UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO

Gabriel Rabello de Abreu Cabral

Óxido nítrico sintase induzida versus arginase 1 em distintas linhagens de macrófagos infectadas com *Toxoplasma gondii*

Campos dos Goytacazes, RJ- Brasil
Março de 2019.

UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO

Gabriel Rabello de Abreu Cabral

Óxido nítrico sintase induzida versus arginase 1 em distintas linhagens de macrófagos infectadas com *Toxoplasma gondii*

Tese apresentada ao Centro de Biociências e Biotecnologia da Universidade Estadual do Norte Fluminense Darcy Ribeiro como parte das exigências para obtenção do Título de Doutor em Biociências e Biotecnologia.

Orientador: Dr. Renato Augusto DaMatta

Campos dos Goytacazes, RJ- Brasil
Março de 2019.

FICHA CATALOGRÁFICA
UENF - Bibliotecas
Elaborada com os dados fornecidos pelo autor.

C117 Cabral, Gabriel Rabello de Abreu.

Óxido nítrico sintase induzida versus arginase 1 em distintas linhagens de macrófagos infectadas com Toxoplasma gondii / Gabriel Rabello de Abreu Cabral. - Campos dos Goytacazes, RJ, 2019.

162 f. : il.
Bibliografia: 145 - 162.

Tese (Doutorado em Biociências e Biotecnologia) - Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Biociências e Biotecnologia, 2019.
Orientador: Renato Augusto da Matta.

1. Toxoplasma gondii. 2. Fatores de virulência. 3. Macrófagos. 4. Arginase 1. 5. Óxido Nítrico Sintase Induzida. I. Universidade Estadual do Norte Fluminense Darcy Ribeiro. II. Título.

CDD - 570

UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO

Gabriel Rabello de Abreu Cabral

Óxido nítrico sintase induzida versus arginase 1 em distintas linhagens de macrófagos infectadas com *Toxoplasma gondii*

Tese apresentada ao Centro de Biociências e Biotecnologia da Universidade Estadual do Norte Fluminense Darcy Ribeiro como parte das exigências para obtenção do Título de Doutor em Biociências e Biotecnologia.

Aprovada em 26 de março de 2019.

Banca examinadora:

_____

Prof. Dra. Alba Lucinia Peixoto Rangel
Professora Associada - LBR/CBB/UENF

_____

Prof. Dr. Milton Masahiko Kanashiro
Professor Associado - LBR/CBB/UENF

_____

Prof. Dra. Suzana Passos Chaves
Professora Adjunta - UFRJ/Macaé

_____

Prof. Dr. Renato Augusto DaMatta
Orientador – Professor Associado- LBCT/CBB/UENF

“Eu te amarei, ó Senhor, fortaleza minha. O Senhor é o meu rochedo, e o meu lugar forte, e o meu libertador; o meu Deus, a minha fortaleza, em quem confio; o meu escudo, a força da minha salvação, e o meu alto refúgio”. Salmos 18:1-2.

Agradecimentos

- Agradeço a Deus por me dar saúde, força e determinação para alcançar meus objetivos e sonhos. Tudo que consegui alcançar até hoje foi através da sua Graça, pois Deus nunca desampara seu filho;
- Agradeço ao meu pai Osmani Cabral de Matos, minha mãe Gisela Maria Rabello de Abreu Cabral de Matos e minha irmã Carolina de Fátima Rabello de Abreu Cabral por todo amor e apoio na busca dos meus sonhos. São minha fortaleza em todos os momentos. Agradeço por terem me dado força quando fui para os EUA realizar parte desta Tese;
- Agradeço aos meus avós que mesmo não estando entre nós, estão olhando por mim a todo momento;
- Agradeço a minha noiva Lediana Cardoso Viana por todo amor, carinho, amizade e companheirismo. Me apoiou na busca do meu sonho quando fui para os EUA e sempre ficou ao meu lado, mesmo nos momentos de tristeza e angústia pela distância. Sou eternamente grato por tudo que fez por mim e ainda faz;
- Agradeço ao meu orientador, mentor e amigo Prof. Dr. Renato Augusto DaMatta que desde a Iniciação Científica me orienta na vida acadêmica. Sempre presente, despertou o interesse pela busca de conhecimento e pela pesquisa que me levaram até o doutorado. Agradeço também a Andréa Carvalho César que junto ao Renato me apoiaram e foram minha fortaleza quando estava nos EUA, longe daqueles que amo. Foram uma segunda família. Pessoas na qual tenho enorme carinho, admiração e gratidão por tudo que fizeram por mim;
- Agradeço ao Prof. Dr. David L. Sibley, da *Washington University in Saint Louis - School of Medicine* - EUA, pela oportunidade de trabalhar com um dos melhores pesquisadores do mundo na área de *Toxoplasma gondii*, pela colaboração nesta Tese e por todos os ensinamentos. Também agradeço muito a todo seu grupo de trabalho, em especial a Jennifer Barks, Sumit Kumar, Kevin Brown, Lisa Drewry e Zi T. Wang pela amizade e ajuda nos experimentos;
- Agradeço ao colaborador Prof. Dr. Gustavo Lazzaro Rezende pela colaboração neste trabalho. Tudo que aprendi na biologia molecular foi graças aos seus ensinamentos;

- Agradeço a Ms. Helena Carolina Martins Vargas pela amizade e ajuda com os experimentos de PCR;
- Agradeço as técnicas do LBCT, em especial a Darly Grativol, Adriana Martins, Rosemary Maciel, Giovana Moraes, Beatriz Ribeiro, Márcia Adriana e Luciana Timóteo pelo companheirismo, amizade e ensinamentos;
- Agradeço aos funcionários do Biotério, em especial ao Fábio Conceição, Paulo Sérgio, Maurício de Oliveira e Rodrigo de Souza por toda ajuda e ensinamentos;
- Agradeço aos colegas de laboratório Joaquim Teixeira Xavier Junior, Thiago Torres de Aguiar, Natália de Souza Almeida, Gisela Garcia, Tâmara Carolina Gomes Ribeiro, Frederico Nolasco pela amizade e companheirismo;
- Agradeço a Joaquim Teixeira Xavier Junior pela amizade e oportunidade de coorientação de sua monografia e ajuda no desenvolvimento desta Tese. Sem sua ajuda, certamente teria sido mais difícil alcançar os objetivos deste trabalho;
- Agradeço aos amigos Dr. João Cláudio, Ms. Luciana Lemos, Dra. Fernanda Silva de Souza por todos ensinamentos, companheirismo e amizade;
- Agradeço aos amigos Vinícius Bragança, Bernardo Mota, Matheus Jorge, Guilherme Guzzo, André Gomes, Luiz Felipe, Lucas Rangel, Diego Monteiro, Lucas Batista, David Gomes, Inácio Pestana, Julia Fardin, Bárbara Coelho, Anna Hautequestt e Luisa Sorrentino pela amizade e companheirismo ao longo de todos esses anos;
- Agradeço ao CNPq, FAPERJ, UENF que disponibilizaram a verba necessária para que os experimentos fossem feitos;
- O presente trabalho foi realizado com o apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES-Brasil- Código de Financiamento 001;
- Agradeço a CAPES pelo financiamento da bolsa de doutorado sanduíche no exterior (PDSE) no período de 6 meses, processo 88881.132450/2016-01;
- Agradeço ao *National Institutes of Health* (NIH) pelo suporte financeiro no período de 6 meses, processo AI118426.

Sumário

Lista de abreviaturas

ARG1: Arginase 1

ASP5: Aspartil protease 5

DMEM*:* Meio Eagle Modificado por Dulbecco´s (do inglês *Dulbecco´s Modified Eagle Medium*)

dsDNA: dupla fita de DNA

DSB: quebra da dupla fita

eNOS: Óxido nítrico sintase endotelial

VF: Fatores de virulência

GRA: Proteínas dos grânulos densos

HR: recombinação homóloga

IFN-γ: Interferon-gamma

IL: Interleucina

iNOS: Óxido nítrico sintase induzida

IRGs: GTPases relacionadas com a imunidade

JAK1/2: Janus Quinase 1 e 2

Δ*ku*80: *knockout* para Ku80

LPS: Lipopolissacarídeos

MIC: Proteínas das micronemas

MYR1: proteína de regulação c-myc 1

NF-κB: Fator Nuclear Kappa B

nNOS: Óxido nítrico sintase neuronal

NOSs: Óxido nítrico sintases

NHEJ: junção final não-homóloga

PBS: Tampão Fosfato-Salino (cloreto de sódio tamponado com fosfato de sódio 0,1 M)

PV: Vacúolo parasitóforo

PVM: Membrana do vacúolo parasitóforo

PS: Fosfatidilserina

RONs: Proteínas do pescoço das róptrias

ROPs: Proteínas do bulbo

STAT: Transdutor de Sinal e Ativador de Transcrição

SAGs: Antígeno de superfície 1

TGF-β: Fator de crescimento transformante β

TgIST: inibidor de transcrição de STAT

TLRs: Receptores do tipo Toll

Lista de figuras e tabelas

Figura 2 artigo: Immunofluorescence detection of iNOS in activated J774-A1 macrophages infected with *T. gondii*. (A) Detection of iNOS (green) in non-infected (Control) and in *T. gondii* (red) infected cells (DAPI - blue) at 2, 6, and 24 h post-infection. Scale bar = 10 mm. (B) Analysis of the proportion of iNOS positive or negative macrophages in non-infected (Control) and *T. gondii* infected cells at 2 and 24 h post-infection. Mean ± SEM (n = 4 experiments, each with 8 replicates). (C) Western blot detection of iNOS expression in non-infected (Control) and *T. gondii* infected (Infected) cells. β-actin was used as loading control. (D) Densitometry of western blots normalized to b-actin at 2 h post-infection. Mean ± SD (n = 3 experiments, each with 1 replicate). *P ≤ 0.05, two-way ANOVA with Tukey post-test, n.s (not significant).	
Figura 3 artigo: Immunofluorescence detection of iNOS in activated RAW 264.7 macrophages infected with *T. gondii*. (A) Detection of iNOS (green) in non-infected (Control) and in *T. gondii* (red) infected cells (DAPI - blue) at 2, 6, and 24 h post-infection. Scale bar - 10 mm. (B) Analysis of the proportion of iNOS positive or negative macrophages in non-infected (Control) and *T. gondii* infected cells at 2 and 24 h post-infection. Mean ± SEM (n = 4 experiments, each with 8 replicates). (C) Western blot detection of iNOS expression in non-infected (Control) and *T. gondii* - infected (Infected) cells at different time intervals post-infection. β-actin was used as loading control. (D) Densitometry of western bolts normalized to b-actin at 2 h post-infection. Mean ± SD (n = 3 experiments, each with 1 replicate), n.s (not significant).	
Figura 4 artigo: Analysis of ROP and ASP5, MYR1, and IST deletion mutants on the ability of *T. gondii* to inhibit NO production (nitrite in mM) of activated macrophages. (A) NO production of non-infected (Control) or activated J774-A1 cells infected with parental (RH1ku80 strain) or various ROP deletion strains of *T. gondii* at 24 h post-infection. Mean ± SEM (n = 3 experiments, each with 12 replicates). ****P ≤ 0.0001, one-way ANOVA with Tukey post-test. (B) NO production of non-infected (Control) or activated J774-A1 cells infected with parental (RH1ku80 strain) or Δasp5, Δmyr1, or Δist mutant strains of *T. gondii* at 24 h post-infection. Mean ± SEM (n = 3 experiments, each with 12 replicates). ****P ≤ 0.0001, one-way ANOVA with Tukey post-test. (C) NO production of non-infected (Control) or activated RAW 264.7 cells infected with parental (RHΔku80 strain) or various ROP deletion strains of *T. gondii* at 24 h post-infection. Mean ± SEM (n = 3 experiments, each with 12 replicates). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, one-way ANOVA with Tukey post-test. (D) NO production of non-infected (Control) or activated RAW 264.7 cells infected with parental (RH1ku80 strain) or Δasp5, Δmyr1, and Δist mutant strains of *T. gondii* at 24 h post-infection. Mean ± SEM (n = 3 experiments, each with 12 replicates). *P ≤ 0.05, **P ≤ 0.01, one-way ANOVA with Tukey post-test.	
Figura 1 manuscrito: Immunofluorescence detection of ARG1 expression (red) in non-activated (M0) or macrophages (DAPI - blue) activated with IL-4 and 8-Br-AMPc (M2) at 24 h post-activation in RAW 264.7 and mouse peritoneal (Swiss, C57BL/6) macrophage cells lines. Scale bar = 10 μm.	

Figura 2 manuscrito: Immunofluorescence detection of ARG1 expression in mouse macrophage cell lines at 24 h post-infection with *T. gondii*. Immunofluorescence and DIC images. (A) ARG1 expression (red) in non-activated macrophages (M0), non-infected or *T. gondii* (RH) infected cells (DAPI - blue). (B) ARG1 expression (red) in macrophages activated with IL-4 and 8-Br-AMPc (M2), non-infected or *T. gondii* (RH) infected cells (DAPI - blue). Scale bar = 10 µm.	
Figura 3 manuscrito: . Arginase 1 (ARG1) activity of macrophages activated with IL-4 and 8-Br-AMPc (M2), or non-activated (M0) infected or not with T. gondii. ARG1 activity in RAW 264.7 (A), Swiss (B) and C57BL/6 (C) peritoneal mice macrophages. Mean ± standard deviation (n = 3 independent experiments, each with 3 replicates). Significantly different by two-way ANOVA with Tukey post-test, *P=0.0109; **P=0.0062; ***P=0.0002; ****P<0.0001; NS = Not significant.	
Figura 4 manuscrito: Evaluation of T. gondii growth in RAW 264.7 and mouse peritoneal (Swiss, C57BL/6) macrophages cells lines activated with IL-4 and 8-Br-AMPc cultured with normal medium or medium supplemented with L-arginine (+ 1280 mg/L L-arginine). Percentage of infected macrophages (A, D and G), mean number of tachyzoites per infected macrophages (B, E and H) and infection rate (C, F and I). Mean ± SD (n = 3 independent experiments, each with 3 replicates). *P ≤ 0.05, two-way ANOVA with Tukey post-test, ns (not significant).	
Figura 5 manuscrito: Bright-field microscopy of Giemsa stained non-activated (M0) or alternatively activated (M2) RAW 264.7 macrophages infected with *T. gondii* for 24h, non-treated (control) or treated with 50 µM of Nor-NOHA. Rosette formed by the multiplication of parasites (arrow). Scale Bar = 10 µm**.**	
Figura 6 manuscrito: Evaluation of T. gondii growth in non-activated (M0) or activated with IL-4 and 8-Br-AMPc (M2) RAW 264.7 macrophages treated or not with Nor-NOHA. . Percentage of infected macrophages (A and B), number of tachyzoites per infected macrophages (C and D) and infection rate (E and F). Representative experiment (n = 3). Significantly different by One Way ANOVA test, *P=0.0147; **P=0.0028; ****P<0.0001; NS = not significant.	

Resumo

Toxoplasma gondii é um parasito intracelular obrigatório capaz de infectar virtualmente todas as células de vertebrados de sangue quente. Macrófagos são importantes células do sistema imune, extremamente heterogêneas e com alta capacidade de responder a infecções por patógenos. Essas células são moduladas por *T. gondii* através de mecanismos evasivos que alteram a expressão de óxido nítrico (NO) sintase induzida (iNOS) e arginase 1 (ARG1) na célula infectada. Nesse trabalho estudamos mecanismos evasivos desse parasito principalmente os que envolvem a inibição da produção de NO e modulação de ARG1. Durante a invasão da célula hospedeira *T. gondii* secreta fatores de virulência (VF); alguns migram para o núcleo da célula hospedeira controlando a expressão gênica. Aqui avaliamos diferentes VF na modulação da expressão da iNOS e produção de NO de linhagens de macrófagos J774-A1 e RAW 264.7 ativadas previamente com IFN-γ e LPS usando *T. gondii* tipo selvagem (cepa RH) ou *knockout* para alguns VF. A infecção reduziu a produção de NO nas duas linhagens, porém J774-A1 apresentou redução na expressão da iNOS, enquanto, na RAW 264.7 a expressão da iNOS não foi modulada. Nenhum dos VF testado foi responsável pelo fenótipo de inibição de NO, indicando que um novo VF pode estar envolvido na modulação deste mecanismo microbicida. Ademais, estudos recentes mostram que ARG1 é modulada durante a interação *T. gondii*-macrófagos, porém, ainda permanece em discussão se sua expressão e atividade em macrófagos ativados sofre alteração após infecção. Macrófagos RAW 264.7 e peritoneais de camundongos Suíços e C57BL/6, não ativados (M0) ou ativados previamente com IL-4 e 8-Br-AMPc (M2) foram infectados com *T. gondii* cepa RH e a expressão e atividade de ARG1, assim como crescimento de *T. gondii*, foram avaliados. Os macrófagos M0 ou M2 apresentaram padrões de expressão de ARG1 similares, sugerindo que expressam ARG1 constitutivamente. Ademais, a infecção de macrófagos com *T. gondii* aumentou a expressão da ARG1 nas linhagens avaliadas, independentemente do perfil de ativação celular. No entanto, a atividade de ARG1 variou de acordo com o perfil de ativação dos macrófagos. Após infecção por *T. gondii*, houve aumento na atividade de ARG1 em macrófagos M0 RAW 264.7 e peritoneais de camundongos Suíços. No entanto, macrófagos M2 não apresentaram diferenças na atividade de ARG1 após infecção por *T. gondii*, em nenhuma das linhagens celulares avaliadas. A suplementação de L-arginina e inibição farmacológica de ARG1 em macrófagos RAW 264.7 M0 e M2 aumentou e reduziu o crescimento de *T. gondii*, respectivamente. Esses resultados sugerem que ARG1 desempenha papel importante na replicação e sobrevivência do parasito. Os resultados obtidos nessa tese contribuem na elucidação de pontos importantes da complexa interação entre *T. gondii* e macrófagos no paradigma iNOS versus ARG1.

