

## Article

# Modulation of Arg1, iNOS, and CD86 Gene Expression During Experimental *Toxoplasma gondii* Infection in Mice

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**Abstract:** Background: *Toxoplasma gondii* is an obligate intracellular protozoan that causes severe disease in humans and animals. Macrophage-mediated host responses are important to understand pathogenesis and protective immunity. Objectives: To determine the relative mRNA expression of Arg1, iNOS and CD86 in peripheral blood of *T. gondii* infected mice. Methodology: Fifty-two BALB/c mice were in the infected group and twenty BALB/c mice were in the uninfected control group. Tissue cysts were inoculated intraperitoneally to establish infection. Whole blood was collected at 40 days post-inoculation, total RNA extracted and cDNA synthesized. Transcript levels were quantified by RT-qPCR and normalized to a validated reference gene by the  $2^{-\Delta\Delta Ct}$  method. Results: All three genes were significantly upregulated (~2-fold) by infection with large effect sizes (Hedges'  $g = 0.81-1.01$ ): Arg1 ( $3.058 \pm 1.647$  vs.  $1.513 \pm 1.147$ ; 2.02-fold;  $p = 0.0031$ ), iNOS ( $3.159 \pm 1.967$  vs.  $1.685 \pm 0.895$ ; 1.87-fold;  $p = 0.0024$ ), CD86 ( $3.792 \pm 1.797$  vs.  $2.094 \pm 1.009$ ; 1.81-fold;  $p = 0.0008$ ). The ratio of Arg1 to iNOS was not different between controls and infected mice (0.90 vs. 0.97), suggesting co-induction of competing L-arginine pathways in a balanced manner, rather than classical M1/M2 polarization. Two-way ANOVA revealed a significant effect of infection (all  $p \leq 0.032$ ) but no effect of sex (all  $p \geq 0.37$ ) or infection  $\times$  sex interaction (all  $p \geq 0.96$ ). Conclusions *T. gondii* infection elicits a sex-independent co-regulated transcriptional signature in peripheral blood that integrates microbicidal, co-stimulatory and immunoregulatory myeloid pathways, and represents a promising minimally invasive biomarker panel for monitoring host immune status.

**Keywords:** *Toxoplasma gondii*; Arginase-1 (Arg1); inducible nitric oxide synthase (iNOS); murine model; macrophage polarization.

## Introduction

*Toxoplasma gondii* is an obligatory intracellular protozoan parasite that infects several warm-blooded species, such as mice and humans, and is a significant contributor to zoonotic diseases globally, and is estimated to chronically infect roughly one-third of the world's population[1,2]. Although

primary infection is typically self-limited or asymptomatic in immunocompetent hosts, the parasite persists lifelong as latent bradyzoite cysts, and reactivation or primary infection in immunocompromised individuals, as well as transplacental transmission during pregnancy, can produce severe ocular, neurological, and systemic disease[2]. Toxoplasmosis continues to be an active, unevenly distributed public health burden. The largest cumulative incidence was in eastern and western Europe and in groups with frequent animal interaction [1].

The murine model remains the primary experimental system for dissecting the host-parasite immunological interface in toxoplasmosis, as the kinetics of parasitaemia, tissue cyst formation and innate immune activation following a blood-borne (parenteral) challenge in mice recapitulates key features of acute human infection. The mononuclear phagocyte system is important to this response, with macrophages serving both as a replicative habitat for tachyzoites and as first-line effectors in parasite management. The functional path taken by a macrophage upon encountering *T. gondii* is often presented according to M1/M2 polarization: classically activated (M1) macrophages execute a pro-inflammatory, microbicidal program driven by interferon- $\gamma$  and Toll-like receptor signaling, while alternatively activated (M2) macrophages adopt an anti-inflammatory, tissue-remodelling phenotype linked to resolution of inflammation and, in the case of chronic infection, persistence of the pathogen [3].

Three markers are commonly employed to distinguish these polarization states at the transcriptional and protein level. Canonical M1 indicators are inducible nitric oxide synthase (iNOS, Nos2) and cluster of differentiation 86 (CD86). iNOS catalyzes the generation of a lethal effector chemical, nitric oxide, whereas CD86 is a co-stimulatory surface glycoprotein that is up-regulated on classically activated macrophages and dendritic cells involved in antigen presentation. Conversely, arginase-1 (Arg1) is a prototype M2 marker that competes with iNOS for the common substrate L-arginine and so inhibits nitric oxide production. It promotes polyamine and proline biosynthesis associated with tissue repair. Arg1 and iNOS represent two opposite arms of arginine metabolism. Thus, the relative expression of these two enzymes is often utilized as a quantitative measure of the M1/M2 balance in infected tissue. CD86 is an independent confirmation of the M1 skewing at the receptor level [3,4].

