Toxoplasma gondii infection in the peritoneal macrophages of rats treated with glucocorticoids

Tao Wang · Jiang-Mei Gao · Si-Qi Yi · Guo-Qing Geng · Xiao-Jie Gao · Ji-Long Shen · Fang-Li Lu · Yan-Zi Wen · Geoff Hide · Zhao-Rong Lun

T. Wang · J.-M. Gao · S.-Q. Yi · G-Q. Geng · X.-J. Gao · Y.-Z. Wen · Z.-R. Lun (✉)
Center for Parasitic Organisms, State Key Laboratory of Biocontrol, School of Life Sciences and Key Laboratory of Tropical Disease Control of the Ministry of Education, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510275, China
e-mail: lsslzr@mail.sysu.edu.cn

J.-L. Shen
Department of Parasitology and The Key Laboratories of Pathogen Biology and Zoonoses, Anhui, Anhui Medical University, Hefei 230032, China

F.-L. Lu
Department of Parasitology and Key Laboratory of Tropical Disease Control of the Ministry of Education, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, China

G. Hide · Z.-R. Lun

Ecosystems and Environment Research Centre and Biomedical Research Centre, School of Environment and Life Sciences, University of Salford, Salford, M5 4WT, UK

T. Wang

Department of Parasitology, School of Basic Medical Sciences, Jiujiang University, Jiujiang 332000, China

Abstract It is well known that toxoplasmosis can be life-threatening to immunocompromised individuals such as AIDS and organ transplantation patients. Glucocorticoids (GCs) are widely used in the clinic for the treatment of autoimmune diseases and organ transplantation resulting in acute toxoplasmosis in these patients. However, the interaction and mechanism between the development of acute toxoplasmosis and GC therapy are still unknown. The aims of this study were to investigate the infection of *T. gondii* in the peritoneal macrophages of rats treated with glucocorticoids. Our results showed that the growth rate of *T. gondii* RH strain was significantly increased in the peritoneal macrophages of rats treated with glucocorticoids *in vivo*. For instance, 242 (±16) tachyzoites were found in 100 macrophages from the rats treated with methylprednisolone (MP), while only 16 (±4) tachyzoites were counted in the macrophages from the non-treated control rats 24 hrs after infection (P<0.01). We also demonstrated that a significant inhibition of nitric oxide (NO) production was detected in the macrophages collected from the rats post-treated with GCs with 12.90 μM (±0.99 μM) of nitrite production from the rats treated with MP, while 30.85 μM (±1.62μM) was found in the non-treated control rats 36 hrs after incubation (P<0.01). Furthermore, glucocorticoids could significantly inhibit the expression of inducible nitric oxide synthase mRNA and its protein in the rat peritoneal macrophages. Our results strongly indicate that the decrease of NO in the rat peritoneal macrophages is closely linked to the cause of acute toxoplasmosis in the host. Additionally, there was a significant increase in the number of cysts produced by the naturally cyst forming, *T. gondii* Prugniaud strain with an average of

2795 (±422) cysts of the parasite being detected in the brains of the rats treated with dexamethasone, while only 1356 (±490) cysts were found in the non-treated control animals ($P<0.01$). As rats and humans are both naturally resistant to *T. gondii* infection, these novel data could lead to a better understanding of the development of acute toxoplasmosis during glucocorticoid therapy in humans.

Introduction

As one of the most severe opportunistic infectious diseases, toxoplasmosis caused by *Toxoplasma gondii* is life-threatening to immunocompromised patients with AIDS, cancer and those who are under immunosuppressant treatment following transplantation. A high mortality rate has been documented in immunocompromised patients due to severe toxoplasmosis (caused by the re-activation of chronic infection) (Montoya and Liesenfeld, 2004). However, the association between immunosuppression using glucocorticoids and the occurrence of severe toxoplasmosis is not well understood (Chandrasekar and Momin, 2003; De-Medeiros et al. 2001).

Glucocorticoids (GCs) are important modulators of immune cell functions (Sternberg, 2006). Due to their well-known immunosuppressive and anti-inflammatory properties, synthetic GCs are widely used in clinical practice and are effective in the treatment of autoimmune disease (Sathasivam, 2008), immunological rejection (Everson *et al*. 2003), atopic inflammatory disease (Tarpataki , 2006) and acute critical illness (Minneci et al. 2004; Rady et al. 2006). It has also been well documented that GCs can mediate immune responses by

affecting viability and function of various important immune cells including macrophages, B cells, monocytes and granulocytes (Chen et al. 2010; Tsianakas et al. 2012). Amongst these cells, macrophages are considered the most ubiquitous in mammalian tissues and play a central role in both innate and acquired immune responses.