Palavras chaves: *Toxoplasma gondii*, fatores de virulência, macrófagos, arginase 1, óxido nítrico sintase induzida, óxido nítrico.

Abstract

Toxoplasma gondii is an obligate intracellular parasite able to infect virtually any nucleated cells from warm-blooded vertebrate animals. Macrophages are important cells of immune system, extremely heterogeneous and with high capacity to respond to pathogens infections. These cells are modulated by *T. gondii* through evasion mechanism that alter expression of inducible nitric oxide synthase (iNOS) and arginase 1 (ARG1) in the infected cell. In this work we studied evasion mechanisms of this parasite especially those involved in inhibition of NO production and ARG1 modulation. During host cell invasion *T. gondii* secrete virulence factors (VF); some migrates to host cell nucleus controlling gene expression. Here we evaluated differentes VF in the modulation of iNOS expression and NO production in J774-A1 and RAW 264.7 macrophages lineages previously activated with IFN-γ and LPS using wild type *T. gondii* (RH strain) or knockout for some VF. The infection reduced NO production in both lineages, however J774-A1 presented reduction in iNOS expression, while, in RAW 264.7 the iNOS expression was not modulated. None of the VF tested were responsible for the NO inhibition phenotype, indicating that a new VF could be involved in the modulation of this microbicide mechanism. Furthermore, recent studies shows that ARG1 is modulated during the *T. gondii*-macrophage interaction, however, still remain in discussion whether ARG1 expression and activity in activated macrophages is modulated after infection. RAW 264.7 and peritoneal Swiss and C57BL/6 mice macrophages, non activated (M0) or previously activated with IL-4 and 8-Br-AMPc (M2) were infected with *T. gondii* RH strain and the expression and activity of ARG1, as well as the growth of *T. gondii* were evaluated. The M0 and M2 macrophages presented similar expression patterns, suggesting constitutive ARG1 expression. Furthermore, the infection of macrophages with *T. gondii* enhanced ARG1 expression in the evaluated lineages, independently of the cellular activation profile. However, the ARG1 activity varied with the activation profile of the macrophages. After *T. gondii* infection, there was an increase in the activity of ARG1 in the M0 RAW 264.7 macrophages and Swiss peritoneal macrophages. However, M2 macrophages did not present differences in the ARG1 activity after *T. gondii* infection, in none of the cellular lineages evaluated. The L-arginine supplementation and pharmacological inhibition of ARG1 in M0 and M2 RAW 264.7 macrophages enhanced and reduced *T. gondii* growth, respectively. These results suggest that ARG1 plays a major role in the replication and survival of the parasite. The results obtained in this work contribute to the elucidation of key points in the complex interaction between *T. gondii* and macrophages in the iNOS x ARG1 paradigm.

Key words: *Toxoplasma gondii*, virulence factors, macrophage, arginase 1, inducible nitric oxide synthase, nitric oxide.

1- Introdução

Toxoplasma gondii é o agente etiológico da toxoplasmose, doença de abrangência mundial (Tenter et al, 2000). A infecção por *T. gondii* é altamente prevalente em humanos e outros animais em todos os continentes (Dubey & Beattie, 1988; Jones & Dubey, 2010; Montoya & Liesenfeld, 2004), ressaltando a importância deste parasito em estudos de infecções por protozoários. *Toxoplasma gondii* é transmitido principalmente pela via fecal-oral, através da ingestão de carne crua infectada, água contaminada ou transmissão pela placenta da mãe para o feto (Dubey & Beattie, 1988; Frenkel et al, 1970). Com ciclo de vida extremamente complexo, este parasito possui inúmeros hospedeiros intermediários, sendo os felídeos seus hospedeiros definitivos (Dubey et al, 1970; Frenkel et al, 1970; Weiss & Kim, 2011). Durante seu ciclo de vida, possui quatro formas infectivas (Ferguson, 2004; Frenkel, 1973; Weiss & Kim, 2011) 1- taquizoítos, forma de rápida de multiplicação; 2- bradizoítos, forma lenta de replicação contida nos cistos teciduais; 3- esporozoítos, contidos em oocistos esporulados liberados nas fezes dos hospedeiros definitivos; 4- merozoitos, forma infectiva com proliferação limitada entre 2-4 gerações dentro dos enterócitos do intestino dos felídeos. Embora na maioria dos casos *T. gondii* cause infecções assintomáticas ou com sintomas brandos (Remington, 1974), a infecção por este parasito pode levar à morte indivíduos saudáveis (Demar et al, 2012; Franco et al, 2016), imunocomprometidos (Luft & Remington, 1992) ou os congenitamente infectados (Montoya & Remington, 1996).

Parasitos do filo Apicomplexa, como *T. gondii*, invadem ativamente células hospedeiras utilizando componentes do citoesqueleto como actina e miosina (Carruthers, 2002; Dobrowolski & Sibley, 1996; Morisaki et al, 1995). Taquizoíto de *T. gondii* não possui especificidade por célula alvo, infectando praticamente todas as células de animais vertebrados de sangue quente (Tenter et al, 2000). Entretanto, estudos *in vivo* indicam que macrófagos e células dendríticas são alvos preferencias, funcionando como “cavalo de tróia” para disseminação do parasito no hospedeiro (Bierly et al, 2008; Courret et al, 2006; Da Gama et al, 2004). Ao invadir ativamente a célula hospedeira, *T. gondii* estabelece um nicho especializado, o

vacúolo parasitóforo (PV), para obtenção de nutrientes, replicação e proteção contra mecanismos microbicidas do hospedeiro (Mercier et al, 2005; Sinai & Joiner, 1997).

O sucesso no estabelecimento da infecção por *T. gondii* na célula hospedeira é dependente da via secretora do parasito, que compreende organelas como micronemas, róptrias e grânulos densos (Bradley & Sibley, 2007; Carruthers et al, 1999; Carruthers & Sibley, 1997). Estas organelas secretam proteínas que desempenham papel essencial na adesão, invasão, estabelecimento do PV e evasão de mecanismos microbicidas do hospedeiro (Hakimi et al, 2017; Hunter & Sibley, 2012). Através do avanço nas técnicas de biologia molecular como o mapeamento genético por polimorfismo de fragmentos de restrição (RFLP) (Sibley et al, 1992) e a mais recente e avançada técnica de *Clustered Regularly Interspaced Short Palindromic Repeat* (CRISPR) e do CRISPR-*associated gene* 9 (Cas9) levou-se a identificação de fatores de virulência secretados pelas róptrias e grânulos densos, proteínas que estão envolvidas na evasão e manipulação da imunidade celular autônoma e do sistema imune do hospedeiro (Coffey & Sleebs, 2015; Curt-Varesano et al, 2016; Etheridge et al, 2014; Gay & Braun, 2016; Hammoudi et al, 2015; Olias et al, 2016; Saeij et al, 2006; Sibley & Boothroyd, 1992; Taylor et al, 2006).

Macrófagos são importantes células do sistema imune e desempenham inúmeras funções em diferentes tecidos do corpo, sendo essenciais para manutenção da homeostase tecidual (Geissmann et al, 2010). De acordo com o perfil de expressão gênica, macrófagos podem assumir distintos perfis de ativação, sendo classificados basicamente em macrófagos residentes (M0), classicamente ativados (M1) e macrófagos alternativamente ativados (M2) (Biswas et al, 2012; Mills et al, 2000; Mosmann et al, 1986). Macrófagos M0 possuem baixa capacidade microbicida e estão disseminados em praticamente todos os tecidos do corpo (Gomez Perdiguero et al, 2015; Sorokin et al, 1992). Macrófagos M1 assumem perfil classicamente ativado quando estimulados com citocinas pró-inflamatórias como interferon-gamma (IFN-γ) (Mills et al, 2000; Stuehr & Marletta, 1987) e lipopolissacarídeos (LPS) (Poltorak et al, 1998; Stuehr & Marletta, 1985) presentes

na parede celular de bactérias gram-negativas. Assumindo um perfil M1 de ativação, macrófagos expressam óxido nítrico sintase induzida (iNOS), enzima responsável pela síntese de oxido nítrico (NO), molécula microbicida essencial no controle de parasitos como *T. gondii* (Khan et al, 1997). Em contraste, macrófagos M2 assumem perfil alternativamente ativado quando estimulados por interleucina (IL)-4, IL-10, Fator de crescimento transformante β (TGF-β) e 8-Br-AMPc (Barksdale et al, 2004; Munder et al, 1999). Caracterizam-se pela expressão de arginase 1 (ARG1), enzima que catalisa a síntese de L-ornitina e ureia a partir do aminoácido L-arginina (Ash, 2004).

O papel de macrófagos com diferentes perfis de ativação na infecção com *T. gondii* tem sido estudado (Butcher et al, 2011; Jensen et al, 2011; Li et al, 2017). Sabe-se que macrófagos M1 agem controlando a infecção pelo parasito através de inúmeros mecanismos, principalmente produção de espécies reativas de oxigênio (Murray & Cohn, 1979) e NO (Khan et al, 1997). Em contrapartida, macrófagos M2 beneficiam *T. gondii*, pois a infecção pelo parasito induz a expressão de ARG1 na célula infectada, levando a competição entre ARG1 e iNOS pelo substrato L-arginina (Chang et al, 1998; Tenu et al, 1999). Dessa forma, a dualidade entre as vias de ativação macrofágica no processo infectivo de *T. gondii* e as lacunas existentes sobre esses mecanismos de modulação reforçam a importância de novos estudos.

Esta tese foi realizada no Centro de Biociências e Biotecnologia (CBB) da Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF) e no Departamento de Biologia Molecular da *Washington University in Saint Louis-School of Medicine*, *Saint Louis*, Estados Unidos. Está dividida em um (1) capítulo de livro publicado, um (1) artigo publicado, e um (1) manuscrito em finalização. Esses documentos abordam a problemática da modulação de células hospedeiras, principalmente macrófagos, por *T. gondii*, dando ênfase ao paradigma iNOS x ARG1.

2- Revisão Bibliográfica

2.1- Toxoplasmose

Toxoplasmose é uma importante zoonose de abrangência global causada pelo parasito *T. gondii* (Hill & Dubey, 2014; Tenter et al, 2000). Cerca de um terço da população mundial apresenta anticorpos contra *T. gondii*, sendo considerada uma doença tropical negligenciada (Wallon & Peyron, 2018). Nos Estados Unidos, por exemplo, a toxoplasmose é a quarta causa mais comum de hospitalização e a segunda maior causa de morte por infecção de origem alimentar (Scallan et al, 2011). Com relação aos aspectos clínicos da doença, a infecção por *T. gondii* pode ser assintomática, ou causar sinais e sintomas que variam dependendo da capacidade imunológica do indivíduo infectado (Remington, 1974), sendo categorizada em: a) toxoplasmose em indivíduos imunocompetentes, b) toxoplasmose adquirida ou reativada em indivíduos imunocomprometidos, c) toxoplasmose congênita e d) toxoplasmose ocular.

Em indivíduos imunocompetentes, a doença normalmente é benigna e assintomática na maioria dos pacientes, e em apenas 10% dos casos geram alguma doença que necessite de tratamento médico, onde a manifestação clínica típica é a linfadenopatia (Remington, 1974). Também podem ocorrer casos de miocardite (Cunningham, 1982), hepatite ou encefalite, porém, são muito infrequentes (Greenlee et al, 1975; McCabe et al, 1987). Muito diferente dos casos assintomáticos em indivíduos imunocompetentes, a toxoplasmose pode ser extremamente severa em pacientes imunocomprometidos com HIV (Luft & Remington, 1992), e na maioria dos casos acontece por uma reativação da fase crônica da doença (Luft & Remington, 1992). O sistema nervoso central é o local mais afetado, causando a encefalite toxoplásmica, onde as manifestações clínicas podem variar desde distúrbios mentais e motores, fraqueza, convulsões e até mesmo a morte se não tratada corretamente (Luft & Remington, 1992). A toxoplasmose também pode causar graves implicações em fetos congenitamente infectados (Montoya & Remington, 1996). Os primeiros relatos de infecção congênita em humanos foram inicialmente descritos por Wolf et al. (1939) em uma criança que ficou doente três dias após o nascimento e desenvolveu distúrbios

respiratórios, convulsões e problemas na espinha dorsal, morrendo com 31 dias de vida. Exames de ultrassonografia podem revelar calcificações intracraniais, dilatação ventricular, e hepatomegalia no feto em desenvolvimento (Remington et al, 2001). Em neonatos, as manifestações clínicas podem variar desde hidrocefalia, calcificações intracranianas, cegueira, retardo mental ou psicomotor, trombocitopenia e anemia (Remington et al, 2001; Wilson et al, 1980). Outro fator importante atrelado às manifestações clínicas é que a severidade das complicações depende da idade gestacional em que a doença é adquirida (Remington et al, 2001): transmissões ao feto entre 10 e 24 semanas de gestação resultam em quadros mais graves da doença, enquanto entre 26 e 40 semanas de gestação o feto poderá manifestar apenas quadros subclínicos em algum período da sua vida (Daffos et al, 1988; Desmonts, 1982). A toxoplasmose ocular, também conhecida como coriorretinite toxoplásmica, ocorre normalmente em indivíduos congenitamente infectados ou como consequência de uma reativação da doença em indivíduos imunocompetentes (Montoya & Remington, 1996; Nussenblatt & Belfort, 1994). Como consequência, lesões focais e forte inflamação podem causar perda parcial ou total da visão nos indivíduos infectados (Holland et al., 1996).

Diversos estudos foram realizados ao longo dos anos para averiguar a soroprevalência anti *T. gondii* a nível global (Figura 1), onde tem-se produzido resultados variáveis, fato que está diretamente relacionado com localização geográfica (Pappas et al, 2009), fatores de risco, hábitos alimentares (Bojar & Szymanska, 2010) e fatores socioeconômicos (Jones & Dubey, 2010). Estudo recente indica que a soroprevalência para *T. gondii* continua aumentando globalmente (Pappas et al, 2009). Nos Estados Unidos e no Reino Unido, estima-se que 10-40% das pessoas são infectadas, enquanto que na América do Sul e Central, e Europa estima-se que os níveis de infecção variam entre 50-80% (Dubey & Beattie, 1988; Dubey et al, 2012; Jones et al, 2007; Pappas et al, 2009). Estudo recente nos EUA utilizando estratégia de grande análise de dados revela informações precisas acerca da toxoplasmose no país, incluindo a quantidade de indivíduos acometidos pela doença, parâmetros de distribuição espaço-temporal e padrões de manifestação da doença (Lykins et al, 2016). O estudo revelou 9260

casos de toxoplasmose entre 2003 e 2012 nos EUA, com maior prevalência no sul do país, e uma média de idade dos indivíduos acometidos entre 17 e 33 anos de idade (Lykins et al, 2016). Ademais, 38% dos indivíduos acometidos possuem toxoplasmose ocular e 12% possuem alguma manifestação grave da doença, como acometimento do sistema nervoso central (Lykins et al, 2016). É importante ressaltar que o método de análise utilizado analisa apenas os registros de indivíduos acometidos pela toxoplasmose que possuem seguro de saúde privado, o que sugere fortemente que o número de indivíduos pode ser muito maior, se incluído os indivíduos sem seguro privado no país. Para gerar estas estimativas, o importante órgão americano no controle de doenças, Centro de Controle e Prevenção para Doenças, baseia-se nos dados aferidos pela Pesquisa Nacional de Exame de Saúde e Nutrição, no qual a primeira avaliação foi feita entre 1988 e 1994 (Hughes & Colley, 2000) e a segunda entre 1999 e 2000 (Jones et al, 2003; Jones et al, 2007). Em relação à Ásia e Oceania, dados sugerem baixa soroprevalência para toxoplasmose, enquanto que na África os índices podem chegar a 80% (Montoya & Liesenfeld, 2004). Apesar do grande número de novas infecções em humanos, e do fato de ser uma das mais prevalentes e disseminadas infecções parasitárias de ampla distribuição animal e geográfica (Pappas et al, 2009; Tenter et al, 2000; Hill & Dubey, 2014), a toxoplasmose continua sendo uma doença negligenciada, revelando a falta de políticas de saúde no combate à doença.

