**Toxoplasma gondii** Inhibits Covalent Modification of Histone H3 at the IL-10 Promoter in Infected Macrophages

**Jin Leng, Eric Y. Denkers**

Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York, United States of America

---

**Abstract**

Infection of macrophages with the protozoan parasite *Toxoplasma gondii* results in inhibition of a large panel of LPS-responsive cytokines, including TNF-α, while leaving others such as IL-10 intact. Recent studies provide evidence that the parasite interferes with chromatin remodeling at the TNF-α promoter that is normally associated with LPS stimulation, but that is not required for TLR4 induction of IL-10. Here, we examined the effect of *Toxoplasma* on IL-10 induced by simultaneous signaling through TLR4 and FcγR, a combined stimulus that triggers histone H3 covalent modification at the IL-10 promoter resulting in high level IL-10 cytokine production. We show that the parasite inhibits high level IL-10 production and prevents histone H3 Ser^10^ phosphorylation and Lys^9/14^ acetylation at the IL-10 promoter. These results provide compelling evidence that *T. gondii* targets the host cell chromatin remodeling machinery to down-regulate cytokine responses in infected macrophages.

---

**Introduction**

The protozoan *Toxoplasma gondii* is an opportunistic apicomplexan parasite with worldwide distribution in humans and animals. Although normally causing an asymptomatic infection, the parasite can emerge as a dangerous pathogen in immunodeficient hosts [1,2]. Previous work by us and others has found that macrophages and dendritic cells, important reservoirs of in vivo infection, become nonresponsive to parasite infection [1,2]. Previous work by us and others has found that macrophages and dendritic cells, important reservoirs of in vivo infection, become nonresponsive to Toll-like receptor (TLR) and IFN-γ receptor activation [3,4,5,6,7,8,9]. Thus, *T. gondii*-infected mouse bone marrow-derived macrophages (BMMØ) are strongly inhibited in their ability to produce a large battery of proinflammatory mediators during stimulation with TLR4 ligand lipopolysaccharide (LPS) [10]. Importantly, not all LPS-responsive genes are suppressed by the parasite. In particular, TLR4 stimulation continues to elicit IL-10 production even when macrophages are infected with tachyzoites [10].

Recently, we conducted a detailed analysis of the activity of the gene encoding TNF-α, a cytokine that is strongly suppressed by *Toxoplasma* [11]. We found that transcription factors associated with the TNF-α promoter, such as NFκB, cAMP-responsive element-binding protein (CREB) and c-Jun, were activated and translocated into the nucleus normally during LPS stimulation of infected cells. Nevertheless, using chromatin immunoprecipitation (ChIP), we obtained evidence that these factors were unable to bind to their target sequences on the native TNF-α promoter after parasite infection [11]. TLR4 triggering of BMMØ resulted in Ser^10^ phosphorylation and Lys^9/14^ acetylation on histone H3 at the TNF-α promoter, epigenetic changes associated with increased transcriptional activity [12]. However, *Toxoplasma* infection prevented these covalent modifications. When we examined the IL-10 promoter, we found that the relatively low level of cytokine produced during LPS stimulation occurred in the absence of histone H3 modification, providing a possible explanation for the lack of suppressive effect of the parasite on this particular cytokine.

Other studies have also indicated that LPS activation of macrophages induces only low amounts of IL-10 and no significant modification of histone H3 [13,14]. However, combining FcγR ligation with TLR4 stimulation triggers high level IL-10 production, and this is associated with ERK mitogen-activated protein kinase-dependent Ser^10^ phosphorylation and Lys^9/14^ acetylation of histone H3 on the IL-10 promoter. Based upon our findings at the TNF-α promoter [11], it was our prediction that high level IL-10 synthesis stimulated by LPS and immune complex (IC) would be suppressed by the parasite, even though low level LPS-induced IL-10 was not affected. Here, we tested this prediction. We found that, as with the TNF-α promoter during LPS stimulation, *Toxoplasma* blocked histone H3 covalent modification at the IL-10 promoter during stimulation with LPS and IC. These combined data provide strong support for a new model in which *T. gondii* targets histone modification rather than the activity of specific transcription factors, and in this way the parasite silences multiple host genes using a common mechanism of suppression.