Recent studies have shown that the virulence mechanisms of *T. gondii* are not passively waiting for infection but are actively directed against this axis. Zhao et al. (2024) demonstrated that iNOS is specifically recruited to guanylate-binding protein (GBP)-decorated parasitophorous vacuoles in murine macrophages and is mechanistically needed for collapse of the intravacuolar membrane network and parasite elimination via nitric oxide-dependent nitration. This ties iNOS expression directly to a particular, chromosome-3 GBP-dependent killi. Moreover, An et al. (2025) demonstrated that the *T. gondii* kinase CDPK3 physically interacts with host Arginase-1 and drives infected microglia/macrophages toward an M1-biased CD86-high phenotype, suggesting that the parasite actively manipulates the Arg1-iNOS balance to modify the immunological milieu. *T. gondii* macrophage migration inhibitory factor homologue has exhibited a transitory increase in CD80, CD86 and iNOS with no and little change in Arg1 and other M2 markers [5].

Despite this progress, most mechanistic data on the regulation of Arg1, iNOS and CD86 come from in vitro macrophage cultures or localized tissue compartments such as brain, placenta or lung and few studies have systematically profiled the temporal and dose-dependent expression of all three markers in peripheral blood and lymphoid tissue after systemic (blood-borne) inoculation in an intact murine host. Blood-borne challenge models are vital to translational research since they reflect the pathway of tachyzoite dissemination during acute parasitaemia in natural and congenital infection and give a longitudinal, accessible readout of the systemic M1/M2 balance that solid-organ biopsies cannot. Therefore, a better understanding of how Arg1, iNOS, and CD86 transcripts and proteins co-vary during blood-borne infection would help determine whether the host initially mounts an M1-dominant response that is then offset by parasite-driven Arg1 induction and at what point this balance tips toward chronic cyst formation.

The present study extends this work by defining the gene expression profiles of Arg1, iNOS and CD86 in a murine model of *T. gondii* infection to describe the kinetics of M1/M2 macrophage

polarization markers during chronic systemic infection and associate this transcriptional signature to parasite burden and disease progression. The definition of this profile is expected to provide mechanistic insight into the means by which *T. gondii* modulates the human arginine-metabolic checkpoint to enhance its survival, and to identify possible sites where the M1/M2 balance can be therapeutically changed to promote more effective parasite clearance.

## Methodology

### Study period and location

The experimental investigation was conducted from February 2025 to January 2026. The parasite maintenance and other animal treatments were carried out in the animal house of College of Veterinary Medicine, Tikrit University, Tikrit, Salah Al-Din Governorate, Iraq. The Molecular Biology Laboratory, Department of Biology College of Science, Tikrit University was used to undertake molecular analyzes, including RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR).

### Parasite isolate and preparation of the inoculum

In the present study, a local cystogenic isolate of *T. gondii* was used. The isolate was isolated from the placenta of a woman with spontaneous abortion from Tikrit Teaching Hospital, Tikrit, Salah Al-Din Governorate, Iraq. All participants gave written informed consent, and the study protocol was approved by the Institutional Research Ethics Committee of Tikrit University (approval no. 12947, dated January 2025). Maternal infection was serologically confirmed by detection of antibodies against *T. gondii* IgM and IgG by immunochromatographic assay. The parasite was recovered by acid-pepsin digestion with slight modification described by Dubey [6]. The isolate was then maintained by serial intraperitoneal passage in laboratory mice, and tissue cysts were recovered from brain homogenates and counted microscopically with a haemocytometer. Each mouse was inoculated intraperitoneally with 100 tissue cysts in 0.2 mL sterile phosphate-buffered saline (PBS, pH 7.2).

### Experimental animales

A total of 52 BALB/c mice of both sexes (26 males and 26 females) aged 6 weeks and weighing 14-24 g were obtained from animal house, Tikrit city, Iraq. The animals were kept under standard laboratory conditions ( $22 \pm 2$  °C, 12-h light/dark cycle) with free access to food and water, and were acclimated for 7 days before the experiment. Mice were randomly divided into two groups: an infected group ( $n = 42$ ) in which each mouse was inoculated intraperitoneally with 100 tissue cysts suspended in 0.2 mL of sterile PBS, and a control group ( $n = 10$ ) that received an equal volume of sterile PBS by the same route.

### Blood sample collection

At 14 days post-infection (dpi), mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. Blood (~0.5–1.0 mL/animal) was obtained by terminal cardiac puncture [7] into sterile K<sub>2</sub>EDTA tubes. All samples were taken between 08:00 and 11:00 h to reduce the effect of circadian fluctuation in gene expression. Immediately after collection, 250 µL of whole blood were combined with 750 µL of TRIzol™ LS Reagent (Invitrogen, Thermo Fisher Scientific, USA) and lysates were either processed immediately or kept at –80 °C for further RNA extraction. Animals were euthanized via exsanguination while still under profound anesthesia. All inoculated mice were proven infected by microscopic identification of tissue cysts in brain squash preparations.