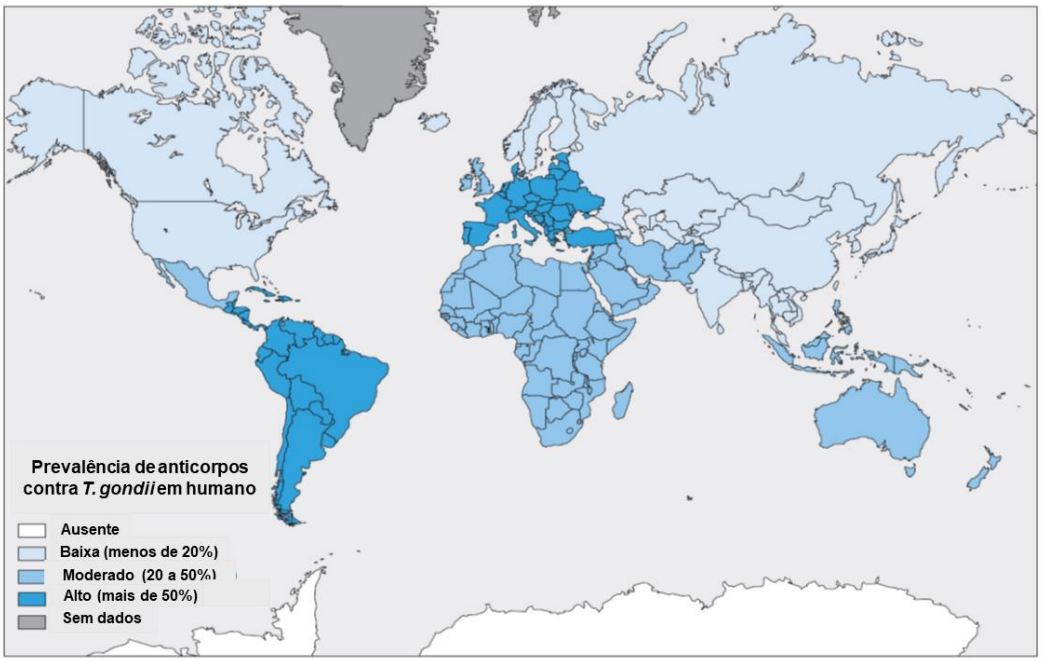


Figura 1: Distribuição geográfica global da soroprevalência para *Toxoplasma gondii* em humanos. Adaptado de Hill & Dubey (2014).

2.2- *Toxoplasma gondii*

Toxoplasma gondii, agente causador da toxoplasmose, é um dos parasitos mais bem estudados no mundo devido a sua importância médica e veterinária, facilidade de cultivo e manipulação gênica (Shen et al, 2014; Sidik et al, 2014). Dessa forma, *T. gondii* tornou-se um modelo de estudo em diversas áreas, principalmente aquelas relacionadas à biologia celular e molecular (Dubey et al, 2007). A descoberta do parasito é atribuída a Nicolle e Manceaux em 1908 (Nicole & Manceaux, 1908) após analisar os tecidos de um roedor infectado, *Ctenodactylus gundi*, utilizado como um hospedeiro para pesquisa em leshimaniose no laboratório de Charles Nicolle no Instituto Pasteur na Tunísia. Baseado na morfologia do parasito (Latim: toxo = arco e plasma = molde, forma), e no hospedeiro no qual foi identificado, nomearam-no *T. gondii*. A descoberta desse parasito também é atribuída a Splendore, no Brasil, que o descobriu em coelhos, porém não o nomeou (Splendore, 1908). *Toxoplasma gondii* viáveis foram primeiramente isolados por Sabin e Olitsky (Sabin & Olitsky, 1937) em camundongos, que se mostraram idênticos aos parasitos isolados em humanos por Wolf et al. (1939) . Desde então, *T. gondii* se tornou um parasito modelo de estudo em diversas áreas.

Toxoplasma gondii é um parasito intracelular obrigatório pertencente ao Filo Apicomplexa*,* possui ciclo de vida complexo, diversos hospedeiros intermediários e quatro estágios infectivos: taquizoítos, bradizoítos, merozoítos e esporozoítos (Dubey et al, 1998; Ferguson, 2004; Frenkel, 1973; Weiss & Kim, 2011). O complexo ciclo de vida de *T. gondii* (Figura 2) pode ser dividido em duas fases: uma fase sexual de replicação que ocorre exclusivamente nos hospedeiros definitivos, espécies da família *Felidae*, e uma fase assexual que ocorre nos hospedeiros intermediários, virtualmente todos os animais vertebrados de sangue quente e também nos hospedeiros definitivos (Dubey et al, 1970; Frenkel et al, 1970; Weiss & Kim, 2011). A fase taquizoíto define a forma de multiplicação rápida do parasito, encontrada durante a fase aguda da toxoplasmose em hospedeiros intermediários e definitivos (Frenkel, 1973; Nicolle, 1909). Taquizoítos podem infectar virtualmente todas as células do corpo (Frenkel, 1973). Dentro da célula hospedeira, taquizoítos multiplicam-se entre 6 às 8h (*in vitro*) dividindo-se por endodiogenia, processo descrito inicialmente por Goldman (Goldman et al, 1958). Após gerar 64 a 128 parasitos, realizam um processo conhecido como ciclo lítico, no qual rompem a célula hospedeira para infectar novas células (Black & Boothroyd, 2000). Sob pressão do sistema imunológico do hospedeiro, taquizoítos se diferenciam em bradizoítos, forma lenta de multiplicação de *T. gondii* que ocorre dentro de cistos teciduais (Dubey et al, 1998) que possuem alta afinidade pelos tecidos neuronal e muscular, como olhos, cérebro músculos esqueléticos e cardíacos (Dubey et al, 1998). A formação dos cistos teciduais no hospedeiro marca a fase crônica da infecção (Dubey & Beattie, 1988). Ao serem ingeridos pelos hospedeiros definitivos através de carnivorismo ou ingestão de água contaminada, os cistos teciduais são digeridos por enzimas proteolíticas no estômago, liberando bradizoítos (Dubey & Beattie, 1988). Ao serem liberados, parte dos bradizoítos infectam as células da lâmina própria do intestino, se diferenciam em taquizoítos e disseminam para outros tecidos. Outros bradizoitos penetram nos enterócitos do tecido epitelial intestinal e iniciam o desenvolvimento de numerosas formas assexuais chamadas de esquizontes que geram merozoítos (Dubey & Frenkel, 1972). Esse merozoítos se dividem assexuadamente via esquizogonia por

algumas poucas gerações garantindo amplificação no número de parasitos infectando os enterócitos dos hospedeiros definitivos (Ferguson, 2004). Estas formas assexuais de reprodução formam gametas; os masculinos têm dois (2) flagelos que são utilizados para movimentar e encontrar os gametas femininos, que após fertilização constituem um zigoto dando início a formação da parede do oocisto (Dubey et al, 1998). O oocisto não esporulado, sem capacidade infectiva, é liberado nas fezes dos felídeos. Os oocistos esporulam cerca de 1-5 dias após liberação no ambiente, podendo variar de acordo com a temperatura e oxigenação, esse é um processo de meiose seguido de mitose formando dentro do oocisto dois esporocistos, cada um deles com quatro esporozoítos (Dubey et al, 1998). Os hospedeiros intermediários e definitivos podem ingerir os oocistos esporulados através da água (bebidas variadas) ou alimentos contaminados. No intestino, oocistos liberam esporozoítos que infectam enterócitos e se diferenciam em taquizoítos novamente, fechando o ciclo (Figura 2) (Dubey et al, 1998).

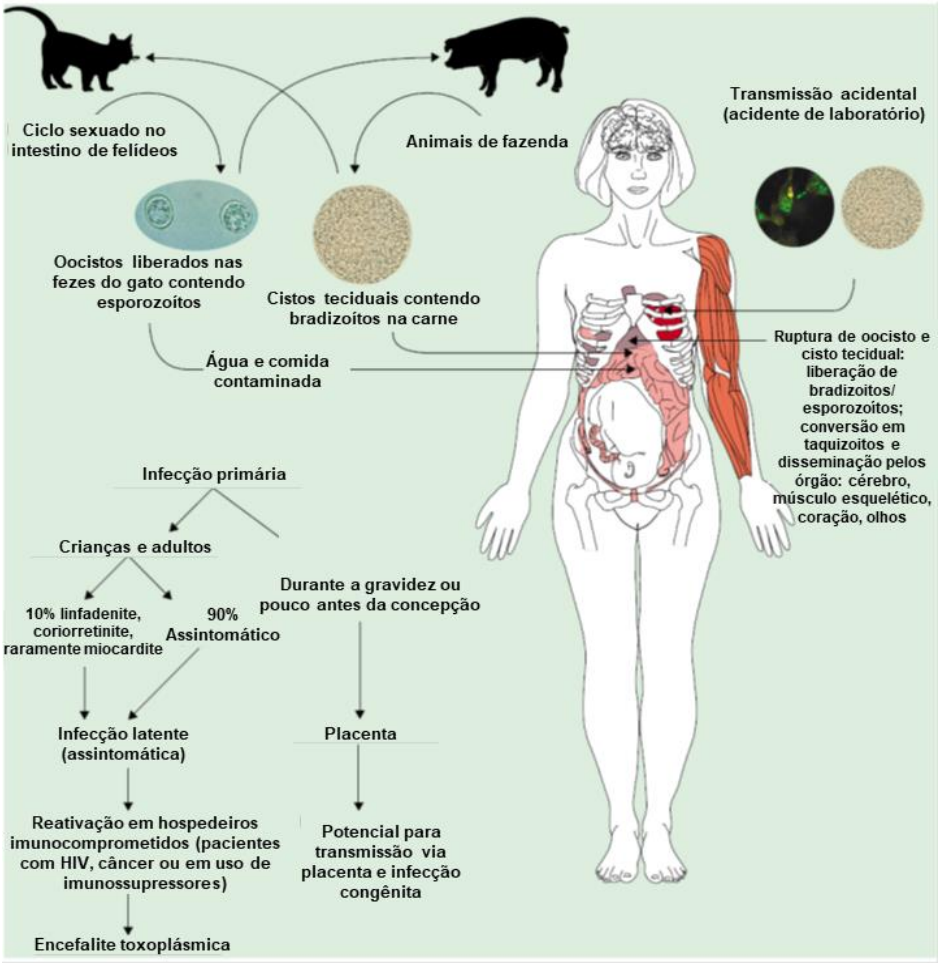


Figura 2: Ciclo de vida de *Toxoplasma gondii* e manifestações clínicas da toxoplasmose em humanos. Adaptado de Montoya & Liesenfeld (2004).

Os estágios infectivos de *T. gondii*, taquizoíto, bradizoíto e esporozoíto, exibem uma morfologia básica, com pequenas variações, todos ligados pelo complexo ciclo de vida do parasito (Dubey et al, 1998). De modo geral, *T. gondii* possui corpo em forma de arco medindo cerca de 2 x 6 µm de tamanho, sendo a região anterior do seu corpo mais alongada (Dubey et al, 1998). Ultraestruturalmente, na região apical do corpo do parasito localizam-se dois anéis apicais de material elétron denso, ainda não caracterizado a nível molecular, onde o superior mede 160 nm e o posterior 200 nm (Dubey et al, 1998). O conóide, estrutura formada por tubulina possui uma organização estrutural única de fibras em espiral (Hu et al, 2002), rodeada no topo por dois anéis polares formados por

microtúbulos; do conóide partem microtúbulos que se estendem da região apical até 2/3 do corpo do parasito (Nichols & Chiappino, 1987). Também são encontrados feixes de microtúbulos intraconoidais que se estendem da região apical ao citoplasma do parasito (Hu et al, 2002). Ademais, um conjunto de membranas formado por plasmalema, membrana interna e membrana externa formam uma estrutura conhecida como película (Vivier & Petitprez, 1969). Todo esse complexo sistema de citoesqueleto associado à película permite a locomoção do parasito e penetração nas células hospedeiras (Keeley & Soldati, 2004).

O mecanismo de invasão das células hospedeiras é extremamente conservado em parasitos do filo Apicomplexa (Baum et al, 2006; Kappe et al, 1999; Kappe et al, 2004). Estudos utilizando inibidores farmacológicos com citocalasina D, um potente inibidor da polimerização de actina e parasitos mutantes mostraram que a invasão da célula hospedeira por *T. gondii* ocorre por um processo de penetração ativa (Morisaki et al, 1995) e requer a polimerização dos filamentos de F-actina do parasito (Carruthers, 2002; Dobrowolski & Sibley, 1996). Foi demonstrado que o processo de invasão da célula hospedeira pode ocorrer em menos de 20 segundos (Morisaki et al, 1995). *Toxoplasma gondii* é desprovido de organelas especializadas em motilidade, como cílios ou flagelos (com exceção de gametas masculinos), e todo processo de invasão da célula hospedeira ocorre através de um mecanismo dinâmico e conservado entre membros do filo Apicomplexa, conhecido como *gliding* (Dobrowolski & Sibley, 1996; Fayer, 1972). O mecanismo de *gliding* é um processo de múltiplas etapas que se inicia com a ligação inicial entre a membrana do parasito com a membrana da célula hospedeira (etapa 1). *Toxoplasma gondii* possui inúmeros antígenos de superfície ancorados a sua membrana plasmática por glicosilfosfatidilinositol. O papel fundamental das SAGs (*surface antigen 1*) é mediar a interface de ligação entre *T. gondii*-célula hospedeira (Lekutis et al, 2001). Anticorpos anti-SAG reduzem a capacidade de invasão do parasito, mostrando sua importância no processo infectivo (Mineo & Kasper, 1994).

Em seguida, *T. gondii* reorienta seu corpo de modo a realizar a ligação da sua região apical com a membrana da célula hospedeira (etapa 2) através de

proteínas micronemais (MICs) adesivas secretadas por organelas especializadas, as micronemas (Carruthers et al, 1999). Estas proteínas possuem domínios adesivos conservados que medeiam a interação proteína-proteína ou proteína-carboidratos, sendo sua secreção bloqueada por inibidores de cálcio, ocasionando a perda de adesão à célula hospedeira e consequente redução da capacidade de invasão (Carruthers et al, 1999). Estudos de purificação e afinidade de proteínas mostraram a associação entre AMA1 e uma outra classe de proteínas provenientes das organelas róptrias, as proteínas do pescoço das róptrias (RONs), formando um complexo AMA1-RONs em forma de anel entre a membrana da célula hospedeira e do parasito (etapa 3). Este complexo origina a junção móvel (etapa 4), uma íntima associação entre as membranas de *T. gondii* e célula hospedeira, formada por RON 2, RON 4 e RON 5 (Alexander et al, 2005; Lebrun et al, 2005). Com a formação da junção móvel, *T. gondii* invade a célula hospedeira (etapa 5) e durante esse processo, secreta outra classe de proteínas das róptrias (ROPs), que são injetadas no ponto da invasão e possuem diversos destinos na célula hospedeira, como núcleo e PV ((Bradley & Sibley, 2007). Com a invasão da célula hospedeira, forma-se um compartimento vacuolar especializado, o PV (etapa 6) derivado da membrana de *T. gondii* e da célula hospedeira (Suss-Toby et al, 1996), que é delimitado por uma membrana, conhecida como membrana do PV (PVM) (Martin et al, 2007). Após completa internalização e formação da PVM e PV, ocorre a secreção de proteínas dos grânulos densos (GRAs) (Achbarou et al, 1991; Carruthers & Sibley, 1997). Essa organela secreta diversas GRAs, incluindo GRA 3 (Bermudes et al, 1994), GRA 5 (Lecordier et al, 1999), GRA 7 (Sehgal et al, 2005), GRA 8 (Carey et al, 2000), GRA 9 (Adjogble et al, 2004) e GRA 10 (Ahn et al, 2005) que são endereçadas para a PVM. As funções exercidas por essa classe de proteínas ainda não estão completamente elucidadas, porém, tem sido proposto que GRA 3 sofre oligomerização para formar um poro na PVM, permitindo a passagem de nutrientes para *T. gondii* (Ossorio et al, 1994). Ademais, também tem sido sugerido que essas proteínas participam do processo de construção e manutenção da parede cística, pois estas proteínas permanecem presentes dentro do PV durante a replicação do parasito (Ferguson, 2004; Torpier et al, 1993).

Deste modo, o PV é um compartimento altamente especializado e dinâmico que forma um nicho para replicação do parasito, evitando sua fusão com o sistema endolisossomal da célula hospedeira e consequente destruição (Jones et al, 1972; Lingelbach & Joiner, 1998; Sibley et al, 1985). Ademais, a PVM dá início a formação da parede cística (Weiss & Kim, 2000), e acredita-se que as funções exercidas pela PVM podem variar de acordo com as diferentes fases do ciclo de replicação do parasito (Lingelbach & Joiner, 1998). Estudos demonstram que PVM desenvolve papel essencial na sobrevivência de *T. gondii*, pois mantêm a integridade estrutural do PV e está envolvida na aquisição de nutrientes e manipulação de funções celulares do hospedeiro (Lingelbach & Joiner, 1998; Sinai & Joiner, 1997). Esses achados demonstram a importância do PV e das organelas secretoras do parasito na evasão imune e sobrevivência de *T. gondii*.