---

**Results**

*Toxoplasma* blocks high level IL-10 production stimulated by combined LPS and immune complex stimulation

We previously reported that *Toxoplasma*-infected BMMØ were suppressed in their ability to produce TNF-α after LPS/TLR4
stimulation, but that low level IL-10 production was not affected by the parasite [11]. Here, we asked if high-level IL-10 induced by LPS in combination with IC was blocked by the parasite. As expected, stimulation with LPS alone induced low levels of IL-10, and this response was not affected by *T. gondii* (Fig. 1A). However, BMMØ produced approximately three-fold more IL-10 when the cells were triggered with LPS + IC, although IC alone failed to elicit this cytokine. Notably, this response was down modulated to levels obtained with LPS alone when cells were pre-infected with *Toxoplasma* (Fig. 1A).

We also measured IL-10 mRNA levels following stimulation with the combination of LPS and IC. Previously, we found that the parasite has no effect on increased IL-10 mRNA levels following LPS stimulation [11]. Here, we found an approximately 10-fold increase in IL-10 mRNA after stimulation with LPS + IC relative to triggering with LPS alone (Fig. 1B). Infection of cells with *T. gondii* resulted in suppressed IL-10 mRNA induction following LPS + IC stimulation (Fig. 1B). We conclude that *Toxoplasma* does not block low-level IL-10 induced by LPS, but that high level IL-10 production triggered by the combination of LPS and IC is sensitive to suppression by the parasite.

We examined the effect of LPS + IC on TNF-α levels in BMMØ. In this case, FcγR ligation did not further enhance TNF-α production over levels obtained with LPS alone (Fig. 1C). Unlike the case of IL-10, infection with *T. gondii* inhibited TNF-α production during LPS and LPS + IC stimulation to background levels.

**Discussion**

Superinduction of IL-10 in macrophages requires two signaling cascades. One emanates from TLR4 and results in activation of IL-10 transcription factors such as STAT3 and Sp1 [15]. The second pathway is activated through FcγR and results in chromatin remodeling at the IL-10 promoter to allow access of
transcription factors [13,14]. The results of this study suggest that Toxoplasma inhibits the superinduction response by interfering with the chromatin remodeling pathway. These data reinforce recent findings from our laboratory indicating that *T. gondii* has a similar inhibitory effect on LPS-initiated histone H3 modification at the TNF-α promoter [11]. In that case, we found that although transcription factors were activated normally during LPS stimulation, histone H3 phosphorylation and acetylation were blocked by *T. gondii*. Recent studies have found that histone H3 phosphorylation, but not acetylation, is the proximal event to IL-10 gene transcription [14]. Our finding that *Toxoplasma* possesses inhibitory effects on total levels of histone H3 Ser10 phosphorylation suggests that this may be the relevant target of suppression.

The IL-10 molecule is an anti-inflammatory mediator that is important in down-modulating proinflammatory responses to avoid pathology. Its relevance during *Toxoplasma* infection was shown in studies employing IL-10 deficient mice, because animals infected with this parasite succumb during acute infection in the absence of IL-10. Death is associated with a runaway proinflammatory cytokine response that, despite controlling the parasite, leads to lethal immunopathology during systemic and oral infection [16,17]. Similarly, IL-10 has an important role in limiting inflammation during toxoplastic encephalitis [18]. From the perspective of the parasite, IL-10 production is important to keep the host alive to maximize chances of transmission. Nevertheless, the ability of IL-10 to down-modulate activation of innate immune effector cells such as macrophages suggests that overproduction of this cytokine must be avoided. In this case, uncontrolled parasite replication would rapidly lead to host death, minimizing the chances of transmission to a new host. We previously reported that low-level IL-10 induction triggered by TLR4 was not inhibited by *Toxoplasma*. Here, we now demonstrate that macrophages triggered through TLR4 and FcyR produce much higher amounts of IL-10, and that *T. gondii* blocks this superinduction response to levels achieved by LPS stimulation alone. We hypothesize that inhibition of high but not low level IL-10 is a reflection of the parasite’s need to ensure appropriate amounts of IL-10 that avoid immunopathology and permit cyst formation, favoring long-term persistence in the host.

In addition to TNF-α and high level IL-10, many other LPS-responsive cytokines and chemokines are down regulated by *Toxoplasma* infection of macrophages [10]. Similar effects occur in LPS-stimulated dendritic cells, where infection blocks upregulation of MHC class II and costimulatory molecules in addition to TNF-α and IL-12 [3]. It is also known that the parasite inhibits the ability of macrophages and fibroblasts to respond to IFN-γ [8,19]. For the case of fibroblasts, a large family of IFN-γ-responsive genes is inhibited during *T. gondii* infection [20]. The ability of the parasite to simultaneously down-regulate large subsets of genes during activation with stimuli such as LPS and IFN-γ suggests that targeting chromatin remodeling may be the mechanism that mediates these profound effects.