Activated macrophages acquire competence for enhanced anti-microbial activity including nitric oxide (NO) production (Cape and Hurst, 2009; Severin et al. 2010; Silva, 2010). NO is recognized as a major regulatory molecule of the immune system and a principal cytotoxic mediator of activated immune effector cells which plays a critical role against pathogen infections (Adams et al. 1990; Li et al. 2012)

However, what is still not clear is the relationship between the effects of GCs on the function of macrophages and the development of acute toxoplasmosis from chronic infections. Infection of humans, with *T. gondii*, usually shows a chronic profile which is similar to that found in rats (Sumyuen et al. 1996; Da-Silva et al. 2010; Dubey, 2011; Li et al. 2012). Therefore, the rat model has been suggested to be an ideal model to study the development of acute toxoplasmosis from chronic infection in humans. The aims of this study were to understand the effect of GCs on the infection of rat peritoneal macrophages with the *T. gondii* RH strain and the effect on cyst formation of the naturally cyst forming *T. gondii* Prugniaud strain in the brains of rats treated with GCs. Results from this work will provide useful data to better understand human toxoplasmosis from the individuals who were treated with GCs.

Materials and methods

Animals and parasites

Special pathogen free Sprague Dawley (SD) rats weighing from 180 to 220 g were used for the source of peritoneal macrophages and BALB/c mice used for the maintenance of the *T. gondii* RH were purchased from the experimental animal center of Guangdong province, China. Special pathogen free F344 rats were purchased from Vital River Laboratories (Beijing, China). BALB/c mice were intraperitoneally (i.p.) inoculated with 1×10^6 tachyzoites of *T. gondii* RH-GFP (Nishikawa et al. 2003). Tachyzoites were harvested and isolated from the infected animals and were resuspended in RPMI-1640 medium supplemented with 10% FBS. *T. gondii* Prugniaud strain was maintained in NIH mice. Cysts were isolated from the brains of infected animal when they were required. Protocols for the use of animals were approved by the Institutional Review Board of Animal Care of Sun Yat-Sen University (973 project, #2010CB530000).

Peritoneal macrophage isolation and cultivation

Methods for isolation of macrophages from GC treated and non-treated rats were used as previously described (Li et al. 2012; El-Mahmoudy et al. 2002). The pelleted peritoneal macrophages were resuspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and penicillin (100 U/ml) after washing. Final concentrations of 2×10^5 cells in 200 μl and of 5×10^6 cells in 2.5 ml were seeded into each well in 96-and 6-well tissue culture plates (Corning, USA) respectively and

were incubated at 37°C, 5% CO_2, 95% air for 2 hrs. The macrophages were then carefully washed three times with FBS free RPMI-1640 medium to remove the non-adherent cells and incubated with the test compounds indicated in the results.

Animal treatments

For the experimental treatment with GCs, 8 rats in each of four groups were used in this study. In the control group, each rat was intramuscularly (i.m.) injected with 0.2 ml saline solution, while in the other three groups, each animal was intramuscularly injected with dexamethasone (DXM), hydrocortisone sodium succinate (HSS) and methylprednisolone (MP) at 1.5 mg/kg/d, 20 mg/kg/d and 20 mg/kg/d respectively for 7 days (Da-Silva et al. 2010; Dimitriu et al. 2008).

T. gondii infection in macrophages collected from rats treated with GC

The macrophages harvested from the rats treated with GCs mentioned above, were challenged with tachyzoites at a ratio of tachyzoite/macrophage of 1:1 at 12 hrs post incubation. Extracellular tachyzoites were then washed out after 1 hr of contact with macrophages and the time was then defined as 1 hr. The macrophages were continuously cultured in RPMI-1640 contained FBS and penicillin. Macrophages were observed under an inverted fluorescent microscope at 1, 12 and 24 hrs and the numbers of cells infected with *T. gondii* as well as the number of parasites per 100 macrophages were counted. Controls were carried out using the macrophages collected from the non-GC treated rats using an identical protocol.

142

143 Measurement of nitrite concentration

144 The NO production was evaluated indirectly by measuring nitrite concentration in

145 macrophage cultural supernatants as described previously (Green *et al*. 1982; Li et al.,

146 2012). Supernatant (150 μl) was collected from each well at intervals of cultivation

147 and mixed with an equal volume of Griess reagent on a 96-well flat-bottomed plate

148 (Corning, USA). Absorbance at 540/550 nm was measured in an ELISA reader.

149 Nitrite concentration was calculated based on a standard curve using sodium nitrite

150 diluted with the same medium used for the cultivation of macrophages.

151

152 RNA extraction and assessment of iNOS mRNA expression by reverse transcription

153 polymerase chain reaction (RT-PCR)

154 Total RNA from macrophages collected from rats treated or non-treated with GCs

155 were extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the

156 manufacturer's instructions. Total RNA was reversed to cDNA using a set of oligo

157 (dT) primer and Super Script^{TM} III First-Strand Synthesis System (Invitrogen,

158 Carlsbad, USA) and the cDNA (1 μl) was used as template for amplification of iNOS

159 and GAPDH gene (used as internal standard) by PCR with the following primers:

160 iNOS, 5'-CTA CCT ACC TGG GGA ACA CCT GGG-3' and 5'-GGA GGA GCT

161 GAT GGA GTA GTA GCG G-3'，producing a fragment of 442 bp； GADPH, 5'-AAT

162 GCK TCC TGY ACC ACC AAC TGC-3' and 5'-TTA GCC AWA TTC RTT GTC

163 RTA CCA GG-3, producing an amplified fragment at 513 bp. The PCR products were

analyzed by agarose gel electrophoresis.