2.3- Descoberta de fatores de virulência de *Toxoplasma gondii* e seu papel na evasão imune de células hospedeiras: técnicas de mapeamento genético e edição genômica

O avanço na criação de ferramentas genéticas para alteração eficiente e sítio-específica do genoma é chave para se decodificar e entender a biologia de um organismo (Cong et al, 2013; Di Cristina & Carruthers, 2018; DiCarlo et al, 2013; Grzybek et al, 2018; Ma & Liu, 2015; Shen et al, 2014). *Toxoplasma gondii* possui um genoma com aproximadamente 65 mb de tamanho distribuídos em 14 cromossomos que codificam cerca de 8300 genes, e se tornou um modelo no estudo de parasitos Apicomplexa devido ao seu fácil cultivo *in vitro* e as inúmeras ferramentas moleculares disponíveis para sua manipulação (Shen et al, 2014; Sidik et al, 2014). Na pesquisa com *T. gondii*, os primeiros estudos na tentativa de aumentar a especificidade da modificação gênica foram em 1993; pela primeira vez reportou-se a inserção e expressão de DNA exógeno através de um marcador positivo de seleção e geração do parasito *knockout* estável (Kim et al, 1993). Nos anos seguintes, inúmeros outros trabalhos reportaram o sucesso na geração de parasitos *knockout* utilizando diferentes marcadores positivos de seleção como *chloramphenicol acetyl transferase* (Cat), que confere resistência ao clorafenicol

(Soldati & Boothroyd, 1993) e *dihydrofolate reductase-thymidylate synthase* (DHFR-TS*) que confere resistência a pirimetamina (Donald & Roos, 1993).

Apesar do grande avanço para a época e o primeiro passo para o início do que seria uma revolução na pesquisa de engenharia genética em *T. gondii*, as mutações realizadas eram extremamente ineficientes, pois menos de 0,1 % eram mutações sítio-específicas no gene de interesse (Fox et al, 2009). Essa baixa eficiência se deve a alta taxa de inserção randômica de DNA exógeno no parasito (Roos et al, 1994). Existem duas vias principais de reparo em quebras de dupla fita de DNA (dsDNA): 1- junção final não-homóloga (NHEJ) e 2- recombinação homóloga (HR). A via de reparo por NHEJ é dita não-homóloga pois a quebra na dsDNA é diretamente ligada sem a necessidade de uma sequência molde homóloga para o reparo (Moore & Haber, 1996). Estas quebras na dsDNA são detectadas por heterodímeros conhecidos como Ku70/80, composto por subunidades de 70 e 80 kDa que se ligam e formam um anel em torno do final da dsDNA, recrutando a DNA ligase IV para reparo (Downs & Jackson, 2004; Teixeira-Silva et al, 2017). Esta via é ativa em todo ciclo celular, porém predomina na fase G1 do ciclo celular, onde a via HR é ausente (Mao et al, 2008). A via HR usa uma fita molde de DNA não danificada para reparar a dsDNA, levando a reconstituição da fita original (Mao et al, 2008; Thompson & Schild, 2001). Esta via predomina na fase S do ciclo celular. Deste modo, ao gerar um parasito *knockout* para Ku80 (Δ*ku*80) é possível aumentar a proporção de recombinação homóloga, aumentando a eficiência para mutações pontuais no gene alvo, já que Ku80, componente essencial da via NHEJ não está funcional (Downs & Jackson, 2004; Teixeira-Silva et al, 2017). Usando linhagens Δ*ku*80, dois grupos independentemente produziram *T. gondii* Δ*ku*80 utilizando marcadores positivos de seleção flanqueados por regiões homólogas (Fox et al, 2009; Huynh & Carruthers, 2009). Assim como a técnica para gerar parasitos *knockouts* estabelecida em 1993, a geração de *T. gondii* Δ*ku*80 também possui desvantagens e limitações: está disponível apenas em parasitos da cepa tipo I RH (Fox et al, 2011; Huynh & Carruthers, 2009) e cepas tipo II PRU (Fox et al, 2011) e tem sido reportado que mutantes Δ*ku*80 possuem alta instabilidade nos cromossomos (Zhang et al, 2011). Sendo assim,

uma ferramenta de edição genômica que possa ser usada em diferentes cepas do parasito e que seja precisa, é altamente desejada, e se tornou disponível após adaptação do CRISPR e do Cas9 para tecnologia de edição genômica em *T. gondii* (Shen et al, 2014; Sidik et al, 2014).

O desenvolvimento do CRISPR/Cas9 revolucionou a manipulação genética em diversos organismos, como *Saccharomyces cerevisiae* (DiCarlo et al, 2013), células de mamíferos (Cong et al, 2013) e parasitos protozoários como *Plasmodium falciparum* (Ghorbal et al, 2014), *Trypanosoma cruzi* (Lander et al, 2015) e *T. gondii* (Shen et al, 2014; Sidik et al, 2014). CRISPR é um sistema de autodefesa encontrado em bactérias e archaea que age contra o DNA exógeno de alvos como fagos (Barrangou et al, 2007). Após invasão por um fago, sequências curtas de DNA do invasor são armazenadas no *lócus* CRISPR e serve como um mecanismo de “memória” para rastrear as infecções. Em infecções subsequentes, essas informações armazenadas são usadas para defender as bactérias de infecções secundárias pelo mesmo fago (Ishino et al, 1987; Jansen et al, 2002; Marraffini, 2015).

Atualmente, o sistema CRISPR/Cas9 tipo 2 desenvolvido a partir de *Streptococcus pyogenes* é a ferramenta de edição genômica mais utilizada em estudos de biologia molecular devido a sua simplicidade (Jinek et al, 2012; Mali & Esvelt, 2013). Todo o sistema necessita apenas da endonuclease Cas9 guiada por RNA e duas moléculas pequenas de RNA, conhecidas como crRNA e tracrRNA para realizar a quebra da dupla fita (DSB) do DNA alvo de maneira extremamente precisa. As moléculas de crRNA e tracrRNA se hibridizam para formar um complexo quimérico conhecido como RNA guia único (sgRNA) que é reconhecido pela Cas9, onde a molécula de crRNA guia a endonuclease Cas9 ao DNA alvo, causando a DSB (Jinek et al, 2012). A introdução da DSB pode ser reparada por NHEJ, mecanismo randômico de reparo da dsDNA levando a inserção e deleção de mutações para inativar genes, ou por HR para alterar um *lócus* alvo usando um doador molde (Mali & Esvelt, 2013). Nos estudos de engenharia genética em *T. gondii*, a endonuclease Cas9 é normalmente expressa a partir de uma região promotora de um gene codificador de proteína, como o antígeno de superfície 1 ou

alfa tubulina, enquanto o sgRNA é normalmente expresso a partir de um promotor endógeno como U6 ou exógeno como T7. Esta estratégia se mostrou eficiente na indução da DSB sítio específica e na HR na região alvo DNA quando guiada por um doador molde (Shen et al, 2014; Sidik et al, 2014).

A aplicação desta importante ferramenta de edição genômica e de técnicas de mapeamento genético tem gerado grandes descobertas na pesquisa com *T. gondii* como: 1- papel de fatores de virulência secretados pelo parasito na manipulação do hospedeiro (Behnke et al, 2011; Bougdour et al, 2013; Curt-Varesano et al, 2016; Etheridge et al, 2014; Franco et al, 2016; Hakimi et al, 2017; Olias et al, 2016; Reese et al, 2011; Saeij et al, 2006; Taylor et al, 2006); 2- mecanismo de invasão da células hospedeira (Long et al, 2017); 3- sinalização celular (Brown et al, 2017); e 4-formação de cistos na fase crônica da toxoplasmose (Jones et al, 2017). A adaptação do sistema CRISPR/Cas9 tem permitido grandes avanços no estudo das funções de importantes proteínas de *T. gondii*, caracterização de vias metabólicas e uma compreensão mais aprofundada da biologia do parasito, demonstrando o papel central da engenharia genética na elucidação de pontos chave na pesquisa com parasitos Apicomplexa.

2.3.1- ROPs

O filo Apicomplexa compreende um grande número de protozoários intracelulares obrigatórios. Estima-se que o filo possua cerca de 5000 espécies, embora estudos de genética populacional revelem um número muito maior em diversidade de espécies (Pawlowski et al, 2012). O nome do filo é associado a presença de uma estrutura chamada de complexo apical, que compreende uma rede de microtúbulos do parasito que são essenciais para o processo de invasão da célula hospedeira e abriga organelas secretoras como as róptrias (Carruthers & Sibley, 1997).

ROPs foram inicialmente identificadas em extratos de róptrias purificadas utilizando-se anticorpos monoclonais (Sadak et al, 1988). Estas proteínas são sintetizadas e conduzidas do retículo endoplasmático para o complexo de Golgi por uma via conservada de tráfego intracelular, onde posteriormente são endereçadas as róptrias (Hoppe et al, 2000). *Toxoplasma gondii* secreta ROPs no citoplasma

das células hospedeiras durante o processo de invasão, onde em seguida, essas proteínas são guiadas para diversos locais, fazendo destas proteínas possíveis candidatos na manipulação da sinalização celular do hospedeiro (Hakansson et al, 2001). Essa possibilidade foi confirmada após identificação de fatores de virulência por técnicas de mapeamento genético como polimorfismo de fragmentos de restrição (RFLP), causados por polimorfismo de nucleotídeo único (SNP) (Sibley et al, 1992) onde foram aplicadas a parasitos obtidos de cruzamentos genéticos entre diferentes cepas de *T. gondii* descrito em trabalhos pioneiros de 1980 (Pfefferkorn & Kasper, 1983; Pfefferkorn & Pfefferkorn, 1980).

O trabalho pioneiro de Elmer Pfefferkorn demonstrou que era possível aplicar genética clássica em *T. gondii* para mapear diferenças genéticas entre as linhagens clonais do parasito e os *lócus* responsáveis por essas diferenças (Pfefferkorn & Kasper, 1983; Pfefferkorn & Pfefferkorn, 1980). Para tal, cepas distintas de *T. gondii* com marcadores de resistência a drogas eram utilizadas para infectar camundongos, de onde posteriormente os cistos teciduais eram obtidos e utilizados para infectar gatos, os quais produziam oocistos recombinantes por reprodução sexual (Sibley, 2009). Após cruzamentos entre as diferentes cepas de *T. gondii*, a progenia F1 era isolada e utilizada para identificação de traços fenotípicos (Sibley, 2009; Sibley & Ajioka, 2008). Traços fenotípicos mais complexos, como a identificação de fatores de virulência, foram identificados pela técnica de mapeamento de *lócus* de característica quantitativa (QTL). A técnica permite identificar através de análises estatísticas, a contribuição de diversos *lócus* para um determinado fenótipo e a interação entre eles (Churchill & Doerge, 1994). Esta técnica permitiu a identificação de inúmeros fatores de virulência como ROP18 (Saeij et al, 2006; Taylor et al, 2006), ROP16 (Saeij et al, 2007), ROP5 (Behnke et al, 2011; Etheridge et al, 2014; Reese et al, 2014) e ROP17 (Etheridge et al, 2014) e seus respectivos fenótipos, assim como os respectivos alvos na célula hospedeira e a importância de cada um deles para as diferentes cepas de *T. gondii* (Barragan & Sibley, 2003).

Na Europa e América do Norte, as três principais cepas de *T. gondii* são classificadas geneticamente como Tipo I, II e III (Howe & Sibley, 1995) e

apresentam fenótipos distintos em camundongos como, virulência, persistência, capacidade migratória e expressão de citocinas (Saeij et al, 2005). Essa classificação é baseada na capacidade do parasito em causar doença em camundongos, onde as cepas tipo I são as mais virulentas, pois o inóculo de um taquizoíto é capaz de matar camundongos (Sibley & Boothroyd, 1992). Em contraste, cepas tipo II e III são consideradas não virulentas em camundongos, pois somente inóculos acima de $1 \times 10^{3-5}$ causam a morte na fase aguda da doença (Howe et al, 1996). A alta identidade genética entre as cepas, sendo considerada como 98% clonais, sugere que as diferenças fenotípicas entre as cepas dependem de um pequeno número de genes (Howe & Sibley, 1995), grande parte localizados no cromossomo VIIa, responsável por cerca de 50% do fenótipo de virulência e 10% no cromossomo IV (Su et al, 2002). Estudos posteriores de cruzamentos genéticos entre cepas tipo I x III e mapeamento por QTL identificaram a ROP18, fator de virulência essencial para sobrevivência de *T. gondii* e um dos principais responsáveis pela diferença fenotípica entre as cepas do parasito (Saeij et al, 2006; Taylor et al, 2006).

ROP18 é um fator de virulência com atividade serina/treonina quinase que ao ser secretado durante a invasão da célula hospedeira é direcionado a PVM, decorando sua superfície voltada para o citoplasma da célula (Molestina & Sinai, 2005). É uma proteína polimórfica, isto é, seu nível de expressão varia de acordo com o tipo da cepa de *T. gondii* (Saeij et al, 2006; Taylor et al, 2006), na quais cepas tipo I e tipo II expressam altos níveis de ROP18, enquanto cepas tipo III expressam baixos níveis deste fator de virulência (Saeij et al, 2006; Taylor et al, 2006). Ademais, estudos posteriores de expressão transgênica da ROP18 de cepas do tipo I em cepas tipo II e III aumentaram fortemente a virulência destes parasitos, confirmando seu papel na virulência de *T. gondii* (Saeij et al, 2006; Taylor et al, 2006). A atividade quinase da ROP18 desempenha papel chave na virulência, pois mutações no aminoácido aspartato no domínio quinase da proteína gera perda de virulência de parasitos tipo III expressando trangenicamente ROP18 de parasitos tipo I (Taylor et al, 2006).

Tem sido demonstrado que a atividade quinase da ROP18 é chave na evasão do sistema imune do hospedeiro mediado por GTPases relacionadas com a imunidade (IRGs) (Etheridge et al, 2014; Zhao et al, 2009). IRGs são proteínas fortemente induzidas por IFN (Bekpen et al, 2005; Li et al, 2009) que se ligam a PVM contendo cepas menos virulentas de *T. gondii* (cepas tipo II e III). IRGs são capazes de realizar, através de um mecanismo ainda não completamente elucidado, a ruptura da PVM (Khaminets et al, 2010), deixando o parasito mais susceptível a imunidade celular autônoma da célula hospedeira (Zhao et al, 2009). Porém, cepas virulentas (tipo I) de *T. gondii* evadem desse mecanismo microbicida, pois ROP18 é capaz de fosforilar e inativar a atividade das proteínas IRGs (Etheridge et al, 2014; Zhao et al, 2009).

Estudos de cruzamentos genéticos entre cepas tipo II x III de *T. gondii* e mapeamento genético por QTL levaram a identificação da ROP16, importante fator de virulência que é capaz de alterar a transcrição gênica do hospedeiro (Butcher et al, 2011; Saeij et al, 2007). Essa alteração na transcrição gênica foi identificada através da análise da expressão gênica da célula hospedeira infectada com diferentes cepas de *T. gondii* (Saeij et al, 2007). Cepas tipo I e III do parasito são capazes de ativar e sustentar a ativação de Transdutor de Sinal e Ativador de Transcrição (STAT) STAT3 e STAT6 via ROP16, pois compartilham a mesma variante do fator de virulência (Butcher et al, 2011; Saeij et al, 2007). Em contraste, cepas tipo II de *T. gondii* falham em sustentar a ativação de STAT3 e STAT6 (Saeij et al, 2007). Estudos comparando as variantes de ROP16 nas três cepas do parasito mostram que uma mutação única no aminoácido de posição 503 é responsável pela diferença na ativação das STATs e consequente diferença na capacidade de alteração da expressão gênica do hospedeiro (Yamamoto et al, 2009). Ativação prolongada de STAT3 e STAT6 reduz a indução de IL-12, limitando a capacidade de resposta imune do hospedeiro contra *T. gondii*, consequentemente reduzindo a inflamação e patologia, em detrimento do aumento da carga parasitária (Saeij et al, 2007).

Mapeamento genético por QTL em cruzamento genéticos entre cepas tipo I x II e cepas tipo II x III de *T. gondii* levaram a identificação de um grupo de genes

repetidos altamente polimórficos no cromossomo XII de *T. gondii*, que codifica o fator de virulência ROP5 com atividade pseudoquinase (Behnke et al, 2011; Reese et al, 2011). ROP5 age de maneira cooperativa se ligando a ROP18 e aumentando sua atividade kinase (Behnke et al, 2012), e ao mesmo tempo se liga às proteínas IRGs (Reese et al, 2014), o que facilita a fosforilação e inativação das IRGs pela ROP18. Ademais, parasitos da cepa tipo I *knockout* para ROP5 possuem sua virulência fortemente atenuada, demonstrando a importância desse fator de virulência associado a ROP18 para evasão de *T. gondii* aos IRGs e sobrevivência do parasito (Reese et al, 2011; Saeij et al, 2007). Estudos posteriores mostraram que ROP5 se liga à ROP18 formando um complexo com outras proteínas efetoras, o que levou a identificação de um novo fator de virulência, ROP17 (Etheridge et al, 2014) (Figura 3).