The mechanism used by *Toxoplasma* to interfere with host chromatin remodeling is presently unclear. The parasite is known to inject rhoptry kinases and phosphatases into the host cytoplasm during infection, and although these molecules relocate to the host cell nucleus, whether they are involved in the effects reported here is not presently known [21,22,23,24]. Nevertheless, it is possible that such molecules interfere with host histone kinases and...
acetylatases. Alternatively, it is possible that parasite-derived effector molecules directly dephosphorylate and deacetylate histone H3.

The findings reported in the present manuscript in combination with other recent results from our laboratory together provide strong evidence for a new view of *Toxoplasma* as an intracellular pathogen that targets chromatin modification rather than (or in addition to) targeting specific transcription factors. The finding that proinflammatory TNF-α and anti-inflammatory IL-10 cytokines are both subject to the same type of regulation by *T. gondii* reinforces the concept that interference with inducible histone modification is a common strategy used by the parasite to influence host gene transcription. These combined studies are the first to demonstrate these effects during protozoan infection, but recent data suggest that some bacterial pathogens, such as *Shigella flexneri*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis* adopt similar strategies during infection [25,26,27,28]. Together, these data support an emerging view of host cell chromatin structure as an important target during microbial pathogenesis.

**Materials and Methods**

**Ethics Statement**

All work with animals received approval from the Cornell University Institutional Animal Care and Use Committee.

**Mice and Parasites**

C57BL/6 mice (6-8 wk of age) were purchased from The Jackson Laboratory. The mice were kept under specific pathogen-free conditions at the Transgenic Mouse Facility, Cornell University College of Veterinary Medicine. The facility is overseen by an Institutional Animal Care and Use Committee. The Type I *T. gondii* parasite strain RH was maintained by bi-weekly passage on human foreskin fibroblast monolayers (American Type Tissue Collection) in DMEM supplemented with 1% Bovine Growth Serum (BGS), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Parasite cultures were tested every 6-8 wk using a PCR-based ELISA (Roche Diagnostics).

**Cell culture**

Bone marrow cells were flushed from femur and tibia of C57BL/6 mice and cultured in macrophage medium consisting of DMEM supplemented with 10% BGS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20% supernatant from L929 cells, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The cells were supplemented with fresh macrophage medium on Day 3 after culture initiation. After 5 days of culture, nonadherent cells were removed, adherent monolayers were washed with PBS and cells were harvested by gentle pipeting with ice-cold PBS. Macrophage infection was accomplished by adding tachyzoites to cell cultures followed by brief centrifugation (200 x g, 3 min) to initiate contact between cells and parasites. Immune complex and LPS were added 18 hr after infection. Cells were recovered at varying times depending upon the assay performed.

**Immune complex preparation**

IgG-opsonized erythrocytes (E-IgG) were generated by incubating sheep red blood cells (SRBC, Lampire Biological Laboratories) with anti-SRBC IgG (MP Biomedicals) at non-agglutinating titers for 30 min at room temperature while rotating. Opsonized cells were washed once in Hank’s Buffered Saline Solution (Invitrogen Life Technologies) and resuspended in macrophage medium. E-IgG were added to cells at a ratio of 10 E-IgG to 1 macrophage.

**Semiquantitative real-time PCR**

RNA was isolated from cells using a commercial kit (RNaseasy Mini-kit; Qiagen) and cDNA synthesized according to standard protocols. Real-time PCR was performed with a Power SYBR green kit according to the manufacturer’s instructions (Applied Biosystems). Amplification was carried out on an Applied Biosystems 7700 Sequence Detector. The sequences of primers used are indicated in Table 1.

**Cytokine ELISA**

IL-10 in culture supernatants was measured using a commercial kit according to the manufacturer’s recommendations (eBioscience).