### RNA Extraction and cDNA Synthesis

Total RNA was isolated from whole blood samples with TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's protocols. The concentration and purity of RNA were evaluated by measuring the absorbance at 260 and 280 nm using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only the samples with A<sub>260</sub>/A<sub>280</sub> ratio of 1.8–2.1 were used for the next cDNA synthesis. A total of 1 µg of RNA was reverse transcribed to first-strand cDNA using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech) according to the manufacturer's procedure, and kept at –20 °C until use.

### Primer design and quantative real -time PCR(qRT-PCR)

Gene specific primers (Table 1) were designed based on *Mus musculus* reference mRNA sequences in NCBI RefSeq using Primer-BLAST [8]. All primers were 20 nucleotides in length with a GC content of 45-60% and generated short amplicons for SYBR Green detection, following current primer design guidelines [9]. Primer pairs were screened for hairpins, self-dimers and cross-dimers and verified in silico on the mouse RefSeq mRNA database. Oligonucleotides were synthesized by MacroGen (Seoul, South Korea), dissolved to 100  $\mu$ M in nuclease-free water and stored at -20 °C. Amplifications were performed with working solutions of 10  $\mu$ M.

Reverse transcription quantitative PCR (RT-qPCR) was performed on a *tepOnePlus™* RealTime PCR System (Applied Biosystems, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Each 20  $\mu$ L reaction contained 10  $\mu$ L of 2 $\times$  master mix, 0.5  $\mu$ L each of the forward and reverse primers ([10  $\mu$ M] stock; final concentration, [250 nM] each), 2  $\mu$ L of cDNA template (equivalent to [100 ng] of input RNA) and 7  $\mu$ L of nuclease-free water. The thermal cycling was performed with an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s and extension at 72°C for 30 s; fluorescence was read at the end of each extension step. After the last cycle, melting-curve analysis was conducted (65–95 °C at 0.5 °C increments, [5 s] per step) to ensure that only one specific product had been amplified and that no primer-dimers were present. Relative gene expression was determined using the  $2^{-\Delta\Delta CT}$  method [10,11].

**Table 1.** Primer sequences used for qRT-PCR analysis of target and reference genes in mice.

| Gene                         | Accession no. | Primer sequence (5'→3')                            | GC (%)   | Annealing (°C) | Product size (bp) |
|------------------------------|---------------|----------------------------------------------------|----------|----------------|-------------------|
| <i>Arg1</i>                  | NM_007482     | F: GGGGATTGGCAAGGTGATGG<br>R: AGATAGGCCTCCCAGAACCG | 60<br>60 | 58             | [142]             |
| iNOS ( <i>Nos2</i> )         | NM_010927     | F: AGATCGAGCCCTGGAAGACC<br>R: CTGACCCGTGAAGCCATGAC | 60<br>60 | 58             | [139]             |
| CD86 ( <i>Cd86</i> )         | NM_019388     | F: CCATGGGCTTGGCAATCCTT<br>R: CACGGCAGATATGCAGTCCC | 55<br>60 | 58             | [103]             |
| 18S rRNA<br>( <i>Rn18s</i> ) | NR_003278     | F: GCTCGCTCCTCTCCTACTTG<br>R: TCTGATAAATGCACGCATCC | 60<br>45 | 58             | 103               |

F, forward primer; R, reverse primer; GC, guanine–cytosine content; bp, base pairs; iNOS, inducible nitric oxide synthase; 18S rRNA

### Ethical approval

All treatments involving animals were authorized by the Animal Ethics Committee, College of Veterinary Medicine, Tikrit University, Iraq (permission No. 12947; January 2025) and were performed in line with the Guide for the Care and Use of Laboratory Animals [13]. The study was reported according to ARRIVE 2.0 guidelines [14]. Efforts were made to limit the suffering of animals and to decrease the number of animals used.

### Statistical Analysis

Values are expressed as mean  $\pm$  SD. Statistical comparisons were carried out on  $\Delta Ct$  values and results are provided as fold changes [12]. Normality was tested using the Shapiro–Wilk test. Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or the Kruskal–Wallis test followed by Dunn's post hoc test when data were not normally distributed. Analyses were conducted using [GraphPad Prism version 9 (GraphPad Software, USA)].  $P \leq 0.05$  was regarded statistically significant.

## Result

### 3.1. Overview of the transcriptional response in peripheral blood

Relative transcript levels of *Arg1*, *iNOS* (encoded by *Nos2*) and *CD86* were quantified by quantitative reverse-transcription PCR (qRT-PCR) in peripheral blood collected from 40T. gondii-

infected mice (20 males and 20 females) and 20 uninfected control mice (10 males and 10 females). Descriptive statistics and the results of the between-group comparisons are summarized in Table 1. Infection was associated with a significant up-regulation of all three transcripts, with mean fold increases of 1.81–2.02 relative to uninfected controls and large standardized effect sizes (Hedges'  $g = 0.81$ – $1.01$ ) (Table 1; Figures 1–3).