Expression analysis of iNOS by western blotting

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

Infection of *T. gondii* Prugniaud strain in F344 rats treated with or without GC

Twenty F344 rats were used in this experiment. Each animal was infected by feeding 300 cysts of the *T. gondii* Prugniaud strain. They were randomly divided into two groups. Animals in Group I were treated with DXM (2 mg/kg/every two days), while rats in Group II (control) were treated with PBS only. Animals in the groups were treated with DXM or PBS for 4 weeks starting after infection. All animals were sacrificed two months after infection and cysts were counted from the homogenized brain tissue of each rat. The reason that DXM was chosen for this work was this compound is more commonly used in the clinic than other compounds.

Statistical analysis

All data were obtained from the experiments repeated at least three times and were shown as the mean ±S.D. Statistical analysis of the data for multiple comparisons was performed by one-way ANOVA. $P<0.05$ was considered statistically significant and $P<0.01$ was considered highly significant.

Results

Replication of *T. gondii* RH strain in the peritoneal macrophages of rats treated with GCs

Table 1 shows the replication of *T. gondii* RH strains tachyzoites in the peritoneal macrophages isolated from the rats injected intramuscularly with GCs (dexamethasone, DXM; hydrocortisone sodium succinate, HSS and methylprednisolone, MP). A difference in infection rate was not found across all 4 groups at 1 hr post infection. However, significant differences were observed between the GC treated and non-GC treated group at 12 and 24 hrs post infection ($P<0.01$).

An example fluorescent micrograph demonstrating the presence of more tachyzoites in the macrophages of rats treated with DMX than in those from the non-treated control rats is shown in Figure 1B and 1D (at 1 and 24hrs). Similar results were also observed in the macrophages collected from rats treated with HSS and MP respectively (data not shown).

Nitrite production in the peritoneal macrophages of rats treated with GCs

The effect of GCs on NO production by peritoneal macrophages from rats was

208 determined by the measurement of nitrite concentration in cell culture supernatants as
209 a reliable marker for NO production. As shown in Table 2, by comparison to the
210 non-treated control, a significantly lower NO production was detected in the
211 peritoneal macrophages collected from the rats treated with GCs for 7 days ($P<0.01$).
212
213 Synergistic effects of *T. gondii* infection and GC treatment on NO production in rat
214 peritoneal macrophages
215 Table 3 shows the synergistic effect on the production of NO by rat peritoneal
216 macrophages when they were treated with GCs *in vivo* and infected with *T. gondii in*
217 *vitro*. Significantly less NO production was detected in the peritoneal macrophages of
218 rats treated with GCs and infected with *T. gondii* than that found in GC treatment or *T.*
219 *gondii* infection only ($P<0.01$). These results indicate that GC treatment and *T. gondii*
220 infection may have a synergistic effect on inhibiting NO production in the rat
221 peritoneal macrophages.
222
223 The mRNA expression and protein levels of iNOS in the peritoneal macrophages of
224 rats treated with GCs
225 Figure 2(A) shows the analysis of iNOS mRNA levels of the peritoneal macrophages
226 of rats treated with GCs by RT-PCR. The size of PCR products was estimated at the
227 correct size of 442 bp. Band intensities show lower levels of iNOS expression in the
228 peritoneal macrophages of rats treated with GCs. Figure 2(B) displays the analysis of
229 iNOS protein levels in the peritoneal macrophages of rats treated with GCs as

determined by Western blotting. Lower expression levels of iNOS were detected in the peritoneal macrophages treated with GCs. Down-regulation of iNOS expression by GC treatment was consistent with the reduction in nitrite level in the culture supernatants from these macrophages.

Effects of DXM on cyst burden of the *T. gondii* Prugniaud strain in the brains of F344 rats

The Prugniaud strain of *T. gondii* is a type II strain and more readily forms cysts in rodent brains than the type I RH strain. Figure 3 shows the number of cysts found in the brains of F344 rats infected with the *T. gondii* Prugniaud strain and treated with DXM. An average of 2795±422 cysts was found in the brain tissues from the rats treated with DXM, while only 1356±490 cysts were detected from the control rats. This result clearly indicated that DXM could significantly increase the number of cysts in the brains of rats treated with this GC ($P<0.01$). Although obvious differences in clinical signs were not observed between these two groups of rats, the significant increase in cysts found in the brain of the rats treated with DXM clearly demonstrates the effect of DXM on *T. gondii* cysts in F344 rats.