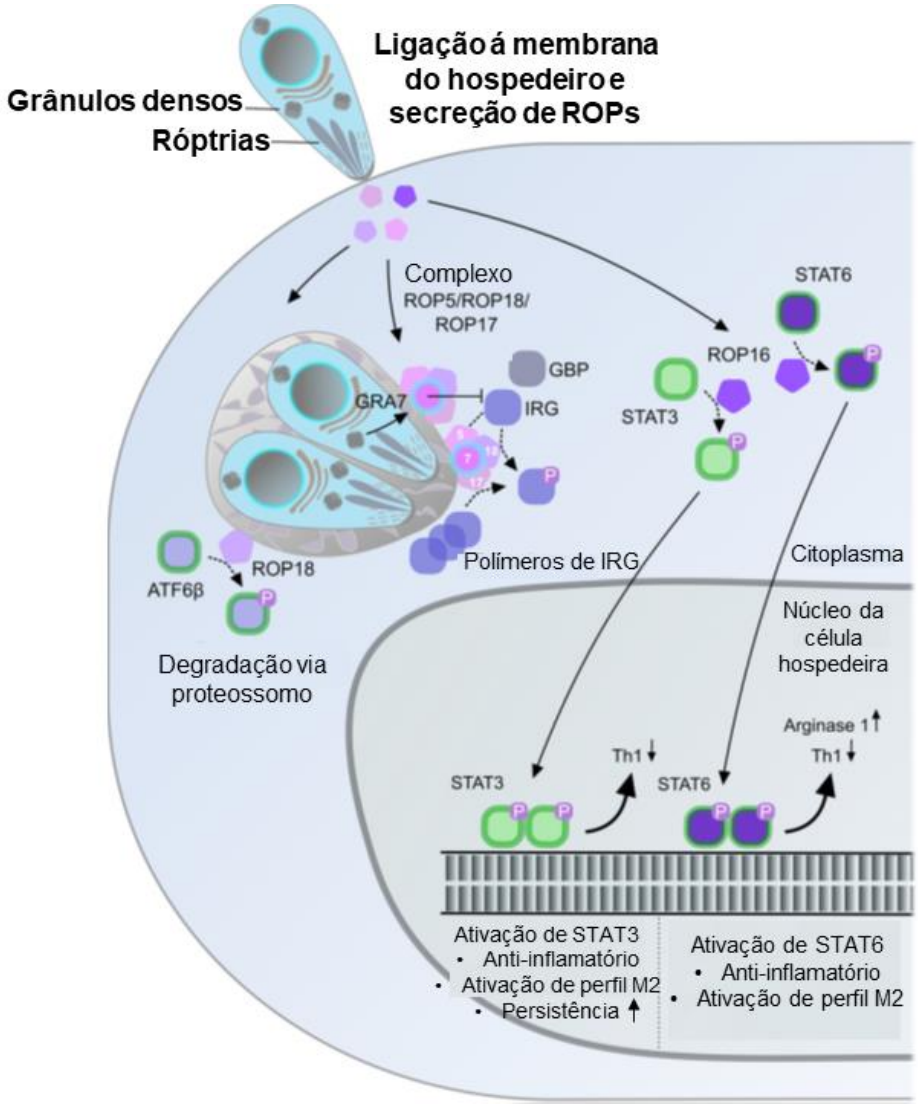


Figura 3: Proteínas efetoras ROPs secretadas durante o processo de invasão da célula hospedeira e seu papel na evasão imune de IRGs. IRG: GTPases relacionadas com a imunidade; GBP: Proteína ligadora de guanilato; STAT3/STAT6: Transdutor de sinal e ativador de transcrição 3/6; ATF6b: Fator de transcrição ativador 6 beta. Adaptado de Hakime et al. (2017).

ROP17 é um fator de virulência que assim como ROP18, possui atividade quinase, porém, diferente da ROP18, ROP17 não foi mapeada em nenhum dos cruzamentos genéticos até então (Etheridge et al, 2014). Outro fato interessante é que a deleção de ROP17 é sinérgica com a perda de ROP18 e ROP5, sugerindo fortemente a formação de um complexo de fatores de virulência ROP5-ROP17-ROP18, no qual posteriormente mostrou-se a participação de proteínas dos grânulos densos, GRA7, nessa formação (Alaganan et al, 2014). ROP17 realiza

papel central na virulência de *T. gondii*, pois fosforila oligômeros de IRGs, acelerando o *turnover* destas proteínas. Deste modo, o complexo ROP5-ROP17-ROP18-GRA7 e outros fatores de virulência que ainda não foram identificados e que participam da formação deste complexo são essenciais na sobrevivência do parasito e um dos principais responsáveis pelas diferenças fenotípicas encontradas entre as cepas de *T. gondii* (Figura 3) (Hakimi et al, 2017; Sibley & Ajioka, 2008).

2.3.2- TgIST

IFN-γ é uma citocina pró-inflamatória que desempenha papel central na imunidade contra *T. gondii* (Nathan et al, 1983; Suzuki et al, 1989). A ligação de IFN-γ ao seu receptor de membrana induz a dimerização de JAK e consequente fosforilação e ativação de STAT1 (Shuai et al, 1993). Após fosforilação, STAT1 sofre dimerização e translocação para o núcleo da célula, onde se liga a uma região conservada conhecida como sequencia ativadora gama (GAS), resultando na transcrição de genes essenciais na resposta imune (Darnell et al, 1994). Estudos mostram que *T. gondii* bloqueia a transcrição via STAT1, mesmo após fosforilação, dimerização e translocação deste fator de transcrição para o núcleo da célula hospedeira (Rosowski et al, 2014; Schneider et al, 2013). Ademais, o bloqueio da transcrição por STAT1 na célula hospedeira infectada, mesmo em associação ao GAS, ocorre independente das linhagens clonais de *T. gondii* (Kim et al, 2007; Rosowski et al, 2014), isto é, cepas tipo I, II, e III (Rosowski et al, 2014). Esses achados indicam que o bloqueio é independente de todos os fatores de virulência descritos até recentemente (Hunter & Sibley, 2012), sugerindo o papel de um novo efetor na evasão deste mecanismo microbicida. Apenas recentemente elucidou-se o mecanismo de inibição de STAT1 em células infectadas com *T. gondii* (Gay & Braun, 2016; Olias et al, 2016).

Durante a invasão da célula hospedeira, *T. gondii* secreta dos grânulos densos o fator de virulência identificado como inibidor de transcrição de STAT (TgIST). Em células infectadas com *T. gondii*, TgIST é translocado através do PVM e se acumula no núcleo da célula hospedeira, onde se liga firmemente a STAT1 recrutando proteínas modificadoras de cromatina (Olias et al, 2016) (Figura 4).

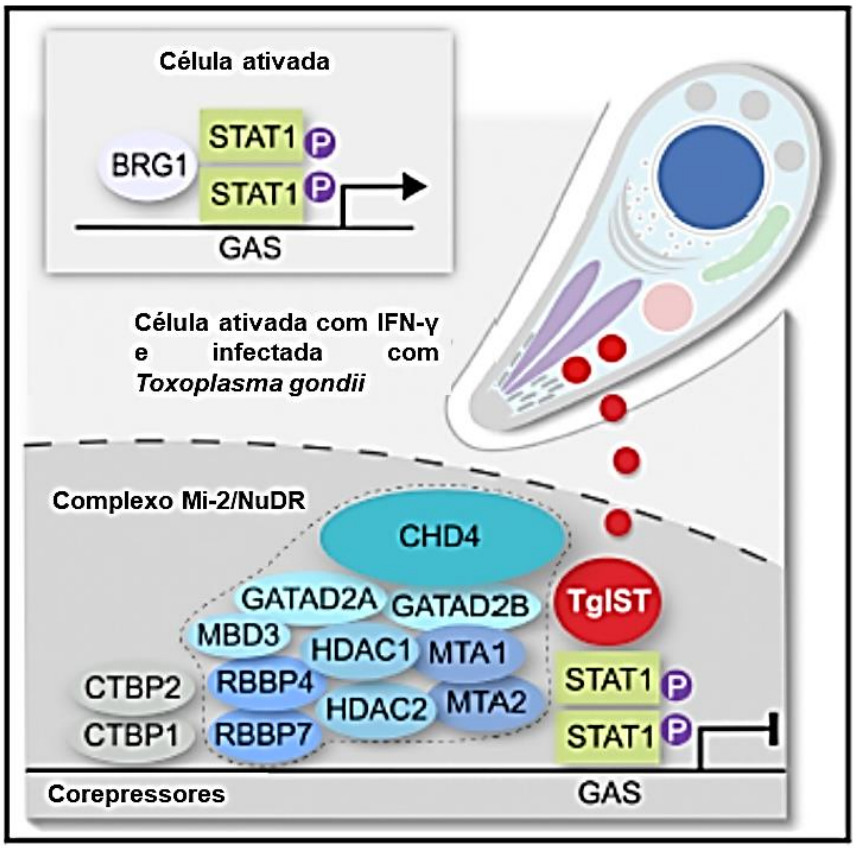


Figura 4: Mecanismo molecular de inibição da STAT1 por TgIST. STAT1: Transdutor de sinal e ativador de transcrição 1; GAS: Sequência ativadora gama; BRG1: Coregulador transcricional BRG1; CHD4: Proteína ligadora de DNA cromodomínio helicase 4; GATAD2A/2B: Domínios de dedo de zinco GATA 2A/GATA2B. HDAC1/2: Histona desacetilase 1/2; MTA1/2: Proteína associada a metástase 1/2; RBBP4/7: Proteína ligadora de histona 4/7; CTBP1/2: Proteína ligadora c terminal 1; TgIST: inibidor de transcrição de STAT. Adaptado de Olias et al. (2016).

Esse recrutamento forma um complexo composto de proteínas desacetilase e remodelador de nucleossomo (Mi-2/NuRD) que bloqueia a transcrição de STAT1, resultando no aumento da sobrevivência e virulência de *T. gondii*. Ademais, tem sido demonstrado que TgIST se liga a histonas desacetilases para prevenir a acetilação de STAT1 e dissociação do DNA, deste modo, comprometendo a reciclagem de STAT1 (Kramer & Heinzel, 2010). A nível celular, TgIST tem papel essencial durante os estágios iniciais de infecção, pois protege os taquizoítos que invadem células não estimuladas da ação de genes ativados por IFN, prevenindo a ativação destas células (Olias et al, 2016). No entanto, em células previamente estimuladas com IFN-γ, TgIST não é capaz de bloquear a transcrição de STAT1,

sugerindo que outras proteínas efetoras são responsáveis por garantir a sobrevivência de *T. gondii* (Olias et al, 2016).

2.3.3- ASP5 e MYR1

Parasitos do filo Apicomplexa, como *T. gondii* e *Plasmodium* spp. se desenvolvem dentro do PV cercado por uma PVM (Lingelbach & Joiner, 1998). Esta estrutura é formada durante a invasão da célula hospedeira, onde o parasito reside, se protege, obtêm nutrientes e altera o perfil de expressão gênica na célula infectada (Bougdour et al, 2014; Hiller et al, 2004). Embora o PV represente uma barreira de proteção contra ações microbicidas do hospedeiro, a sobrevivência de *T. gondii* é dependente da secreção e exportação de fatores de virulência através da PVM, alcançando diferentes alvos, como a própria PVM e núcleo da célula hospedeira (Bougdour et al, 2013; Bougdour et al, 2014; Braun et al, 2013). Estudos de bioinformática identificaram genes de *T. gondii* e *Plasmodium* spp. codificando proteínas que expressam sinais de localização nuclear, tornando estes, possíveis candidatos na modulação da transcrição gênica do hospedeiro (Bougdour et al, 2013; Braun et al, 2013). Porém, um fator importante ainda precisava ser elucidado: como proteínas do parasito são secretadas e alcançam diferentes alvos, como a PVM e núcleo da célula hospedeira? Nos últimos anos, grande esforço tem sido feito para elucidar o tráfego e exportação de proteínas através da via secretora de parasitos do filo Apicomplexa, principalmente em *T. gondii* e *Plasmodium* spp. um grande avanço foi obtido.

Atualmente, o maior avanço na compreensão de tráfego de proteínas na via secretora e sua exportação para a célula hospedeira além do espaço vacuolar é em *Plasmodium* spp. (Boddey et al, 2013). Neste parasito, proteínas da via secretora possuem um *motif* na região N-terminal chamado de elemento de exportação de *Plasmodium* (PEXEL), também conhecido como sinal de transporte nuclear. A exportação destas proteínas é dependente de maturação e clivagem no retículo endoplasmático do parasito por uma protease conhecida como plasmepsina V (Boddey et al, 2010; Russo et al, 2010; Sleebs et al, 2014). Após sofrer modificações pós-traducionais pela plasmepsina V, as proteínas da via secretora são transportadas através da PVM por proteínas especializadas,

podendo então alcançar seu destino final na célula hospedeira (de Koning-Ward et al, 2009; Spillman et al, 2015). Partindo do princípio de que este mecanismo de edição pós-traducional e tráfego de proteínas a partir do VP via proteínas na PVM é conservado ao longo do filo, estudos similares foram realizados a fim de se elucidar o mecanismo de tráfego de proteínas efetoras em *T. gondii*. Estes estudos levaram a identificação da aspartil protease 5 (ASP5) (Coffey & Sleebs, 2015; Curt-Varesano et al, 2016; Hammoudi et al, 2015) homólogo da plasmepsina V em *Plasmodium* spp. (Boddey et al, 2010) e proteína de regulação c-myc 1 (MYR1) (Franco et al, 2016) na clivagem e exportação de inúmeros fatores de virulência proveniente dos grânulos densos (Figura 5).

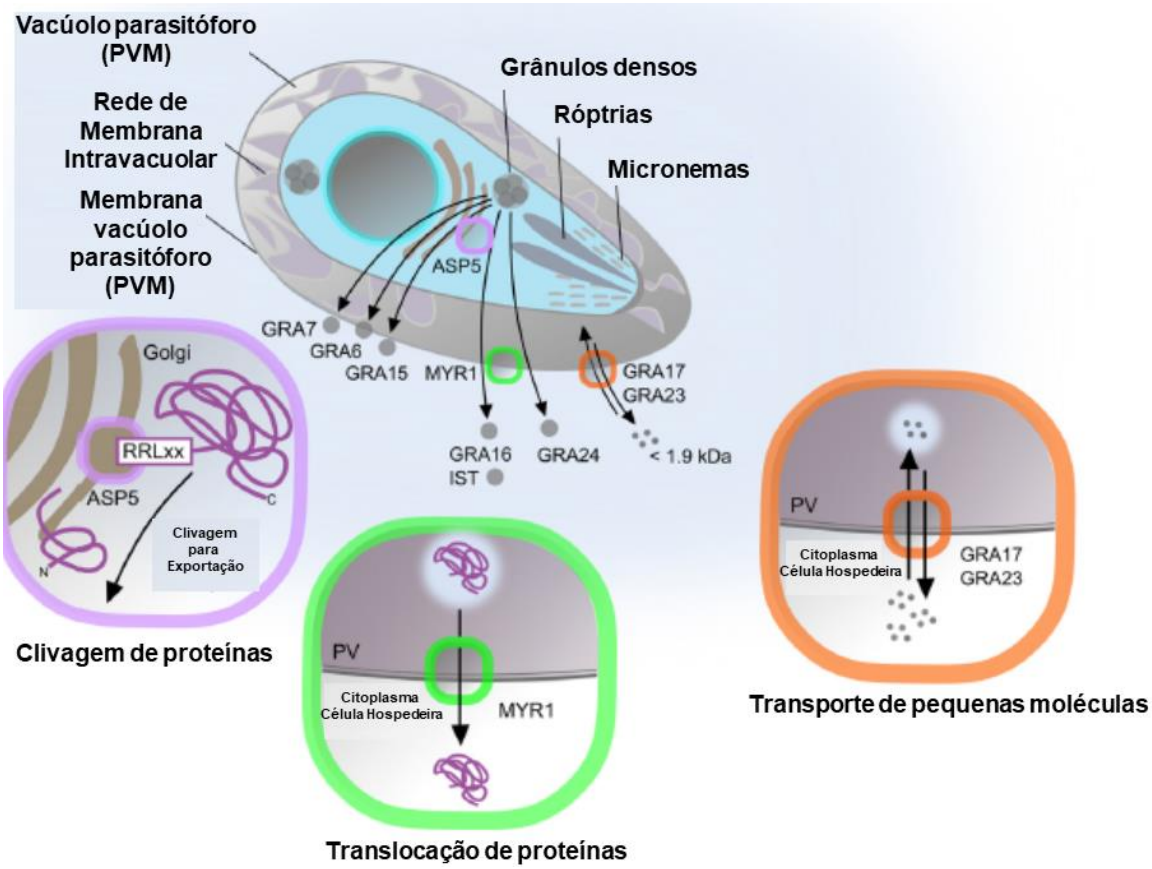


Figura 5: Mecanismo de exportação e tráfego de fatores de virulência dos grânulos densos através do vacúolo parasitófaro (VP). ASP5 (aspartil protease 5) é localizada no complexo de Golgi e realiza a clivagem de proteínas com domínio PEXEL (elemento exportador de *Plasmodium*), enquanto MYR1 (proteína de regulação c-myc 1) é situada na membrana do VP e é responsável pelo transporte de proteínas com destino na célula hospedeira. Adaptado de Hakimi et al. (2017).