#### Expression of Arg1

Arg1 expression was significantly higher in infected mice compared to uninfected controls ( $3.058 \pm 1.647$  vs.  $1.513 \pm 1.147$ , mean  $\pm$  SD), a 2.02-fold increase (mean difference = 1.545; 95% CI: 0.580–2.510; Welch's  $t = 3.322$ ,  $df = 21.73$ ;  $p = 0.0031$ ) (Figure 1A). The median changed from 1.628 (interquartile range [IQR]: 0.322–2.479) in controls to 3.250 (IQR: 1.398–4.125) in infected animals, with the upward trend happening throughout the whole distribution. The data were stratified by sex and Arg1 expression was considerably increased in infected compared to control males ( $2.790 \pm 1.757$  vs.  $1.269 \pm 1.147$ ; 2.20-fold;  $p = 0.0468$ ) (Figure 1B) and in infected relative to control females ( $3.326 \pm 1.539$  vs.  $1.757 \pm 1.222$ ; 1.89-fold;  $p = 0.0454$ ) (Figure 1C).

#### Expression of iNOS

iNOS transcripts were almost 2-fold higher in infected mice blood ( $3.159 \pm 1.967$  vs.  $1.685 \pm 0.895$ ; 1.87-fold; mean difference = 1.474; 95% CI: 0.563–2.385;  $t = 3.287$ ,  $df = 34.13$ ;  $p = 0.0024$ ) (Figure 2A). The median increased from 1.427 (IQR: 1.124–2.292) to 3.166 (IQR: 1.089–5.043). Interestingly, the infected group had a much bigger inter-individual variability (range: 0.177 – 6.273) than the control group (range: 0.539 – 3.607). The variances of the two groups were substantially different ( $F = 4.835$ ,  $p = 0.0172$ ), which justifies the adoption of Welch's correction. In the sex-stratified study, iNOS expression was considerably enhanced in infected men ( $3.061 \pm 1.888$  vs.  $1.612 \pm 0.785$ ; 1.90-fold;  $p = 0.0249$ ) (Figure 2B). Females similarly infected showed a comparable magnitude increase ( $3.257 \pm 2.101$  vs.  $1.758 \pm 1.082$ ; 1.85-fold) that just missed significance ( $p = 0.0547$ ; 95% CI of the difference:  $-0.035$  to  $3.032$ ) (Figure 2C).

#### Expression of CD86

Of the three genes tested, CD86 demonstrated the largest variation across groups. Expression was considerably higher in infected animals than in controls ( $3.792 \pm 1.797$  vs.  $2.094 \pm 1.009$ ; 1.81-fold; mean difference = 1.698; 95% CI: 0.775–2.621;  $t = 3.770$ ,  $df = 27.76$ ;  $p = 0.0008$ ), and the median increased from 2.488 (IQR: 1.087–2.926) to 4.117 (IQR: 2.472–5.285) (Figure 3A). This upregulation was confirmed in both sexes, a substantial increase in males ( $3.800 \pm 2.117$  vs.  $2.093 \pm 0.861$ ; 1.82-fold;  $p = 0.0184$ ) (Figure 3B) and in females ( $3.784 \pm 1.482$  vs.  $2.094 \pm 1.245$ ; 1.81-fold;  $p = 0.0358$ ) (Figure 3C).

#### Influence of host sex

To test for a modulation of the transcriptional response by host sex, the data were subsequently analyzed by two-way ANOVA with infection status and sex as factors. There was a significant main effect of infection for Arg1 ( $F(1, 38) = 7.42$ ,  $p = 0.0097$ ), iNOS ( $F(1, 38) = 4.96$ ,  $p = 0.032$ ) and CD86 ( $F(1, 38) = 7.64$ ,  $p = 0.0088$ ), but neither the main effect of sex (all  $p \geq 0.37$ ) nor the infection  $\times$  sex interaction (all  $p \geq 0.96$ ) was significant for any gene. There were no differences in Arg1 ( $p = 0.366$ ), iNOS ( $p = 0.783$ ) or CD86 ( $p = 0.980$ ) expression between infected males and females, consistent with the comparisons between infected males and females. Thus, the degree of induction was very similar between both sexes (fold changes 1.82–2.20 in males and 1.81–1.89 in females; Table 1).

**Table 1.** Relative expression of Arg1, iNOS and CD86 in peripheral blood of *Toxoplasma gondii*-infected and uninfected control mice.