Discussion

Human infection with *T. gondii* can be obtained by ingestion of oocysts from contaminated food and water, by eating undercooked or raw meat containing cysts and by congenital transmission (Dubey, 2011). After the acute stage of disease

252 (tachyzoites), the parasite develops cysts (bradyzoites, latent stage) in a variety of
253 organs, but mainly in the brain and skeletal muscle cells, and establishes a chronic
254 infection (Lang et al. 2007). Immunosuppression found in AIDS, cancer and organ
255 transplantation patients can cause the reactivation of a latent infection resulting in
256 acute infection (Sibley, 1993; Skariah et al. 2010). Additionally, it has been suggested
257 that natural immunosuppression associated with pregnancy may also result in
258 reactivation of maternal infection followed by congenital transmission (Hide et al.
259 2009; Thomasson et al. 2011). However, little is known about the mechanism of the
260 reactivation of chronic infection of *T. gondii* within mammalian hosts although
261 weaker immunity has been suggested to be linked to such reactivation (Chandrasekar
262 and Momin, 2003; De-Medeiros et al. 2001).

263 It is reported that the peritoneal macrophages of rat are naturally resistant to *T.*
264 *gondii* infection (Dubey, 2011) and recent studies have demonstrated that the
265 mechanism of such resistance is strongly linked to higher expression of inducible
266 nitric oxide synthase (iNOS) in the rat peritoneal macrophage (Li et al. 2012).
267 Interestingly, however, lower expression of iNOS was found in alveolar macrophages
268 of rat and they were demonstrated to be more sensitive, than those from the peritoneal
269 cavity, to infection with *T. gondii* RH strain (Zhao et al. 2013). GCs are known to
270 regulate the immunological reactions, mediate many aspects of homeostasis and play
271 a critical role in the systemic stress response. The powerful anti-inflammatory and
272 immunosuppressive properties of GCs have also made them an extremely valuable
273 therapy in patients with severe inflammatory and autoimmune disorders (Everson et al.

2003). Therefore, we proposed that GCs might decrease NO production in rat peritoneal macrophages since it has been shown that the long term use of GCs in mammalian hosts (rodents and humans) can result in the development of acute toxoplasmosis from chronic infection (Montoya and Liesenfeld, 2004; De-Medeiros et al. 2011). Understanding these mechanisms will greatly improve our knowledge of the reasons why and how long term use of GCs in mammalian hosts including humans, can cause acute toxoplasmosis.

In this study, we have adopted three GC agents that are all widely used in the clinic and each has a physiological half-life and pharmacodynamic action. Our results further support previous studies (Li et al. 2012) that *T. gondii* cannot multiply efficiently in the normal rat peritoneal macrophages, but surprisingly we found that it could grow well in the peritoneal macrophages of rats treated with GCs. Furthermore, our results showed that the growth of *T. gondii* in the rat peritoneal macrophages was linked to the reduction of NO production in macrophages treated with GCs. These results are consistent with the results by Li and colleagues (Li et al. 2012) that they demonstrated that a lower concentration of NO in the rodent peritoneal macrophages is strongly linked to their susceptibility to *T. gondii* infection.

Some studies demonstrated that *T. gondii* infection could decrease NO production in the peritoneal macrophages (Nishikawa et al. 2007) and it was suggested as one of the reasons for the development of acute toxoplasmosis from chronic infection. Our results also showed that *T. gondii* infection could partially decrease NO production in the infected cells which was suggested as one of the

296 pathways used by the parasite to enable to escape the immune reaction in the host
297 (Seabra et al. 2002). However, we consider that it may not be the key reason for the
298 development of acute toxoplasmosis from chronic infection. Our data showed that the
299 synergistic interaction between *Toxoplasma* infection and GC treatment may be an
300 important driver for this transition. Based on the above analysis, it is reasonable to
301 propose that the down-regulation of NO could be a shared and generic mechanism
302 that could explain why macrophage resistance to *T. gondii* infection or control of
303 *Toxoplasma* activation is impaired during GC treatment.
304 Furthermore, we demonstrated that GCs could inhibit the expression of iNOS
305 mRNA and protein in rat peritoneal macrophages by RT-PCR and western-blotting
306 analysis. These data are supported by the results from previous studies which
307 demonstrated that DXM could inhibit the expression of iNOS in human hepatocytes
308 and alveolar macrophages (Geller et al. 1993; Xiang et al. 2000). Because of the
309 tremendous differences in iNOS activity between the peritoneal macrophages from
310 mice and rats (Li et al. 2012), the bias towards studies in mouse models needs to be
311 augmented with further studies on rats.
312 A key question that needs to be addressed is: what is the mechanism of NO
313 action that drives the transition from acute toxoplasmosis to the chronic infection? NO
314 is beneficial in minimizing pathogenesis in the host during chronic toxoplasmosis
315 (Hayashi et al.1996; Scharton-Kersten et al. 1997). In the absence of NO, chronically
316 infected rats and mice could develop lethal disease with increased *T. gondii* burden
317 and severe inflammation in the places where the parasites were located (Bohne et al.