**Immunoblotting**

Anti-phospho-histone H3 (Ser^{10}, Cell Signaling) and anti-total histone H3 (Cell Signaling) were used for Western blot analysis. Cells (2 x 10^5/sample) were lysed in reducing SDS-PAGE sample buffer, and DNA was sheared by forcing samples 3 times through a 27-gauge needle. After 3 min at 100°C, samples were separated by 10% SDS-PAGE and proteins were subsequently electrotransferred onto nitrocellulose membranes. The membranes were blocked in 0.1% Tween 20 in Tris-buffered saline, pH 7.6 (TBST) containing 5% nonfat dry milk for 1 hr at room temperature, followed by overnight incubation (4°C) with Ab in 5% BSA in TBST. After washing blots in TBST, Ab binding was detected with a horseradish peroxidase-conjugated secondary anti-rabbit Ab (Jackson Immunoresearch) in TBST containing 5% nonfat dry milk. Following 1 hr incubation, blots were washed in TBST and developed with a chemiluminescence-based detection system (Cell Signaling).

**Chromatin immunoprecipitation (ChIP)**

ChIP-grade Ab to phospho-histone H3 (Ser^{10}) and acetylated histone H3 (Lys^{14}) were obtained from Cell Signaling. Assays were performed using the ChIP-IT enzymatic express kit (Active Motif) according to the manufacturer’s instructions. Briefly, cells (1.5 x 10^7/sample) were fixed in 1% paraformaldehyde at room temperature for 10 min. Fixation was quenched by adding glycine to the mixture. The cells were then collected by scraping in buffer containing PMSF (100 mM). After brief centrifugation, the macrophages were resuspended in cell digestion buffer (Active Motif) and subjected to enzymatic digestion for 10 min at 37°C. The reaction was terminated by addition of 0.5 M EDTA. Ab were added to the sheared chromatin preparations and the mixture was incubated with Protein G magnetic beads (Active Motif) overnight at 4°C. The precipitated DNA-protein-Ab complexes were then washed and the cross linking was reversed by incubation at 65°C for 4 hr. Proteinase K was added to digest

| Table 1. Primers used in this study. |
|--------------------------------------|
| **Primer** | **Sequence (5’ to 3’)** |
| IL-10 forward | CCT GGC TCA GCA CTA T |
| IL-10 reverse | GCT ATT TTC ACA GGG GAG AA |
| GAPDH forward | CCT GAA CAG AAC AGC AAT GGC T |
| GAPDH reverse | GTC TGA CGG TGT CTT TGT CCT |
| IL-10 nucleosome 2 forward | GCA GAA GTT CAT TCC GAC CA |
| IL-10 nucleosome 2 reverse | GGC TCC TCC TCC CTC TTC TA |

doi:10.1371/journal.pone.0007589.t001
protein and DNA was subsequently purified using ethanol extraction, air dried, and redissolved in 100 μL H2O. The retrieved DNA was then subjected to real-time RT-PCR using promoter-specific primers.

Acknowledgments
We thank B. A. Butcher for enlightening discussion and critical review of this manuscript.