ASP5 é uma protease associada ao complexo de Golgi responsável pela clivagem de proteínas com domínio PEXEL a serem exportadas na via secretora de *T. gondii*, seja para a PVM ou célula hospedeira (Coffey & Sleebs, 2015; Curt-Varesano et al, 2016; Hammoudi et al, 2015). Ademais, foi a primeira proteína da via secretora dos grânulos densos a ser identificada (Coffey & Sleebs, 2015). Recentemente, a clivagem e exportação de uma nova classe de fatores de virulência provenientes dos grânulos densos de *T. gondii* foi identificada: 1- GRA16 (Bougdour et al, 2013), exportado para o núcleo da célula hospedeira para bloqueio a progressão do ciclo celular, evitando a apoptose; 2- GRA24 (Braun et al, 2013) responsável pelo controle da expressão gênica do hospedeiro, principalmente aquelas envolvidas na resposta imune; 3- GRA15 (Rosowski et al, 2011), envolvida na ativação do fator nuclear kappa b (NF-κB) em linfócitos B; 4- GRA17 e GRA23 (Gold et al, 2015), responsáveis pelo transporte de pequenas moléculas através da PVM. Parasitos *knockout* para ASP5 (Δasp5) falham em secretar GRA16 e GRA24 para o núcleo da célula hospedeira, onde permanecem retidas no PV (Coffey & Sleebs, 2015; Curt-Varesano et al, 2016; Hammoudi et al, 2015). Ademais, parasitos Δasp5 são incapazes de secretar GRA16, GRA19, GRA20, mostrando que esses fatores de virulência são diretamente processados pela ASP5 (Coffey & Sleebs, 2015; Curt-Varesano et al, 2016; Hammoudi et al, 2015). Também tem sido sugerido uma via alternativa de secreção de proteínas da via secretora que não possuem o domínio PEXEL conservado, como a GRA24, que é processada de maneira independente de ASP5, embora esta protease seja requerida para a exportação da GRA24 (Curt-Varesano et al, 2016) . ASP5 também está envolvida na exportação de TgIST, porém ainda não se sabe se seu domínio PEXEL é diretamente clivado por ASP5 ou por outra proteína ainda não identificada (Gay & Braun, 2016). Ademais, a deleção de ASP5 em *T. gondii* induz a perda da capacidade de recrutar mitocôndrias do hospedeiro para a PVM, diminuição da capacidade de modulação da expressão gênica do hospedeiro e atenuação da virulência em camundongos (Coffey & Sleebs, 2015; Hammoudi et al, 2015), demonstrando o papel essencial de ASP5 para o parasito.

O modelo de translocação de proteínas da via secretora em *Plasmodium* spp. através da PVM também levou a identificação de outra importante proteína da via secretora em *T. gondii*, a MYR1 (Franco et al, 2016). MYR1 é uma proteína localizada na PVM responsável por exportar proteínas dos grânulos densos que sofreram modificações pós-traducionais pela ASP5, através da PVM ao citoplasma da célula hospedeira, sendo provavelmente um dos componentes de um complexo de proteínas responsáveis por este transporte (Franco et al, 2016). MYR1 também é processada por ASP5, sendo clivada em duas porções (Coffey & Sleebs, 2015) que permanecem ligadas a PVM (Franco et al, 2016). Parasitos *knockout* para MYR1 falham em exportar GRA16, GRA24 e TgIST, e consequentemente, perdem a habilidade de modular importantes vias de transcrição da célula hospedeira (Franco et al, 2016). Em contraste, o transporte de GRA15 não foi alterado, indicando que MYR1 age majoritariamente no transporte de proteínas com alvos intracelulares na célula hospedeira, como o núcleo celular (Franco et al, 2016). Estes resultados evidenciam a importância de ASP5 e MYR1 na habilidade do parasito modular importantes vias de transcrição do hospedeiro e consequentemente evadir do sistema imune dessas células, garantindo sua sobrevivência (Coffey & Sleebs, 2015; Curt-Varesano et al, 2016; Franco et al, 2016; Hammoudi et al, 2015). Deste modo, entender como fatores de virulência participam na modulação da resposta imune do hospedeiro e como essas proteínas especializadas são transportadas do *T. gondii* para a célula hospedeira através do PV pode levar ao maior entendimento da biologia do parasito e o desenvolvimento de possíveis alvos para o controle deste importante parasito de abrangência mundial.

2.4- Macrófagos

Macrófagos são células do sistema imune e um dos componentes chave de uma família de células funcionalmente relacionadas ao conceito conhecido como “Sistema Fagocítico Mononuclear”, que além dos macrófagos, inclui monócitos circulantes do sangue e células precursoras localizadas na medula (Hume, 2008; van Furth & Cohn, 1968; van Furth et al, 1972). Durante muitos anos, a origem dos macrófagos se tornou motivo de grande debate entre imunologistas. A definição do

sistema fagocítico mononuclear foi inicialmente proposto por Van Furth et al. (1968; 1972) no final dos anos 60 e início dos anos 70 . Esse conceito propõe que macrófagos se originam, desenvolvem e se renovam a partir de monócitos derivados da medula, os quais realizam diapedese e se diferenciam em macrófagos teciduais (van Furth et al, 1972; Volkman & Gowans, 1965), conceito até hoje aceito. Porém, têm sido proposta algumas modificações nesse conceito, pois ao longo dos anos, inúmeros trabalhos independentes mostraram que nem todos os macrófagos necessariamente são derivados de monócitos (Ginhoux et al, 2010; Lichanska et al, 1999; Schulz et al, 2012; Sorokin et al, 1992). O trabalho pioneiro de del Rio-Hortega mostrou que micróglias, macrófagos teciduais residentes do cérebro, derivam de células provenientes do saco vitelino (del Rio-Hortega, 1932).

Diversos trabalhos recentes também mostraram que diferentes tipos de macrófagos residentes teciduais também derivam do saco vitelino e possuem capacidade de auto renovação na maioria dos tecidos de camundongo (Gomez Perdiguero et al, 2015; Hashimoto et al, 2013). Estas células são formadas nos estágios iniciais do desenvolvimento embrionário, em um estágio conhecido como hematopoese primitiva (Samokhvalov, 2014). Durante esta fase do processo ontogenético, os macrófagos são as únicas células de defesa que são formadas, similar ao que ocorre no sistema imune de *Drosophila*, indicando que a formação dos macrófagos no saco vitelino pode ter uma origem conservada (Makhijani & Bruckner, 2012). Outro indício de que os macrófagos se formam independentemente dos monócitos provenientes da medula, é que as células tronco hematopoiéticas derivadas da medula são formadas apenas no período perinatal, produzindo as demais linhagens de células do sistema imune (Orkin & Zon, 2008). Esses achados introduziram um novo paradigma acerca do entendimento da biologia do desenvolvimento e função dos macrófagos residentes teciduais.

Macrófagos são células evolutivamente conservadas e foram inicialmente descritas por Ilya Metchnikoff no final do século 19 (Cooper & Alder, 2006; Metchnikoff, 1883; Metchnikoff, 1892; Tauber, 2003), formando um grupo

fenotipicamente e funcionalmente heterogêneo de células, os macrófagos residentes teciduais (Gomez Perdiguero et al, 2015; Sorokin et al, 1992). A heterogeneidade destas células pode ser identificada *in situ* por antígenos de diferenciação e padrões de expressão gênica (Andreesen et al, 1988). Neste sentido, o desenvolvimento de anticorpos monoclonais para identificação de antígenos de membrana seletivamente expressos na superfície dos macrófagos permitiu a detecção precisa destas células em diferentes órgãos, assim como sua heterogeneidade (Taylor et al, 2005). O antígeno clássico utilizado na identificação de macrófagos, F4/80, desempenha papel central na detecção destas células em diferentes partes do organismo em camundongos (Hume & Gordon, 1983). Ademais, análises por *microarray* e proteômica permitiram a identificação de proteínas expressas em todos os macrófagos ou que são características de um tipo especializado de macrófago, demonstrando o papel central destas células em diferentes órgãos (Lavin et al, 2014). O Projeto Genoma Imunológico revelou que as diferentes populações de macrófagos teciduais possuem grande diversidade transcricional, sugerindo a presença de diferentes classes de macrófagos no organismo que expressam uma gama de receptores de superfície e intracelular (Gautier et al, 2012).

A maioria dos tecidos dos vertebrados possuem macrófagos residentes que desempenham funções tecido-específicas para manutenção da homeostase (Geissmann et al, 2010). O conceito de homeostase foi inicialmente proposto pelo neurocientista Walter Cannon, baseado nos estudos de Claude Bernard (Cooper, 2008). Na manutenção da homeostase tecidual, o sistema imune possui complexa rede de células e componentes solúveis que age sinergicamente para responder as mudanças fisiológicas dos tecidos (Chorro et al, 2009). Mesmo sendo uma célula completamente diferenciada, as mudanças fisiológicas do tecido são respondidas com alteração específica no perfil de expressão gênica dos macrófagos, revelando sua plasticidade fenotípica (Lavin et al, 2014).

O conceito de plasticidade fenotípica e a nomenclatura de macrófagos têm sido largamente discutido, e muitas vezes gera confusão por serem células versáteis (Chavez-Galan et al, 2015; Martinez & Gordon, 2014; Mills & Ley, 2014).

A classificação dos macrófagos se iniciou em um trabalho com células T *helper*, na qual estas se polarizavam e assumiam funções distintas de acordo com o padrão de citocinas que elas produziam: células T *helper* tipo 1 (Th1) e tipo 2 (Th2) (Mosmann et al, 1986) (Figura 6).

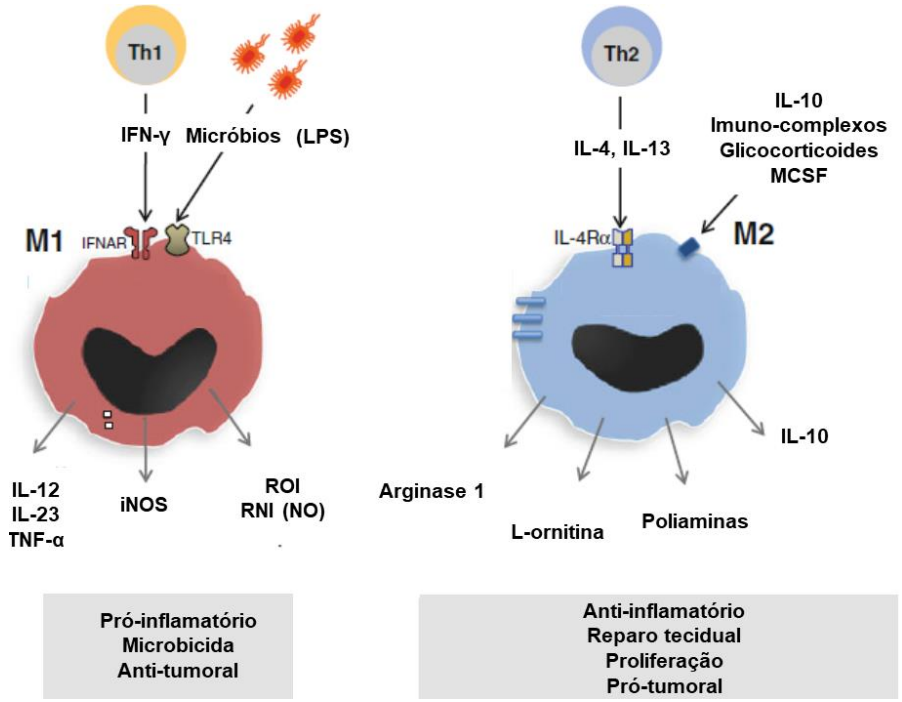


Figura 6: Representação esquemática dos perfis de polarização de macrófagos M1 e M2 e suas respectivas características funcionais e metabólicas. IFN-γ: Interferon-gama; LPS: Lipopolissacarídeo; IL: interleucina; MCSF: Fator estimulador de colônias de macrófagos; IFNAR: Receptor de interferon alfa/beta; TLR4: Receptor do tipo toll; IL-4Ra: Receptor de interleucina 4; ROI: Espécies reativas de oxigênio; RNI: Espécies reativas de nitrogênio; NO: Óxido nítrico; iNOS: Óxido nítrico sintase induzida; TNF-a: Fator de necrose tumoral alfa. Adaptado de Biswas et al. (2012).

Natan et al (1983) mostraram que IFN-γ estimula a produção de moléculas oxidativas nestas células, que se tornam altamente eficientes na eliminação de patógenos intracelulares . Em seguida, foi mostrado que a citocina anti-inflamatória IL-4 induz um perfil de expressão gênica em macrófagos muito diferente quando comparado ao induzido por IFN-γ (Stein et al, 1992). Em 2000, Mills et al. (2000) propuseram a nomenclatura M1-M2 . De acordo com os estímulos recebidos em seu microambiente, os macrófagos podem assumir distintas funções e dois perfis básicos de polarização podem ser definidos: 1- macrófagos classicamente ativados

(M1) e 2- macrófagos alternativamente ativados (M2) (Figura 6). Macrófagos assumem perfil M1 de ativação quando estimulados com citocinas pró-inflamatórias como IFN-γ (Mills et al, 2000), lipopolissacarídeos (LPS) presentes na parede celular de bactérias gram-negativas (Poltorak et al, 1998) e fator estimulador de colônias de granulócitos e macrófagos (Fleetwood et al, 2007). Estas células se caracterizam pela produção de citocinas pró-inflamatórias, fortes propriedades microbicidas com produção de espécies reativas de oxigênio, espécies reativas de nitrogênio, como óxido nítrico (NO), e expressão da óxido nítrico sintase induzida (iNOS), mediando a resistência a patógenos (Khan et al, 1997). Macrófagos assumem perfil M2 quando estimulados com IL-10, IL-4, TGF-β, 8-Br-cAMP e são caracterizados pela expressão de receptor de manose e da enzima ARG1 (Barksdale et al, 2004; Munder et al, 1999). Macrófagos M2 desempenham papel chave no reparo tecidual e angiogênese (Jenkins et al, 2013; Martinez et al, 2008). O balanço entre os perfis de ativação M1-M2 é essencial para a manutenção da homeostase tecidual (Recalcati et al, 2010). Ademais, estudos identificaram outros perfis de ativação destas células para desempenhar funções tecido-específicas, como macrófagos associados a tumor, envolvidos no câncer (Mantovani et al, 2006); macrófagos $CD169^{+}$, subpopulação encontrada nos órgão linfoides e que desempenham papel na tolerância imune e apresentação de antígenos (Crocker & Gordon, 1986; Mebius & Kraal, 2005); e macrófago receptor de células T positivo (Puellmann et al, 2006). Deste modo, a definição dos perfis de ativação dos macrófagos vai além do paradigma “M1-M2”.

Estudos recentes têm reconhecido o papel central dos macrófagos como uma linha primária de defesa na resposta imune em praticamente todos os animais (Cooper, 2010; Mills & Ley, 2014). O papel central destas células na defesa do organismo fica ainda mais evidente, pois 95% do reino animal não possui imunidade adaptativa, ou seja, mediada por linfócitos T e B (Mills & Ley, 2014) Dentre as inúmeras funções desempenhadas pelos macrófagos na resposta imune, a biologia da interação entre macrófagos e parasitos, como *T. gondii*, tem recebido grande atenção (Hakimi et al, 2017; Hunter & Sibley, 2012). Macrófagos são capazes de controlar a replicação de *T. gondii* através de inúmeros mecanismos

microbicidas como: a) fagocitose (Jones et al, 1972), b) espécies reativas de oxigênio (Murray & Cohn, 1979), c) expressão de iNOS e produção de NO (Khan et al, 1997), e d) GTPases relacionadas com a imunidade (Hunn et al, 2011). Estes mecanismos microbicidas mediados pelos macrófagos são capazes de controlar a replicação de *T. gondii* e garantir a sobrevivência do hospedeiro.

2.5- Óxido Nítrico Sintase induzida e óxido nítrico

Óxido nítrico sintases (NOSs) são enzimas que possuem peso molecular que varia entre 110kDa a 160 kDa dependendo da isoforma (Bredt et al, 1991; Chartrain et al, 1994; Marsden et al, 1993). São cataliticamente ativas quando sofrem dimerização e requerem dois substratos, L-arginina e oxigênio molecular em combinação com diversos cofatores como nicotinamida adenina dinucleotídeo fosfato, flavina adenina dinucleotídeo, flavina mononucleotídeo e tetraidrobiopterina para gerar L- citrulina e NO (Klatt et al, 1996). A reação de catálise do NO é realizada em duas fases: o substrato L-arginina é primeiramente hidrolisado em N-hidroxi L-arginina e em seguida oxidado em L-citrulina e NO (Klatt et al, 1996). Existem quatro isoformas da enzima, com distintos padrões de expressão e características: óxido nítrico sintase neuronal (nNOS ou NOS1) expressa nos neurônios (Kishimoto et al, 1992), óxido nítrico sintase induzida (iNOS ou NOS2) expressa em resposta ao LPS (Stuehr & Marletta, 1985) e citocinas pro-inflamatórias (Stuehr & Marletta, 1987), óxido nítrico sintase endotelial (eNOS ou NOS3) expressa em endotélio (Marsden et al, 1993), e uma quarta isoforma de NOS presente em mitocôndrias no retículo sarcoplasmático cardíaco, sendo conhecida como NOS mitocondrial (mtNOS) (Ghafourifar & Richter, 1997; Valdez et al, 2005; Zaobornyj & Ghafourifar, 2012).

iNOS é uma enzima homodimérica que, assim como as outras isoformas de NOS, converte L-arginina em NO (Xie et al, 1992). Porém, diferente das outras isoformas da enzima, a iNOS é cálcio independente e não é constitutivamente expressa (Casado et al, 1997; Saini et al, 2006). Diversos estudos identificam a localização celular da expressão da iNOS enzimaticamente ativa em macrófagos e neutrófilos no citoplasma e núcleo (Saini et al, 2006), pequenas vesículas

(Vodovotz et al, 1995), grânulos primários (Evans et al, 1996) e citoesqueleto de actina (Webb et al, 2001).