1994; Scharton-Kersten et al. 1997). Some studies have shown that NO may serve as a molecular trigger for stage conversion of *T. gondii* tachyzoites to bradyzoites leading to a state of chronic infection in the host (Bohne et al. 1994). In most cases, *T. gondii* causes asymptomatic infection in healthy individuals. However, GCs as immunosuppressive agents could change the immune status of the host resulting in the development of acute toxoplasmosis from a chronic infection (Djurkovic-Djakvoic and Milenkovic, 2001). Our work demonstrated that GCs significantly reduced the synthesis of NO in rat peritoneal macrophages which is similar to the effect of these compounds on the hepatocytes of rats (Geller et al. 1993) and alveolar macrophages of humans (Xiang et al. 2000). Furthermore, these studies provide novel data which could enable a better understanding of the generation of acute toxoplasmosis.

Rats have long been considered the species of choice as a model for human toxoplasmosis on account of the similarity of their resistance to *T. gondii* infection with that found in humans (Derouin et al. 1995; Freyre et al. 2003; Dubey, 2011), although much less work has been done than in the mouse model. It has been demonstrated that activated rat peritoneal macrophages can markedly inhibit the multiplication of *T. gondii in vitro* (Zhao et al. 2009). Cellular immunity appears to be crucial in the control of *Toxoplasma* infection in mammalian hosts and, in particular, the role of activated macrophages has been emphasized (Suzuki et al. 1988). Our results showed that GCs have an enhancing effect on the growth of *T. gondii* in rat peritoneal macrophages which further demonstrates the important role of NO in macrophages in causing the host to be naturally resistant to *T. gondii* infection.

Interestingly, a recent study has revealed that acute toxoplasma infection also increases endogenous production of GCs (Kugler et al. 2013). From the parasite perspective and based on our observations, this mechanism this may serve to promote further parasite growth. From the host perspective, Kugler et al (2013) show that this endogenous GC production serves to invoke a CD4+ T cell response which they propose limits collateral tissue damage and improves host survival. Our data would support the role of GC dependent NO reduction in the parasite side of this host-parasite evolutionary arms race.

In addition, our results demonstrated that significantly more *T. gondii* cysts (Prugniaud strain) were found in the brains of rats treated with the glucocorticoid dexamethasone (DXM). This result was consistent with our expectation. We consider that the treatment of rat peritoneal macrophages with GCs causes changes in some important physiological functions, for instance, a lower concentration of NO which provides a suitable environment for the growth and multiplication of *T. gondii*. This also potentially explains why acute infection of *T. gondii* is frequently found in human patients treated with GCs.

In conclusion, we have analyzed possible mechanisms of the effect of GCs on the resistance of rat peritoneal macrophages to *T. gondii* infection. These results not only provide useful novel data for better understanding the protective mechanisms of the host against this parasite but also reveal, indirectly, that patients who have been administered with GCs following organ transplantation could potentially provide *T. gondii* with a good opportunity to generate a new infection.

Acknowledgments The authors are thankful to Dr. X.N. Xuan for providing *Toxoplasma gondii* RH-GFP strain. The work was supported by National Basic Research Program of China (973 Program) (No. 2010CB530000), http://www.973.gov.cn/English/Index.aspx.

References

Adams LB, Hibbs RR, Krahenbuhl JL (1990) Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. J Immunol 144(7): 2725-2729

Bohne W, Heesemann J, Gross U (1994) Reduced replication of *Toxoplasma gondii* is necessary for induction of bradyzoite-specific antigens, a possible role for nitric oxide in triggering stage conversion. Infect Immun 62(5): 1761-1767

Cape JL, Hurst JK (2009) The role of nitrite ion in phagocyte function-perspectives and puzzles. Arch Biochem Biophys 484(2): 190-196

Chandrasekar PH, Momin F (1997) Disseminated toxoplasmosis in marrow recipients, a report of three cases and a review of the literature. Bone Marrow Transplant 19(7): 685-689

Chen X, Chen S, Li C, Zhu Y, Peng B (2011) Skewed T-cell subsets and enhanced macrophages phagocytosis in the spleen of patients with immune thrombocytopenia failing glucocorticoids. Int J Hematol 94(3): 248-254

Da-Silva RC, Da-Silva AV, Langoni H (2010) Recrudescence of *Toxoplasma gondii*

infection in chronically infected rats (*Rattus norvegicus*). Exp Parasitol 125(4): 409-412

De-Medeiros BC, De-Medeiros CR, Werner B，Loddo G, Pasquini R， Bleggi-Torres LF (2001) Disseminated toxoplasmosis after bone marrow transplantation, report of 9 cases. Transpl Infect Dis 3(1): 24-28

Derouin F, Lacroix C, Sumyuen MH, Romand S, Garin YJ, (1995) Experimental models of toxoplasmosis. Pharmacological applications. Parasite 2(3): 243-256

Dimitriu C, Bacb M, Lagreze WA, Jeble M (2008) Methylprednisolone fails to preserve retinal ganglion cells and visual function after ocular ischemia in rats. Invest Ophthalmol Vis Sci 49(11): 5003-5007

Djurkovic-Djakvoic O, Milenkovic V (2001) Murine model of drug-induced reactivation of *Toxoplasma gondii*. Acta Protozool 40(6): 99-106

Dubey JP (2011) *Toxoplasma gondii* of Animals and Humans. CRC Press.