References
1. Montoya JG, Liesenfeld O (2004) Toxoplasmosis. Lancet 363: 1965–1976.
2. Petersen E, Liesenfeld O (2007) Clinical disease and diagnostics. In: Weiss LM, Kim K, eds. Toxoplasma gondii: The model apicomplexan: Perspectives and methods. Amsterdam: Academic Press. pp 61–100.
3. Birly AK, Shufesky WJ, Sodhi-Singga W, Morrell A, Denkers EY (2008) Dendritic cells expressing plasmacytoid marker PDCA-1 are Trojan horses during Toxoplasma gondii infection. J Immunol 181: 8445–8491.
4. Butcher BA, Kim L, Johnson PF, Denkers EY (2001) Toxoplasma gondii tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NF-kappa B. J Immunol 167: 2193–2201.
5. McKee AS, Dzierzinski F, Boes M, Roos DS, Pearce EF (2004) Functional inactivation of immature dendritic cells by the intracellular protozoan Toxoplasma gondii. J Immunol 173: 2632–2640.
6. Leng J, Butcher BA, Denkers EY (2009) Dysregulation of macrophage signal transduction by Toxoplasma gondii. Past progress and recent advances. Parasite Immunol. In press.
7. Lang C, Algner M, Beinert N, Gross U, Luder CG (2006) Diverse mechanisms employed by Toxoplasma gondii to inhibit IFN-gamma-induced major histocompatibility complex class II gene expression. Microbes Infect 8: 1994–2005.
8. Luder CGK, Algner M, Lang C, Bleicher N, Gross U (2003) Reduced expression of the inducible nitric oxide synthase after infection with Toxoplasma gondii facilitates parasite replication in activated murine macrophages. Internat J Parasitol 33: 833–844.
9. Kim L, Butcher BA, Denkers EY (2004) Toxoplasma gondii interferes with lipopolysaccharide-induced mitogen-activated protein kinase activation by mechanisms distinct from endotoxin tolerance. J Immunol 172: 3005–3010.
10. Lee CW, Bennouna S, Denkers EY (2006) Screening for Toxoplasma gondii-regulated transcriptional responses in lipopolysaccharide-activated macrophages. Infect Immun 74: 1916–1923.
11. Gilbert LA, Ravindran S, Turetsky JM, Boothroyd JC, Bradley PJ (2007) Toxoplasma gondii targets a protein phosphatase 2C to the nuclei of infected host cells. Eukaryot Cell 6: 73–83.
12. Saeij JP, Boyle JP, Coller S, Taylor S, Sibley LD, et al. (2006) Polymorphic secreted kinases are key virulence factors in toxoplasmosis. Science 314: 1780–1783.
13. Saeij JP, Coller S, Boyle JP, Jerome ME, White MW, et al. (2007) Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. Nature 445: 324–327.
14. Taylor S, Barragan A, Su C, Fux B, Fentress SJ, et al. (2006) A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen Toxoplasma gondii. Science 314: 1776–1780.
15. Arshie I, Kim DW, Batsche E, Pedron T, Mateescu B, et al. (2007) An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses. Nat Immunol 8: 47–56.
16. Gazzinelli RT, Wysocka M, Hoey S, Scharton-Kersten T, Cheever A, et al. (1996) In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent upon CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma, and TNF-alpha. J Immunol 157: 786–805.
17. Suzuki Y, Sher A, Yap G, Park D, Ellis Neyer I, et al. (2000) IL-10 is required for prevention of necrosis in the small intestine and mortality in both genetically resistant BALB/c and susceptible C57Bl/6 mice following peroral infection with Toxoplasma gondii. J Immunol 164: 5373–5382.
18. Wilson EH, Wille-Reece U, Dzierzinski F, Hunter CA (2005) A critical role for IL-10 in limiting inflammation during toxoplasmosis encephalitis. J Neuroimmunol 162: 63–74.
19. Luder CGK, Walter W, Beuerle B, Maeurer MJ, Gross U (2001) Toxoplasma gondii down-regulates MHIC class II gene expression and antigen presentation by murine macrophages via interference with nuclear translocation of STAT1alpha. Eur J Immunol 31: 1475–1484.
20. Kim SK, Fouts AE, Boothroyd JC (2007) Toxoplasma gondii dysregulates IFN-gamma-inducible gene expression in human fibroblasts: insights from a genome-wide transcriptional profiling. J Immunol 178: 5154–5163.
21. Gilbert IA, Ravindran S, Turetsky JM, Boothroyd JC, Bradley PJ (2007) Toxoplasma gondii targets a protein phosphatase 2C to the nuclei of infected host cells. Eukaryot Cell 6: 73–83.
22. Gazzinelli RT, Wysocka M, Hoey S, Scharton-Kersten T, Cheever A, et al. (1996) In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent upon CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma, and TNF-alpha. J Immunol 157: 786–805.
23. Saeij JP, Boyle JP, Coller S, Taylor S, Sibley LD, et al. (2006) Polymorphic secreted kinases are key virulence factors in toxoplasmosis. Science 314: 1780–1783.
24. Saeij JP, Coller S, Boyle JP, Jerome ME, White MW, et al. (2007) Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. Nature 445: 324–327.
25. Taylor S, Barragan A, Su C, Fux B, Fentress SJ, et al. (2006) A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen Toxoplasma gondii. Science 314: 1776–1780.
26. Arshie I, Kim DW, Batsche E, Pedron T, Mateescu B, et al. (2007) An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses. Nat Immunol 8: 47–56.
27. Hamon MA, Ravindran S, Turetsky JM, Boothroyd JC, Bradley PJ (2007) Toxoplasma gondii targets a protein phosphatase 2C to the nuclei of infected host cells. Eukaryot Cell 6: 73–83.
28. Wang Y, Curry HM, Zwilling BS, Lafuse WP (2005) Mycobacteria inhibition of IFN-gamma induces HLA-DR gene expression by up-regulating histone deacetylation at the promoter region in human THP-1 monocytic cells. J Immunol 174: 5687–5694.

Author Contributions
Conceived and designed the experiments: JL EYD. Performed the experiments: JL. Analyzed the data: JL EYD. Wrote the paper: EYD.