| Gene | Subset (n)      | Control (mean $\pm$ SD) | Infected (mean $\pm$ SD) | Fold Change | Mean difference (95% CI) | Welch's $t$ (df) | $p$ value     | Hedges' $g$ |
|------|-----------------|-------------------------|--------------------------|-------------|--------------------------|------------------|---------------|-------------|
| Arg1 | All (20/40)     | $1.513 \pm 1.147$       | $3.058 \pm 1.647$        | 2.02        | 1.545 (0.580 to 2.510)   | 3.322 (21.73)    | <b>0.0031</b> | 0.98        |
|      | Males (10/20)   | $1.269 \pm 1.147$       | $2.790 \pm 1.757$        | 2.20        | 1.521 (0.026 to 3.016)   | 2.251 (10.51)    | <b>0.0468</b> | 0.89        |
|      | Females (10/20) | $1.757 \pm 1.222$       | $3.326 \pm 1.539$        | 1.89        | 1.569 (0.040 to 3.098)   | 2.347 (8.39)     | <b>0.0454</b> | 1.02        |
|      |                 |                         |                          |             |                          |                  |               |             |

|             |         |               |         |      |                   |         |               |      |
|-------------|---------|---------------|---------|------|-------------------|---------|---------------|------|
| <i>iNOS</i> | All     | 1.685 ± 0.895 | 3.159 ± | 1.87 | 1.474             | 3.287   | <b>0.0024</b> | 0.81 |
|             | (20/40) |               | 1.967   |      | (0.563 to 2.385)  | (34.13) |               |      |
|             | Males   | 1.612 ± 0.785 | 3.061 ± | 1.90 | 1.449             | 2.463   | <b>0.0249</b> | 0.81 |
|             | (10/20) |               | 1.888   |      | (0.207 to 2.691)  | (16.85) |               |      |
|             | Females | 1.758 ± 1.082 | 3.257 ± | 1.85 | 1.499             | 2.098   | <b>0.0547</b> | 0.74 |
|             | (10/20) |               | 2.101   |      | (-0.035 to 3.032) | (13.85) |               |      |
| <i>CD86</i> | All     | 2.094 ± 1.009 | 3.792 ± | 1.81 | 1.698             | 3.770   | <b>0.0008</b> | 1.01 |
|             | (20/40) |               | 1.797   |      | (0.775 to 2.621)  | (27.76) |               |      |
|             | Males   | 2.093 ± 0.861 | 3.800 ± | 1.82 | 1.706             | 2.607   | <b>0.0184</b> | 0.85 |
|             | (10/20) |               | 2.117   |      | (0.326 to 3.087)  | (17.10) |               |      |
|             | Females | 2.094 ± 1.245 | 3.784 ± | 1.81 | 1.689             | 2.526   | <b>0.0358</b> | 1.13 |
|             | (10/20) |               | 1.482   |      | (0.144 to 3.235)  | (7.91)  |               |      |

Values are relative expression levels. n = number of control/infected mice in each subset. Fold change = mean of the infected group ÷ mean of the control group. Mean difference = infected – control, with its 95% confidence interval (CI). *p* values were obtained with a two-tailed unpaired *t*-test with Welch's correction; values in bold indicate statistical significance (*p* ≤ 0.05). Hedges' *g* = bias-corrected standardized mean difference.

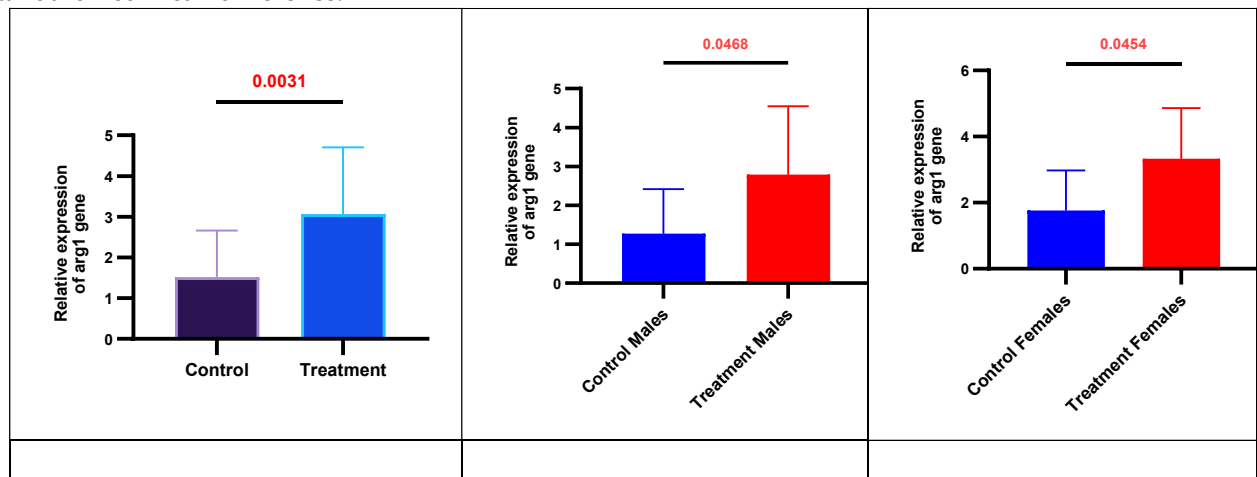


**Figure 1.** Relative expression of *Arg1* in peripheral blood of *T. gondii*-infected and uninfected control mice. (A) All animals (control, n = 20; infected, n = 40); (B) males (control, n = 10; infected, n = 20); (C) females (control, n = 10; infected, n = 20). Bars represent mean ± SD. The *p* value shown above each comparison was obtained with a two-tailed unpaired *t*-test with Welch's correction.