El-Mahmoudy M, Matsuyama H, Borgan MA, Shimizu Y, El-Sayed MG, Minamoto N, Takewaki T (2002) Thymoquinone suppresses expression of inducible nitric oxide synthase in rat macrophages. Int Immunopharmacol 2(11): 1603-1611

Everson GT, Trotter JF, Kugelmas M, Forman L (2003) Immunosuppression in liver transplantation. Minerva Chir 58(5): 725-740

Freyre A, Falcón J, Mendez J, González M, Venzal JM, Morgades D (2003) Fetal *Toxoplasma* infection after oocyst inoculation of pregnant rats. Parasitol Res 89(5): 352-353

Geller DA, Nussler AK, Silvio M, Lowenstein CJ, Shapiro RA, Wang SC, Simmons

406 RL, Billiar TR (1993) Cytokines, endotoxin, and glucocorticoids regulate the
407 expression of inducible nitric oxide synthase in hepatocytes. Proc Natl Acad Sci
408 USA 90(2): 522-526
409 Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR(1982)
410 Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Anal Biochem
411 126(1): 131-138
412 Hayashi S, Chan CC, Gazzinelli R, Roberge FG (1996) Contribution of nitric oxide to
413 the host parasite equilibrium in toxoplasmosis. J Immunol 156(4): 1476-1481
414 Hide G, Morley EK, Hughes JM, Gerwash O, Elmahaishi MS, Thomasson D, Wright
415 EA, Williams RH, Murphy RG, Smith JE (2009) Evidence for high levels of
416 vertical transmission in *Toxoplasma gondii*. Parasitology 136(14): 1877-1886.
417 Kugler DG, Mittelstadt PR, Ashwell JD, Sher A, Jankovic D (2013) CD4+ T cells are
418 trigger and target of the glucocorticoid response that prevents lethal
419 immunopathology in *toxoplasma infection*. J Exp Med 210(10): 1919-1927
420 Lang C, Gross U, Lüder CG (2007) Subversion of innate and adaptive immune
421 responses by *Toxoplasma gondii*. Parasitol Res 100(2): 191-203
422 Li Z, Zhao ZJ, Zhu XQ, Ren QS, Nie FF, Gao JM, Gao XJ, Yang TB, Zhou WL, Shen
423 JL, Wang Y, Lu FL, Chen XG, Hide G, Ayala FJ, Lun ZR (2012) Differences in
424 iNOS and arginase expression and activity in the macrophages of rats are
425 responsible for the resistance against *T. gondii* infection. PLoS One 7(4): e35834
426 Minneci PC, Deans KJ, Banks SM, Eichacker PQ, Natanson C (2004) Meta-analysis,
427 the effect of steroids on survival and shock during sepsis depends on the dose.

Ann Intern Med 141(1): 47-56

Montoya JG, Liesenfeld O (2004) .Toxoplasmosis. Lancet 363(9425):1965-1976

Nishikawa, Y., Kawase, O., Vielemeyer, O., Suzuki, H., Joiner, K.A., Xuan, X. and Nagasawa, H. (2007). *Toxoplasma gondii* infection induces apoptosis in noninfected macrophages, role of nitric oxide and other soluble factors. Parasite Immunol 29(7): 375-85

Nishikawa Y, Xuan X, Makala L, Vielemeyer O, Joiner KA, Nagasawa H (2003) Characterisation of *Toxoplasma gondii* engineered to express mouse interferon-gamma. Int J Parasitol 33(13): 1525-1535

Rady MY, Johnson DJ, Patel B, Larson J, Helmers R (2006) Corticosteroids influence the mortality and morbidity of acute critical illness. Crit Care 10(4): R 101

Sathasivam S (2008) Steroids and immunosuppressant drugs in myasthenia gravis. Nat Clin Pract Neurol 4(6): 317-327

Scharton-Kersten TM, Yap G, Magram J, Sher A (1997) Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen *Toxoplasma gondii*. J Exp Med 185(7): 1261-1273

Seabra SH, Da-Souza W, Da-Matta RA (2002) *Toxoplasma gondii* partially inhibits nitric oxide production of activated murine macrophages. Exp Parasitol 100(1) 62-70

Severin VI, Soliman H, El-Matbouli M (2010) Expression of immune-regulatory genes, arginase-2 and inducible nitric oxide synthase (iNOS), in two rainbow trout (*Oncorhynchus mykiss*) strains following exposure to *Myxobolus cerebralis*.

