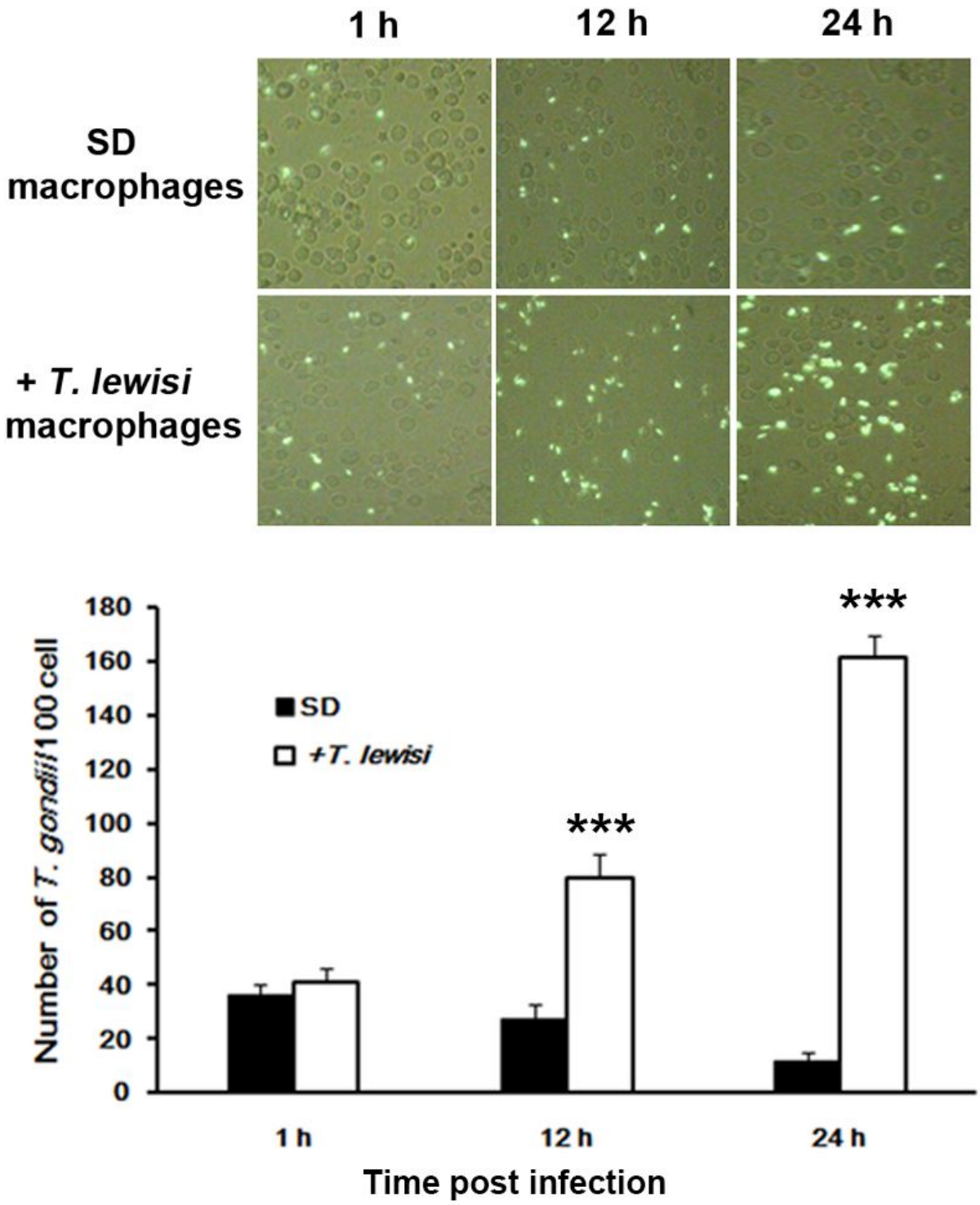

## Figure 3

Inducible nitric oxide synthase (iNOS)/arginase expression and nitric oxide (NO)/urea production in peritoneal macrophages from Swiss Webster mice. A. Comparison of NO production, measured by the Griess reaction, in macrophages of normal and T. musculi pre-infected Swiss Webster mice. B. Comparison of urea production in macrophages of normal and T. musculi pre-infected Swiss Webster mice. Error bars indicate standard deviations of measured values (n=3). Data were analyzed using the unpaired Student's t-test (two-tailed). * $p < 0.05$; ** $P < 0.01$; *** $p < 0.001$ C. RT-PCR analysis of the expression of iNOS and arginase-1 mRNA. D. Western blotting analysis for expression of the iNOS and arginase-1 proteins.




## Figure 4

Inducible nitric oxide synthase (iNOS)/arginase expression and nitric oxide (NO)/urea production in peritoneal macrophages from Sprague-Dawley rats. A. Comparison of NO production, measured by the Griess reaction, in macrophages from normal and T. lewisi infected SD rats. B. Comparison of urea production in macrophages of normal and T. lewisi infected SD rats. Error bars indicate standard deviations of measured values (n=3). Data were analyzed using the unpaired Student's t-test (two-tailed). * $p < 0.05$; ** $P < 0.01$; *** $p < 0.001$ C. RT-PCR analysis for the expression of the iNOS and arginase-1 mRNA. D. Western blotting analysis for expression of the iNOS and arginase-1 proteins.




## Figure 5

Comparison of Toxoplasma gondii proliferation in macrophages of T. lewisi infected Sprague-Dawley rats and control animals. A. Analysis of T. gondii proliferation in macrophages by fluorescent microcopy. SD Macrophages, taken from SD rats infected with T.gondii alone; +T. lewisi macrophages, taken from SD rats infected with T.gondii and T. lewisi. B. Number of T. gondii per 100 macrophages counted 1 hr, 12 hrs and 24 hrs after infection. Error bars indicate standard deviations of measured values (n=3). Data were

analyzed using the unpaired Student’s t-test (two-tailed) and statistical differences were designated by * (p  0.05), **(0.001  p  0.01) and ***(p  0.001).

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