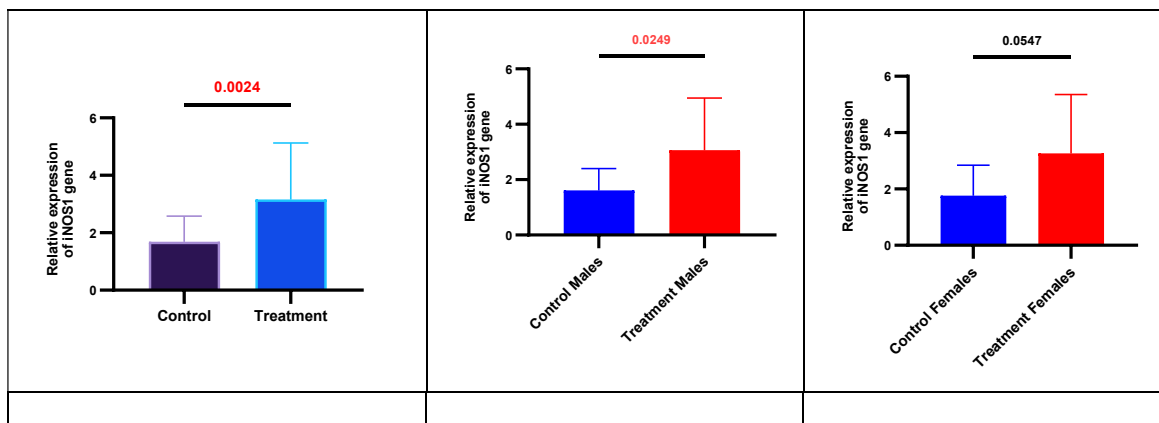


**Figure 2.** Relative expression of *iNOS* in peripheral blood of *T. gondii*-infected and uninfected control mice. (A) All animals (control,  $n = 20$ ; infected,  $n = 40$ ); (B) males (control,  $n = 10$ ; infected,  $n = 20$ ); (C) females (control,  $n = 10$ ; infected,  $n = 20$ ). Bars represent mean  $\pm$  SD. The  $p$  value shown above each comparison was obtained with a two-tailed unpaired  $t$ -test with Welch's correction.

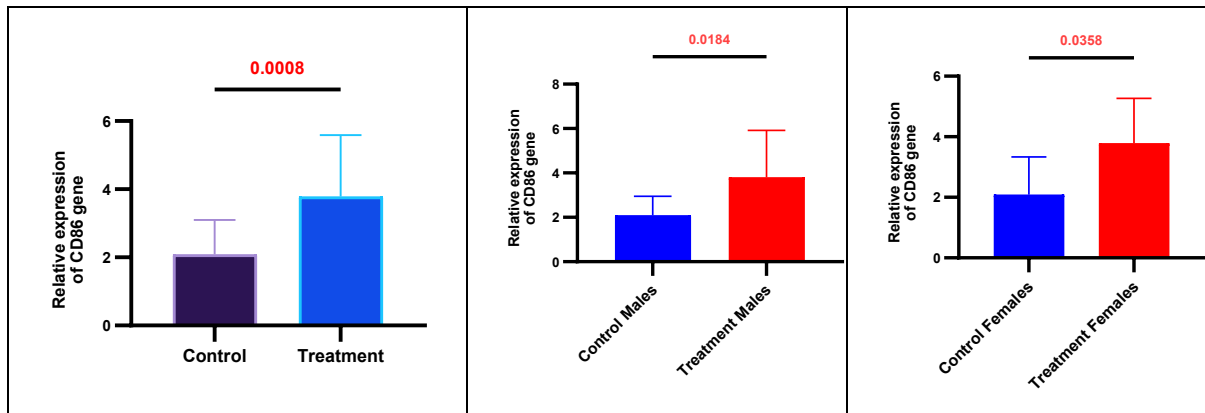


**Figure 3.** Relative expression of CD86 in peripheral blood of *T. gondii*-infected and uninfected control mice. (A) All animals (control,  $n = 20$ ; infected,  $n = 40$ ); (B) males (control,  $n = 10$ ; infected,  $n = 20$ ); (C) females (control,  $n = 10$ ; infected,  $n = 20$ ). Bars represent mean  $\pm$  SD. The  $p$  value shown above each comparison was obtained with a two-tailed unpaired  $t$ -test with Welch's correction.