Parasitol Res 106(2): 325-334

Sibley LD (1993) Interactions between *Toxoplasma gondii* and its mammalian host cells. Semin Cell Biol 4(5): 335-344

Silva MT (2010) When two is better than one, macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. J Leukoc Biol 87(1): 93-106

Skariah S, McIntyre MK, Mordue DG (2010) *Toxoplasma gondii:* determinants of tachyzoite to bradyzoite conversion. Parasitol Res 107(2): 253-260

Sternberg EM (2006) Neural regulation of innate immunity, a coordinated non-specific host response to pathogens. Nat RevImmunol 6(4): 318-328

Sumyuen MH, Garin YJ, Derouin F (1996) Effect of immunosuppressive drug regimens on acute and chronic murine toxoplasmosis. Parasitol Res 82(8): 681-686

Suzuki Y, Orellana MA, Schreiber RD, Remington JS (1988) Interferon gamma, the major mediator of resistance against *Toxoplasma gondii*. Science 240(4851): 516-518

Tarpataki N (2006) Recent developments in canine atopic dermatitis, a review. Acta Vet Hung. 54(4): 473-484

Thomasson D, Wright EA, Hughes JM, Dodd NS, Cox AP, Boyce K, Gerwash O, Abushahma M, Lun ZR, Murphy RG, Rogan MT, Hide G (2011) Prevalence and coinfection of *Toxoplasma gondii* and *Neospora caninum* in *Apodemus sylvaticus* in an area relatively free of cats. Parasitology 138(9): 1117-1123

Tsianakas A, Varga G, Barczyk K, Bode G, Nippe N, Kran N, Roth J, Luger TA, Ehrchen J, Sunderkoetter C (2012) Induction of an anti-inflammatory human monocyte subtype is a unique property of glucocorticoids, but can be modified by IL-6 and IL-10. Immunobiology 217(3): 329-335

Xiang XD, Zhou HY, Chen P (2000) Effects of nitroglycerin and dexamethasone on nitric oxide and endothelin derived from alveolar macrophages in patients with mild and middle asthma. Hunan Yi Ke Da Xue Xue Bao 25(4): 363-366

Zhao YL, Ferguson DJ, Wilson DC, Howard JC, Sibley LD, Yap GS (2009) Virulent *Toxoplasma gondii* evade immunity-related GTPase-mediated parasite vacuole disruption within primed macrophages. J Immunol 182(6): 3775-3781.

Zhao ZJ, Zhang J, Wei J, Li Z, Wang T, Yi SQ, Shen JL, Yang TB, Hide G, Lun ZR (2013) Lower expression of inducible nitric oxide synthase and higher expression of arginase in rat alveolar macrophages are linked to their susceptibility to *Toxoplasma gondii* infection. PLoS One, 8(5):e63650.

Tables and Figure legends

Table 1 Proliferation of *T. gondii* in peritoneal macrophages of rats treated with GCs.

	Number of *T. gondii*/100 cells		
GCs	1 h	12 hrs	24 hrs
DXM	35±3	55±8*	176±15[#]
HSS	39±5	81±12*	227±14[#]
MP	37±6	85±14*	242±16[#]
Control	39±5	23±4	16±4

SD rats were injected intramuscularly with a dose of GCs for 7 days, macrophages were harvested and cultured for 12 hrs and then incubated with *T. gondii* at the ratio of 1:1 (parasites/macrophages = 1:1). The extracellular *T. gondii* were washed from the medium after 1 hr contact and the time was defined as 1 hr and the number of parasites per 100 macrophages was counted at 1, 12 and 24 hrs after infection. * and [#]: $P<0.01$ vs. control analyzed by one-way ANOVA. All values are expressed as the mean ± S.D of each infected group mice (X±SD, n=3). Abbreviations: dexamethasone (DXM), hydrocortisone sodium succinate (HSS) and methylprednisolone (MP).

Table 2 Nitrite production by peritoneal macrophages from rats treated with GCs.

	Nitrite production (μM)		
GCs	12 h	24 hrs	36 hrs
DXM	9.78±0.37*	14.31±1.22*	17.99±1.08*
HSS	8.19±0.32*	11.05±0.74*	13.51±1.45*
MP	7.86±0.46*	10.65±0.98*	12.90±0.99*
Control	14.44±1.04	25.41±1.44	30.85±1.62

Peritoneal macrophages isolated from rats treated with GCs for 7 days were incubated for 12, 24 and 36 hrs. NO_2^- production in the supernatant of the cell culture medium, a reflection of NO production, was measured by the Griess reaction. *: $P<0.01$ vs. control analyzed by one-way ANOVA (X±SD, n=3).

Table 3 Nitrite production by peritoneal macrophages isolated from rats injected intramuscularly with GCs and subsequent infection with *Toxoplasma gondii*.

	Nitrite production (μM)	
Groups	12 h	24 h
DXM+*T. gondii*	7.70±0.79*	9.03±1.17*
HSS+*T. gondii*	6.37±0.97*	7.01±0.85*
MP+*T. gondii*	6.7±0.99*	7.22±0.85*
Control+*T. gondii*	12.02±1.12$^{\#}$	19.28±1.38$^{\#}$
Control	15.97±1.41	27.71±1.04

SD rats were injected intramuscularly with a dose of GCs for 7 days (see details in the Materials and Methods). Peritoneal macrophages collected from the treated rats, were cultured for 12 hrs and were then challenged with *T. gondii in vitro*. At 12 hrs and 24 hrs after infection, NO_2^- production in the supernatants was measured by the Griess reaction.*: $P<0.01$ vs. control+*T. gondii*;$^{\#}$: $P<0.05$; the comparison between control+*T. gondii* and control was analyzed by one-way ANOVA. All values are expressed as the mean ± S.D of each infected group mice (X±SD, n=3).