Estudos das regiões promotoras do gene de iNOS mostram que a expressão desta enzima é fortemente induzida em resposta ao LPS (Stuehr & Marletta, 1985) e citocinas pró-inflamatórias (Stuehr & Marletta, 1987). A via de sinalização mediada por LPS é ativada após reconhecimento desta molécula por receptores do tipo toll (TLRs) na superfície de macrófagos, principalmente TLR4 (Lorenz et al, 2002), induzindo uma cascata de sinalização que ativa duas proteínas adaptadoras da via: IRAK (Swantek et al, 2000) e MyD88 (Kawai et al, 1999). Em seguida, o sistema imune ativa a via de sinalização mediada por NF-κB. Estudos pioneiros em macrófagos de camundongo identificaram o papel central de NF-κB na indução da expressão da iNOS (Lowenstein et al, 1993; Xie et al, 1993). NF-κB é um fator de transcrição que foi identificado em 1986 (Sen & Baltimore, 1986), sendo expresso na sua forma inativa no citoplasma das células, ligadas a proteínas inibitórias de NF-κB (Sen & Baltimore, 1986). Após fosforilação e consequente degradação das proteínas inibitórias pelo proteossomo, NF-κB é translocado para o núcleo da célula onde se liga a regiões promotoras dos genes alvo, como o da iNOS, onde a expressão do seu mRNA ocorre entre 1-2 horas após ativação com IFN-γ e LPS (Lowenstein et al, 1993)Lowenstein et al., 1993) (Figura 7).

A transdução de sinal mediada por citocinas pró-inflamatórias como interferon tipo I (IFN-α/β) e interferon tipo II (IFN-γ) foi uma das primeiras vias identificadas na indução da iNOS em macrófagos de camundongo (Lowenstein et al, 1993; Xie et al, 1993). Esta via de sinalização é mediada por Janus kinase (JAK) e STAT, mais conhecida como via JAK/STAT (Blanchette et al, 2003; Hu & Ivashkiv, 2009). A ligação de IFNs a seu receptor específico na superfície dos macrófagos resulta na ativação de JAK, que fosforila resíduos de tirosina nas proteínas STAT (Blanchette et al, 2003; Lowenstein & Padalko, 2004). Após fosforilação, STAT sofre dimerização e migra para o núcleo celular onde regula a transcrição de genes alvo, como a iNOS (Blanchette et al, 2003; Lowenstein & Padalko, 2004) (Figura 7). Trabalhos pioneiros com camundongos *knockout* para

IFN-γ e seu receptor mostraram que estes animais possuem deficiência na indução de iNOS (Kamijo et al, 1993).

Estudos mostram que LPS e IFN-γ podem agir sinergicamente para máxima ativação gênica da iNOS (Lowenstein et al, 1993; Xie et al, 1993), tanto em células de camundongo quanto em células de humanos. Essa máxima produção se deve a uma mudança conformacional sofrida pelo locus que contém o gene da iNOS permitindo que múltiplos fatores de transcrição se liguem em sua região promotora, aumentando sua transcrição (Perrella et al, 1999). Ademais, outro importante componente da via conhecido como GAS foi identificado na região promotora de iNOS em macrófagos de camundongos, servindo como sitio de ligação para STAT (Gao et al, 1997). A ativação sinergética da iNOS por LPS e IFN-γ resulta em máxima produção de NO em macrófagos, molécula efetora do sistema imune inato que desempenha inúmeras funções no organismo (Gao et al, 1997).

NO é uma molécula gasosa e um radical livre de baixo peso molecular, pesando cerca de 30 Da (Moncada, 1999). Esta molécula é capaz de permear as membranas biológicas e mediar inúmeras funções homeostáticas e imunológicas (Thomas et al, 2008) em diversos sistemas biológicos. Inicialmente, foi descrito como um fator de relaxamento endotelial, isto é, um vasodilatador (Ignarro et al, 1987; Palmer et al, 1987). Atualmente, além do seu papel como vasodilatador do endotélio, sabe-se que NO está envolvido em uma série de funções no organismo, como neurotransmissão (Kuriyama & Ohkuma, 1995), inflamação (Lipton et al, 1993), secreção de insulina (Eckersten & Henningsson, 2012) e angiogênese (Murohara et al, 1998). NO também possui papel central na regulação do sistema imune, ligando a imunidade inata e imunidade adaptativa (Taylor-Robinson et al, 1993), exercendo atividade microbicida, antiviral, antitumoral (Bogdan, 2000; MacMicking et al, 1997), e antiparasitária contra microrganismos (Scharton-Kersten et al, 1997) (Figura 7).

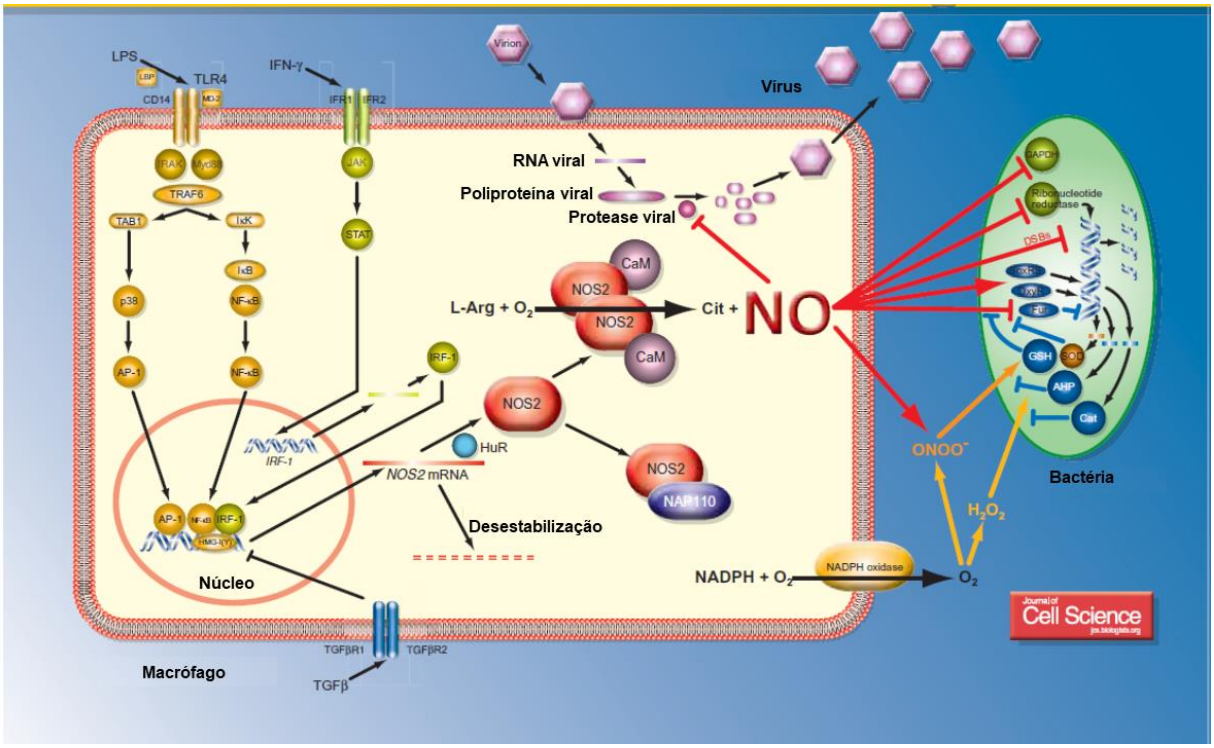


Figura 7: Efeito sinérgico da via de sinalização imune inata mediada por LPS e IFN-γ induz a expressão da óxido nítrico sintase induzida (iNOS ouNOS2) e consequente produção de óxido nítrico (NO) contra microrganismos invasores. LPS: Lipopolissacarídeo; IFN-γ: Interferon-gama; CD14: Grupo de diferenciação 14; TLR4: Receptor do tipo toll 4; IFR1-2: Receptor de interferon-gama 1-2; IRAK: Quinase associada a receptor de interleucina; Myd88: Resposta primária de diferenciação mielóide 88; TRAF6: Receptor de fator de necrose tumoral 6; TAB1: Quinase ativadora de TGF-beta;p38: Proteínas quinases ativadas por mitógeno; AP-1: Proteína ativadora 1, IkK: Proteína inibidora de quinase;IkB: Proteína quinase B; NF-kB: Fator nuclear kappa B; JAK: Janus quinase; STAT: Transdutor de sinal e ativador de transcrição; HuR: Antígeno r humano; CaM: Calmodulina; NAP110: Proteína associada a iNOS; TGF-β: Fator transformante de crescimento β; TGFbR1: Receptor de fator transformante de crescimento β1; TGFbR2: Receptor de fator transformante de crescimento β2. Adaptado de Lowenstein & Padalko (2004).

A atividade microbicida do NO contra microrganismos tem sido amplamente estudada em macrófagos (Bogdan, 2000; Bogdan, 2015; Nathan & Shiloh, 2000; Scharton-Kersten et al, 1997). Esta molécula é capaz de reagir com elementos estruturais, componentes da maquinaria de replicação, ácidos nucleicos e enzimas metabólicas, formando a base da sua ação (Drapier & Hibbs, 1986; Fang, 2004; Stamler, 1994). A ação microbicida do NO contra infecções por *T. gondii* também tem sido reportada (Scharton-Kersten et al, 1997). Dependendo da fase da infecção, NO pode mediar forte processo inflamatório no intestino durante a fase aguda de infecção, ou ser benéfica e minimizar os efeitos da infecção durante a

fase crônica da doença no sistema nervoso central (Scharton-Kersten et al, 1997). Ademais, camundongos *knockout* para iNOS ou tratados com aminoguanidina, um potente inibidor da iNOS, são mais susceptíveis a infecção por *T. gondii,* desenvolvendo alta parasitemia, ao mesmo tempo que reduz a inflamação intestinal nos estágios iniciais da infecção (Khan et al, 1997; Scharton-Kersten et al, 1997). Porém, na fase crônica da doença, NO é essencial para controlar a replicação de *T. gondii*, minimizando os efeitos da inflamação no cérebro de camundongos cronicamente infectados (Khan et al, 1997; Scharton-Kersten et al, 1997). Deste modo, o hospedeiro deve ser capaz de regular os efeitos nocivos do NO durante a fase aguda da doença e os efeitos benéficos durante a fase crônica para sobrevivência (Khan et al, 1997). Apesar dos efeitos nocivos nos estágios iniciais de infecção por *T. gondii*, NO é essencial para a sobrevivência do hospedeiro (Khan et al, 1997), mostrando o papel essencial desta importante molécula microbicida no controle da infecção por *T. gondii*.

2.6- Arginase 1

Arginase (ARG) é uma enzima encontrada em diversos organismos como arqueobactérias, bactérias, plantas, invertebrados e vertebrados (Dowling et al, 2008; Ouzounis & Kyrpides, 1994). Foi inicialmente descoberta em 1904 no fígado de mamíferos(Kossel & Dakin, 1904). Existem duas isoformas de ARG, e ambas são produtos de distintos genes localizados em cromossomos diferentes (Dizikes et al, 1986). ARG1 é citosólica e predominantemente encontrada no fígado, onde realiza a fase final do ciclo da ureia (Herzfeld & Raper, 1976), podendo também ser expressa em tecidos não hepáticos, enquanto arginase 2 é mitocondrial e encontrada em diferentes tecidos como cérebro e músculo esquelético (Morris et al, 1997; Vockley et al, 1996). Estudos de estrutura cristalográfica da ARG1 no fígado de rato (Kanyo et al, 1996) e ARG2 no rim de humanos (Cama et al, 2003) identificaram sua estrutura como sendo um homotrímero, onde cada subunidade possui 35 kDa e um centro binuclear de manganês, totalizando 105 kDa de peso molecular. Característica comum a todas ARG até hoje identificadas nos diferentes organismos é o requerimento de manganês, um cátion divalente que funciona

como um ativador fisiológico (Reczkowski & Ash, 1992), e um pH ótimo alcalino variando entre 9,0 e 9,5 (Roholt & Greenberg, 1956).

ARG é uma metaloenzima manganase binuclear que catalisa a síntese de L-ornitina e ureia a partir da hidrólise de L-arginina (Ash, 2004). L-arginina é um dos vinte aminoácidos necessários para a síntese de proteínas e sua concentração plasmática nas células é mantida majoritariamente pelo catabolismo de proteínas ou pela síntese a partir de outros aminoácidos (Windmueller & Spaeth, 1981). Em humanos, L-arginina é considerado um aminoácido semi-essencial (Flynn et al, 2002) e desempenha papel metabólico extremamente versátil, pois é molécula precursora na síntese de uréia, NO, poliaminas, prolina, glutamato, creatina e agmatina (Morris, 2007). Ademais, L-arginina é utilizada como substrato obrigatório de duas enzimas em vias metabólicas distintas, NOS (Klatt et al, 1996) e ARG (Kossel & Dakin, 1904). ARG1 é fortemente induzida em macrófagos por citocinas anti-inflamatórias como IL-4, IL-6, IL-10, IL-13 e TGF-β (Barksdale et al, 2004; Munder et al, 1999). A ligação de citocinas anti-inflamatórias ao seu respectivo receptor na superfície dos macrófagos induz a fosforilação de STAT6 por JAK, levando a dimerização e translocação de STAT6 para o núcleo dos macrófagos onde regula a transcrição de inúmeros genes, incluindo o de ARG1 (Wei et al, 2000).

ARG é a enzima central no metabolismo de L-arginina no ciclo da ureia (Krebs & Henseleit, 1932), via que envolve também a participação de outras enzimas como carbamil fosfato sintetase-I, ornitina transcarbamilase, argininosuccinato sintetase e argininosuccinato liase. Ademais, a via também requer a participação de diversas outras proteínas *in vivo* para funcionamento eficiente, como glutaminase hepática (Curthoys & Watford, 1995), N-acetilglutamato sintetase, transportadores de ornitina e citrulina mitocondrial, e transportador mitocondrial de aspartato e glutamato (Palmieri et al, 2001). O ciclo da ureia ocorre nos hepatócitos e ao final do ciclo, tem como produto a produção de L-ornitina e ureia (Krebs & Henseleit, 1932). Ademais, estudos mostram o papel central da ARG1 na produção de L-ornitina, pois este é utilizado pela ornitina descarboxilase na síntese de poliaminas em macrófagos ativados com LPS

(Nichols & Prosser, 1980; Tjandrawinata et al, 1994). Nessa via bioquímica, a ornitina descarboxilase catalisa a descarboxilação da L-ornitina, formando poliaminas, principalmente putrescina, espermina e espermidina (Kepka-Lenhart et al, 2000).

Poliaminas são moléculas orgânicas policatiônicas que desempenham inúmeras funções celulares, como cicatrização tecidual (Maeno et al, 1990), regulação de canais iônicos (Lopatin et al, 1994) e proliferação celular (Heby, 1981; Landau et al, 2010). O papel das poliaminas na proliferação celular de *T. gondii* e sua interação com o sistema imune tem sido reportado (Cook et al, 2007; Pfaff et al, 2005) (Figura 8).

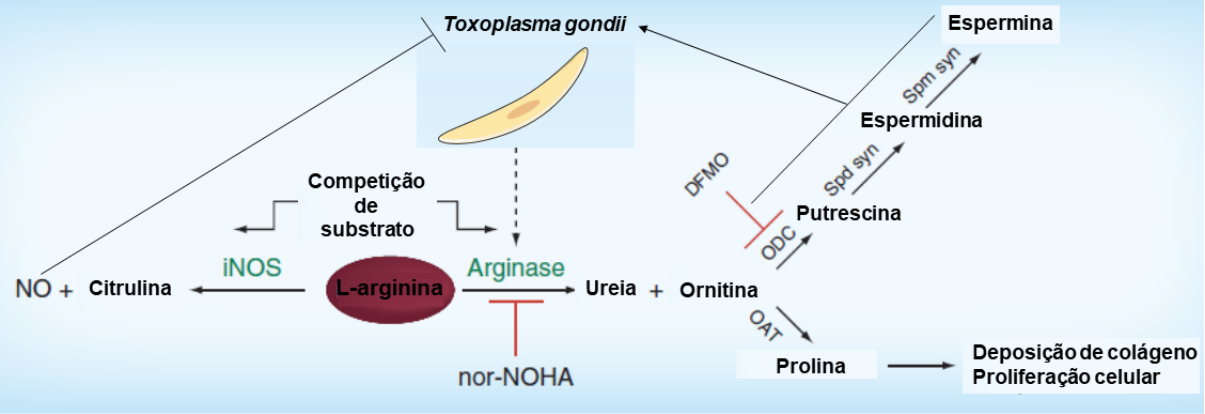


Figura 8: Metabolismo de arginina em mamíferos e interação entre iNOS e ARG1 em infecções com *Toxoplasma gondii* . Adaptado de Ivanenkov & Chufarova (2014). NO= óxido nítrico; iNOS= óxido nítrico sintase induzida, OAT= ornitina amino trasnferase. ODC= ornitina descarboxilase; spd syn= espermidina sintase; Spm dyn:espermina sintase

Estudos mostram que a indução de ARG1 em macrófagos causada pela infecção por *T. gondii* é um mecanismo de evasão do parasito (El Kasmi et al, 2008; Jensen et al, 2011; Li et al, 2012): 1- ARG1 compete com a iNOS pelo substrato L-arginina (Chang et al, 1998; Tenu et al, 1999); 2- ARG1 utiliza L-arginina para produção de L-ornitina, que posteriormente é utilizada pela ornitina descarboxilase para síntese de poliaminas, moléculas utilizadas por *T. gondii* para multiplicação (Cook et al, 2007; Pfaff et al, 2005). Ademais, camundongos C57BL/6 *knockout* para ARG1 apresentam aumento de sobrevida em infecções com *T. gondii* (El Kasmi et al, 2008). O papel central de ARG1 para replicação e

sobrevivência de parasitos também tem sido reportada para *Mycobacterium tuberculosis* (El Kasmi et al, 2008), *Trypanosoma brucei* (Gobert et al, 2000) e *Schistosoma mansoni* (Abdallahi et al, 2001). A interação entre ARG1 e o sistema imune, mais especificamente na relação parasito-célula hospedeira tem despertado o interesse de pesquisadores a fim de se compreender de maneira mais aprofundada a complexa biologia da interação entre macrófago e *T. gondii* e seu consequente impacto para o hospedeiro.