## Discussion

Monocytes, macrophages and dendritic cells are the main host cells infected by *T. gondii* and the major effectors limiting its replication, and the functional state of these cells is usually assessed by the reciprocal L-arginine-metabolizing enzymes inducible nitric oxide synthase (iNOS) and arginase-1, together with co-stimulatory molecules such as CD86 [15– 18]. However, most current knowledge regarding these markers is derived from in vitro-infected cells or tissue-resident populations. Peripheral blood, which is easily accessible and can be sampled repeatedly, integrates the responses of circulating monocytes, granulocytes, dendritic-cell precursors and lymphocytes, has been studied less extensively, although blood transcriptional profiling has unveiled infection-associated immune dysregulation in seropositive humans [19]. In the present investigation, *T. gondii* infection was linked with a ~2-fold concurrent up-regulation of Arg1, iNOS, and CD86 in blood (high effect sizes) of similar magnitude in male and female mice. These data together suggest that infection results in a systemic stimulation of myeloid and antigen presenting cells with a concurrent activation of pro-inflammatory (iNOS, CD86) and counter-regulatory (arginase-1) pathways.

The induction of iNOS is consistent with the interferon- $\gamma$  (IFN- $\gamma$ )-dominated type 1 response that forms the backbone of host resistance to the parasite. Interleukin-12 produced by dendritic cells and macrophages promotes IFN- $\gamma$  production by natural killer cells and T cells, and IFN- $\gamma$  in turn activates STAT1-dependent transcription of *Nos2* in myeloid cells [15, 17]. The resultant nitric oxide (NO) and reactive nitrogen species have a direct anti-parasitic function. iNOS is enriched at guanylate-binding protein (GBP)-coated parasitophorous vacuoles in IFN- $\gamma$ -activated macrophages, where it nitrates the vacuole and collapses the intravacuolar network, and myeloid-specific deletion of iNOS increases parasite burden and reduces host survival, as recently shown by [19]. In mice infected with the type II ME49 strain, serum IFN- $\gamma$ , TNF and IL-12 are still high at 30 and 60 days post-infection [20], demonstrating that a cytokine environment capable of maintaining *Nos2* transcription in circulating leukocytes remains far past the acute phase. NO has a dual role: excessive output of NO can inhibit lymphocyte proliferation and contribute to immunopathology, and iNOS is mostly dispensable for parasite management in acute infection, but is important in chronic infection [17]. The considerable heterogeneity in iNOS expression between infected animals (range 0.18–6.27, with statistically different

variances) shows that the strength of IFN- $\gamma$ -driven activation differed between individuals, possibly reflecting changes in parasite burden or in the kinetics of the response. This heterogeneity is in agreement with the observation that the strength and timing of early IFN- $\gamma$  responses vary greatly amongst parasite genotypes and correlate with disease prognosis [21].

Arginase-1(Arg1), a hallmark of alternatively activated (M2-like) macrophages, is upregulated by IL-4/IL-13 via STAT6 and IL-10 via STAT3 and C/EBP $\beta$  signaling, rendering the concurrent elevation of Arg1 particularly noteworthy [22]. Depending on parasite genotype, secreted effectors such as ROP16 and GRA15 modulate host STAT3/6 and NF- $\kappa$ B signaling pathway. In type II strains, GRA15 promotes classically activated (M1-like) macrophages polarization, although its overall impact *in vivo* remains limited [21]. Furthermore, An *et al.* (2025) demonstrated that the calcium-dependent protein kinase TgCDPK3 of the type II ME49 strain directly interacts with host Arg1, suppressing its expression and enzymatic activity while promoting M1 polarization characterized by elevated iNOS and CD86 levels. Against this backdrop, the systemic elevation in Arg1 is likely a host-derived counter-regulatory reaction rather than parasite-driven M2 polarization. Myeloid-derived suppressor cells (MDSCs)—immature myeloid cells that accumulate during chronic inflammation and infection to suppress T-cell responses by Arg1 and iNOS—represent a plausible cellular source [23].

Some studies showed the upregulation of Arg1, a marker of alternatively activated (M2-like) macrophages, by IL-4/IL-13 via STAT6 and by IL-10 via STAT3 and C/EBP $\beta$ . Secreted effectors such as ROP16 and GRA15 modulate STAT3/6 and NF- $\kappa$ B signaling in a parasite genotype-dependent manner[22]. In type II strains, GRA15 induces classically activated (M1-like) macrophages, but has a minimal effect *in vivo* [24]. In this context, the systemic increase of Arg1 is more likely to be a host-derived counter-regulatory reaction than a parasite-driven M2 polarization. Macrophage polarization toward the M1 phenotype, together with upregulation of iNOS and CD86, has been attributed to the type II ME49 calcium-dependent protein kinase TgCDPK3, which binds host arginase-1 and reduces both its expression and its activity [25].

CD86 is rapidly upregulated on activated monocytes, dendritic cells, and B cells, acting as a key regulator of T-cell activation by driving CD28-dependent priming and undergoing CTLA-4-mediated trans-endocytosis [25]. In *T. gondii* infection, CD86 is elevated on M1-like macrophages and decidual dendritic cells, alongside CD80/CD86-linked induction of indoleamine 2,3-dioxygenase in dendritic cells [26]. Thus, the increase in CD86 transcripts in blood probably reflects the activation of circulating antigen-presenting cells. This is consistent with the priming and persistence of IFN- $\gamma$ -producing T-cell responses and feedback via IFN- $\gamma$  on iNOS expression.