Figure legends:

Fig. 1 Fluorescent micrographs of *Toxoplasma gondii* proliferation in the macrophages of rats treated with DXM.

Macrophages collected from SD rats treated with DXM for 7 days and were cultured *in vitro* for 12 hrs before challenge with *T. gondii* tachyzoites at a ratio of *T. gondii*/ macrophage of 1:1. A and B: *T. gondii* in the normal control SD rat macrophages; and C and D: *T. gondii* infection in the peritoneal macrophages of SD rats treated with DXM. Similar results were also found in the macrophages of rats treated with HSS and MP (Data not shown) (Scale is 50 μm)

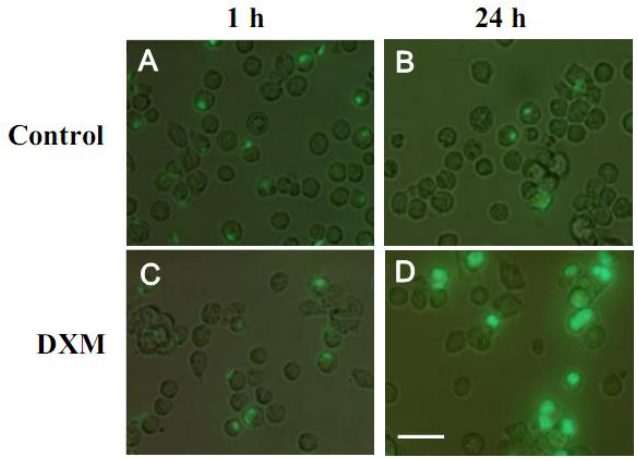
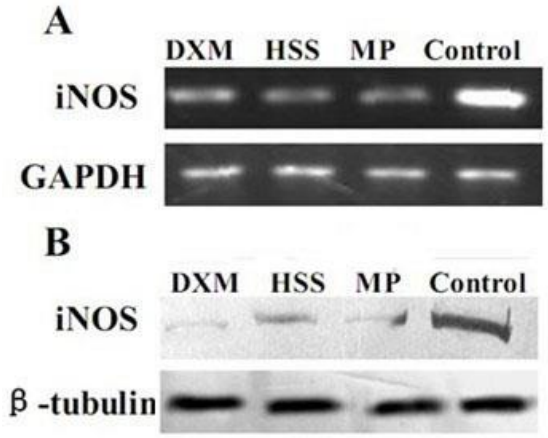


Fig. 2 Comparison of iNOS expression levels from the peritoneal macrophages of rats treated with GCs.

Macrophages collected from SD rats treated with GCs for 7 days and were cultured *in vitro* for 12 hrs. (A) RT-PCR analysis for the expression of iNOS mRNA. (B) Western blotting analysis for the expression of iNOS protein.



570

571 **Fig. 3** Effect of DXM on the cyst burden of the *T. gondii* Prugniaud strain in the brain

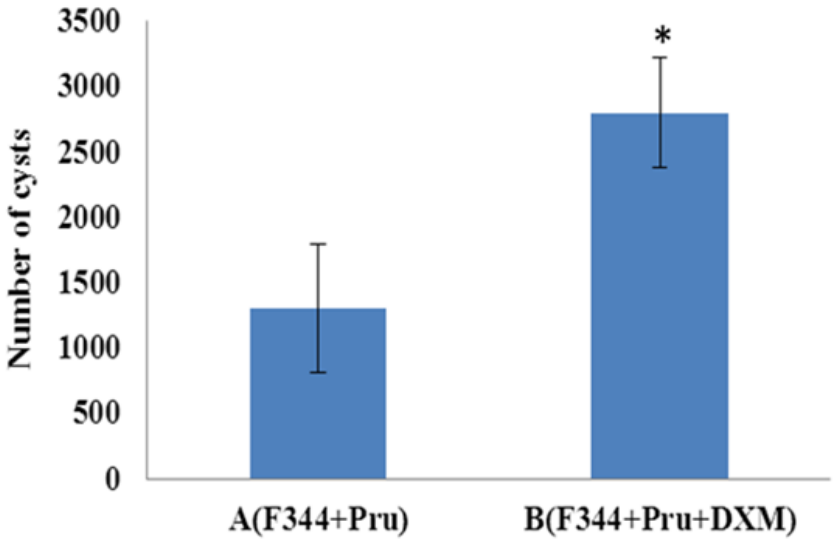
572 of F344 rats.

573 Cyst burden from the brains of F344 rats infected with the Prugniaud (Pru) strain of *T.*

574 *gondii* and treated (B) and non-treated (A) with DXM. * indicates a significance value

575 of $P < 0.05$.

576



577

578