These findings help explain the concurrent induction of CD86 and iNOS observed here. During *T. gondii* infection, circulating myeloid cells play a dual role: GRA28-driven macrophages adopt hypermigratory, dendritic cell-like phenotypes, CCR2<sup>+</sup> monocytes traffic to cerebral replication sites, and infected monocytes and dendritic cells act as "Trojan horses" to cross biological barriers [26]. Circulating CD86<sup>+</sup> myeloid cells may thus function both as immune effectors and as vehicles for parasite dissemination. Notably, CD86 differed most strongly between groups ( $p = 0.0008$ ; Hedges'  $g = 1.01$ ), highlighting co-stimulatory activation as a robust systemic hallmark and a promising blood biomarker of infection.

In this study we show a significant transcriptional upregulation of classical M1 pro-inflammatory markers (iNOS1 and CD86) and the alternative M2 regulatory marker (Arg1) in response to *T. gondii* infection ( $p = .0024$ ,  $p = .0008$  and  $p = .0031$ , respectively) indicating concurrent activation of microbicidal mechanisms and tissue reparative pathways [18]. Stratification by host sex showed a statistically significant induction of iNOS1 in infected male mice ( $3.061 \pm 1.888$  vs.  $1.612 \pm 0.785$  in controls,  $p = .0249$ ). Infected females showed higher mean transcript levels ( $3.257 \pm 2.101$  vs.  $1.758 \pm 1.082$ , close to significance,  $p = .0547$ ) owing to greater inter-individual variation. These results are consistent with known patterns of sexual dimorphism in mouse toxoplasmosis. Males often show a more robust early innate response under the control of the IFN- $\gamma$ /STAT1/iNOS axis to control parasite replication via NO production [27], while females often show a more heterogeneous and dysregulated inflammatory response associated with severe immunopathology [26]. At the same time, infected males ( $2.790 \pm 1.757$ ,  $p = .0468$ ) and females ( $3.326 \pm 1.539$ ,  $p = .0454$ ) had a significant increase in Arg1

expression with females showing increased baseline and post infection transcript levels. This correlates with the observation that females are more susceptible than males to tissue cyst formation, potentially due to increased expression of Arg1 and differential expression of iNOS1 in females. Arg1 directly competes with iNOS1 for the common substrate L-arginine to generate polyamines that are required for intracellular parasite replication [28]. This sex-dependent divergence is mainly mediated by female sex steroids, i.e., 17 $\beta$ -estradiol and progesterone, that regulate macrophage polarization through estrogen receptor alpha (ER $\alpha$ ) and serum-glucocorticoid regulated kinase 1 (SGK1) signaling, respectively. Moreover, X-chromosome-linked regulators like miR-223-3p inhibit uniform M1 polarization to favor an M2-skewed phenotype [28]. Conversely, there was a significant up-regulation of the co-stimulatory molecule CD86 both in infected males ( $p = .0184$ ) and females ( $p = .0358$ ), to a similar extent, indicating that macrophages' antigen-presenting capacity and subsequent T-cell co-stimulation are not affected in either sex [5]. Collectively, these results indicate that *T. gondii* induces a strong co-stimulatory activation that is independent of sex, but the intracellular enzymatic competition between iNOS1 and Arg1 is mainly governed by the biological sex of the host, and therefore shapes sex-dependent disease outcomes [29].

This study has some limitations. Because expression was measured in whole blood, the cellular origins of the transcripts are unknown, and the increases may partly reflect changes in leukocyte composition (e.g., monocytosis) rather than per-cell up-regulation. Only mRNA was measured. Protein levels, arginase activity and NO metabolites (nitrite/nitrate) were not measured. Additionally, single time point sampling precludes conclusions regarding response kinetics, the control group was small ( $n = 10$  per sex) and limited the power of sex-stratified comparisons, and transcript levels were not correlated with parasite burden. Longitudinal studies that combine flow-cytometric phenotyping of circulating myeloid subsets (CD11b+Ly6Chi monocytic and CD11b+Ly6G+ granulocytic cells, including MDSCs), functional arginase and NO assays, and tissue parasite quantification are needed to define the cellular basis and prognostic value of this signature.

## Conclusion

*T. gondii* infection induced a coordinated, approximately two-fold up-regulation of Arg1, iNOS and CD86 in the peripheral blood of mice, with no evidence of sex-dependent modulation. The concomitant activation of the iNOS and arginase-1 axes, together with the increased co-stimulatory potential, reflects a balanced systemic myeloid activation, in which antimicrobial and counter-regulatory mechanisms are both at play. These blood-based markers may provide a practical minimally-invasive tool to monitor host immune status and to evaluate the efficacy of conventional treatments in experimental toxoplasmosis.

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