3- Objetivos

3.1- Objetivos gerais

Estudar mecanismos microbicidas de macrófagos e mecanismos evasivos de *Toxoplasma gondii* com foco em iNOS e ARG1.

3.2- Objetivos específicos

- Redigir revisão dos mecanismos microbicidas em macrófagos e mecanismos evasivos de *T. gondii;*
- Entender a dinâmica de expressão da iNOS e produção de NO em duas linhagens de macrófago murino infectadas com *T. gondii* selvagem e *knockout* para fatores de virulência secretados durante a invasão celular;
- Avaliar expressão e atividade de ARG1 em duas linhagens de macrófagos residentes e ativados M2, infectados com *T. gondii* e verificar o papel dessa enzima para o desenvolvimento do parasito.

4- Trabalhos

Trabalho 1

Chapter in “Antimicrobial Activities of Macrophages and *Toxoplasma gondii* Evasion Mechanisms. Gabriel R. A. Cabral, Renato A. DaMatta. Chapter 3. In *Toxoplasma gondii* : Dangers, Life Cycle and Research. Editor Sean L. Hall, Nova Science Publishers, Inc. Hauppauge, NY, EUA, 2017. pp 39-84. ISBN. 978-15-3611-067-8”

Chapter 3

MACROPHAGES ANTIMICROBIAL ACTIVITIES AND *TOXOPLASMA GONDII* EVASION MECHANISMS

Gabriel Rabello de Abreu Cabral

and Renato Augusto DaMatta[1]*.

Universidade Estadual do Norte Fluminense Darcy Ribeiro, RJ, Brazil.

ABSTRACT

Toxoplasma gondii, the agent of toxoplasmosis, is an obligate intracellular parasite that can infect virtually any nucleated cell from homeothermic vertebrate. Macrophages are important cells of the immune system that, through cell-autonomous microbicidal mechanisms such as phagocytosis, reactive oxygen species, nitric oxide (NO) and immune related GTPases (IRGs), control *T. gondii* replication. After recognition of surface molecules macrophages can internalize *T. gondii* by phagocytosis followed by degradation. Furthermore, sensing of *T. gondii* molecules by macrophages and other cell receptors induce a proinflammatory immune response that produces interferon-gamma classically activating macrophages, inducing reactive oxygen species production and the expression of inducible NO synthase and NO generation. Both microbicidal molecules inactivate crucial enzymes, killing *T. gondii*. IRGs are another class of proteins induced in activated mouse cells that is involved in the control of intracellular parasites. IRGs proteins accumulate at the parasitophorous vacuole harboring *T. gondii*, causing vacuole membrane vesiculation and rupture, and parasite death. However, along its evolutionary history, *T. gondii* evolved evasion mechanisms persisting in the host by: a) active invasion of the host cell, inhibiting lysosomal fusion to the parasitophorous vacuole as in phagocytosis, b) reactive oxygen species decrement, (c) inducible NO synthase degradation and NO inhibition, (d) IRGs arrest, and (e) the switch from proinflammatory to an anti-inflammatory profile of macrophages. These microbicidal

[1]* Corresponding author. Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, 28013-602, Brazil. Tel + 55 22 2739 7310; fax + 55 22 2739 7178; renato@uenf.br

mechanisms and their respective evasions are discussed in this chapter.

1. INTRODUCTION

Toxoplasmosis is a worldwide disease with an estimate of one-third of the human population seropositive (Tenter et al., 2000). This highlights how important *Toxoplasma gondii*, the causative agent of toxoplasmosis, is. Thus, there is need for more studies on this parasite. The epidemiology of this disease has a direct correlation with risk factors, socioeconomic parameters and population habits (Pappas et al., 2009). In general, Europe and North America have low seropositive rates for toxoplasmosis because of the high socioeconomic structure and the public health policy programs against this disease (Benard et al., 2008). Studies in Asia also showed low seropositive rates for toxoplasmosis, but a high seroprevalence was found in the Middle East (Abu-Madi et al., 2008). Little is known about the seroprevalence ratios for toxoplasmosis in Africa and most information comes from studies in the early 1990s showing a high seroprevalence ratio in pregnant women in Gabon (Nabias et al., 1998). In general, countries in South America have high seropositive ratios for toxoplasmosis, including Brazil, and it is directly related to the low socioeconomic structure of the country (Cabral et al., 2008). These data show that (a) the causative agent of toxoplasmosis is adapted to different regions in the world, (b) there is lack of public health policies against toxoplasmosis in many countries, (c) this is a neglected disease, (d) there is need for more efficient monitoring and accurate data collection on the global distribution of toxoplasmosis.

T. gondii is an obligate intracellular parasite belonging to the phylum Apicomplexan, and was first described by Splendore in rabbits, in Brazil (Splendore, 1908) and by Nicolle and Manceaux in the rodent *Ctenodactylus gundi*, in Tunisia (Nicolle and Manceaux, 1908). This parasite has a complex life cycle, with a sexual phase in the definite host, all species of the Felidae family, and asexual phase in the intermediate host that include virtually all homeothermic animals (mammals and birds). The asexual phase occurs also in the definitive host (Dubey et al., 2004).

T. gondii has three highly polarized infective forms, exhibiting a basic morphology between them, consisting of an Arc-shaped structure with 7 µm of length (Carruthers and Sibley, 1997). The anterior region is sharper and contains the conoid, where the secretory organelles micronemes and rhoptries form the apical complex

giving the name to the phylum (Chobotar and Scholtyseck, 1982). These secretory organelles are involved in host cell invasion, parasitophorous vacuole (PV) formation and immune evasions. The host cell invasion is an active complex process that initiates when the parasite binds at the host cell membrane and realizes the gliding movement (Fayer, 1972) that is dependent on the actin and myosin of the parasite (Frenal et al., 2010). Attachment to the host cell membrane creates an intimate association between the parasite apical complex and a moving junction (MJ) is formed (Mitchell et al., 2004) that is composed of proteins secreted by *T. gondii*. The MJ connects the cytoskeleton of both cells allowing traction for active penetration of the parasite, and selectively excludes host transmembrane proteins during PV formation guaranteeing its unique composition. The PV contains secreted proteins first from micronemes, and rhoptries, and later from dense granules (Paredes-Santos et al., 2012). These secreted proteins are addressed to specific regions of the host cells influencing their modulation.

During host cell invasion the first organelle to secrete its content is the microneme that depends on intracellular calcium signaling (Carruthers and Sibley, 1999). Proteins from micronemes (Mital et al., 2005) and from rhoptries (Kessler et al., 2008) form complexes with other proteins that are essential for active penetration of the parasite. Rhoptry is an elongated organelle with a thin electron dense neck and a less electron dense bulbous base (Boothroyd and Dubremetz, 2008). These regions have two classes of proteins: neck proteins of the rhoptries (RONs) that are first secreted, and bulb proteins (ROPs) (Lemgruber et al., 2011). Some RONs are secreted in response to micronemes proteins and form the MJ (Tyler and Boothroyd, 2011). ROPs are essential for PV formation and have specific activity (Miller et al., 2003; Gilbert et al., 2007; Butcher et al., 2011), with kinases and pseudokinase activities crucial for evasion from activated host cells.

Proteins from dense granules (GRA) are secreted during and after the host cell invasion process and can be found associated with the PV performing major roles in *T. gondii* survival and replication (Mercier et al., 2002). Until today, distinct GRAs have been described and most of them are associated with the PV membrane and the PV lumen (Cesbron-Delauw, 1994; Mercier et al., 2005). Recently, it has been shown that GRA24 is involved in the modulation of the host immune response (Braun, 2013). GRA24 traffics from the PV to the host cell nucleus, triggering the phosphorylation and

translocation of host p38α MAP kinase, a class of mitogen-activated protein kinases that controls many cellular responses, as the production of proinflammatory cytokines, including interleukin (IL) 12 and CC-chemokine ligand (CCL) 2 (Pearson et al., 2001).

With the secretion of micronemes, rhoptries, and dense granules, *T. gondii* is able to form the PV that associates with intermediate filaments and microtubules of the host cell (Coppens et al., 2006). Inside the PV, *T. gondii* is able to access nutrients (Coppens et al., 2000) and subvert the host immune response (Sibley, 2003) specially the cell-autonomous effector mechanisms of macrophages.

Macrophages are part of the mononuclear phagocytic system (MPS) proposed to include the precursor cells found in the bone marrow, monocytes and tissue macrophages (Hume, 2006). These cells are functionally and physiologically related and share common characteristics as phagocytic capacity, gene expression pattern and plasticity (Hume, 2008). Changes in the MPS concept have been proposed after the identification of antigen-presenting Dendritic cells (DCs) (Dalod et al., 2014) that have many overlapping functions and expression markers to cells from the MPS (Geissmann et al., 2010). Thus, DCs should be included in the MPS. In addition, it has also been suggested that monocytes are not the immediate precursors of all tissue macrophages with many resident tissue macrophages generated independently from the monocyte population. If these macrophages do not come from monocytes, which cells originate them? Studies have shown that monocytopenic mice do not have any deficiency in the production of tissue resident macrophages, indicating that these cells can self-renew without a precursor monocyte (Bigley et al., 2011). Furthermore, markers have been found discriminating monocyte populations in tissues, with inflammatory monocytes being key cells in the control of pathogens, including *T. gondii* (Dunay et al., 2008). Thus, a new concept of the MPS has been proposed including (Jenkins and Hume, 2014): a) DCs as antigen-presenting cells, sharing similar markers and functions to macrophages (Hume, 2008); b) that the majority of tissue macrophages do not derive from monocytes being self-renewed in adult individuals (Bigley et al., 2011; Schulz et al., 2012); and c) inflammatory monocytes are found in tissues (Geissmann et al., 2010).

Resident macrophages can be found virtually in all tissues of the body and have a major role in the maintenance of tissue homeostasis through the monitoring of invasive microorganisms, responding to physiological changes of the organism (Geissmann et

al., 2010) and uptake of apoptotic cells via phagocytosis (Erwig and Henson, 2008). Initially, phagocytosis was not related to the immune process, but became central as it connects the innate and adaptive immunity by antigen presentation of DCs and macrophages (Flannagan et al., 2012). Macrophages are highly effective antigen-presenting cells monitoring the tissue microenvironment, and presenting processed antigens from the cytosol, through MHC-I, and from the extracellular environment, through MHC-II (Watts, 1997). The antigen presentations through MHC-I molecules are essential to trigger the immune response against virus-infected cells and tumor cells through the immunity mediated by cytotoxic T CD8+ lymphocytes (Mantegazza et al., 2013). However, the antigen presentation through MHC-II molecules are essential to trigger the immune response against invading pathogens; and macrophages respond to microbial stimuli secreting molecules as tumor necrosis factor alpha (TNF-α), IL-12 and CXC chemokine ligand 10 (Gordon and Taylor, 2005). This causes infiltration and activation of other cell types, such as T lymphocytes, which contribute to the inflammatory response (Auffray et al., 2007).

The activation of T lymphocytes by proinflammatory molecules secreted by macrophages induces the production of interferon-gamma (IFN-γ) by DCs that activates macrophages in an M1 proinflammatory profile (Mills et al., 2000; Martinez et al., 2008). The M1 activation profile induces functional changes in macrophages (Mauel, 1982), being able to kill intracellular parasites, as *T. gondii*, by enhancement of synthesis and secretion of lysosomal enzymes (Pantalone and Page, 1977), reactive oxygen species (ROS) production (Murray and Cohn, 1979), nitric oxide (NO) production through inducible NO synthase (iNOS) (Adams et al., 1990; Lowenstein and Padalko, 2004) and expression of immunity related GTPases (IRGs) (Khaminets et al., 2010). However, during the coevolution with the host, *T. gondii* with evasion mechanisms to the microbicidal mechanisms were positively selected and were able to avoid: lysosomal fusion with the PV, ROS production, NO production by degrading iNOS, IRGs impairment caused by specific ROP proteins, and proinflammatory macrophages by inducing an alternative activation profile (M2) of the infected cell. All these evasion mechanisms are essential for *T. gondii* persistence and survival inside the host cell.

The first evasion mechanism described for *T. gondii* was the inhibition of

lysosomes fusion to the PV, preventing phagolysosome formation and consequent degradation of the parasite (Jones and Hirsch, 1972). The capacity of *T. gondii* to subvert the phagolysosomal fusion is directly related to the mechanism of host cell entry, if active penetration or phagocytosis. Secondly, it was shown that ROS was not produced where the parasite contacts macrophages. Another important evasion mechanism evolved by *T. gondii* is the inhibition of NO production (Seabra et al., 2002; Seabra et al., 2004), an important microbicide molecule that controls parasite replication (Adams et al., 1990). After host cell invasion, *T. gondii* induces iNOS degradation, inhibiting the NO production in activated macrophages (Padrão et al., 2014). Recently, the inhibition of IRGs has also been reported (Zhao et al., 2009b; Etheridge et al., 2014). These molecules are expressed after IFN-γ signaling being part of a resistance mechanism that is capable of controlling *T. gondii* with intermediate and low virulence (Khaminets et al., 2010), but highly virulent strains of the parasite evade from this mechanism through virulence factors secreted by rhoptries during host cell invasion (Zhao et al., 2009b). Furthermore, the induction of an alternative activation program in the infected cell has also been reported (Butcher et al., 2011). After host cell invasion, *T. gondii* activates transcription factors that trigger the production of anti-inflammatory cytokines and arginase 1 (ARG1) expression that apparently benefits parasite growth (Butcher et al., 2011). The antimicrobial action that controls *T. gondii* replication and the strategy of the parasite to subvert these cell-autonomous immune actions will be discussed in this chapter.

2. MACROPHAGES AS EFFECTOR CELLS AGAINST *T. GONDII*

Macrophages are one of the oldest cell types in the animal kingdom (Ottaviani et al., 2012). Most tissues of the body have populations of resident macrophages, which are extremely heterogeneous and specialized to perform tissue-specific tasks during the development and the whole life of the individual (Gordon and Plűddemann, 2013). Resident macrophages have a major role in tissue homeostasis by monitoring microorganisms, responding to physiological changes of the organism (Geissmann et al., 2010) and uptake of apoptotic cells by phagocytosis (Erwig and Henson, 2008). In addition, they are involved in inflammation (Henson and Bratton, 2009), tissue remodeling and repair (Brown et al., 2015), development, autophagy and autoimmune

disorders (Brown et al., 2015).

The inflammatory process is one of the most documented functions of macrophages. These cells are able to respond to microbial stimuli, as *T. gondii* profilin, an actin-binding protein involved in host cell invasion (Plattner et al., 2008) and secrete proinflammatory cytokines and chemokines as INF-γ, TNF-α, IL-12, CXC chemokine ligand 10 and CCL2 (Gordon and Taylor, 2005). These factors are able to induce the infiltration and activation of other cell types which, in an orchestrated way, contribute to the inflammatory process. That way, inflammatory macrophages are an example of polarization of these cells in an M1 activated profile (Auffray et al., 2007).

The concept of "activation" means a functional and biochemical change that occurs in macrophages (Mauel, 1982) such as the increase in: a) size, b) protein synthesis (Blanden et al., 1969), c) secretion of lysosomal proteins (Keller et al.,1974), and d) capacity to kill or inhibit intracellular pathogens (Mackaness, 1970). The activated macrophage concept was first proposed by Mackaness in 1962 to describe the increase of their microbicidal capacity when infected by bacillus Calmette-Guerin (BCG) and *Listeria* spp. (Mackaness, 1962). Classically activated macrophages acquire this phenotype in response to IFN-γ, lipopolysaccharide (LPS) and granulocyte-macrophage colony stimulating factor (GM-CSF). IFN-γ is the major molecule associated with the classic activation of macrophages that is recognized by its receptor. After recognition, Janus Kinase 1 and 2 (JAK1/2) are recruited and activate signal transducer and activator of transcription (STAT) 1 (Hu and Ivashkiv, 2009) that controls the gene expression of cell activation markers, cell adhesion molecules and proteins involved in the host cell response against microorganisms (Nathan and Hibbs, 1991). Another potent activator of macrophages in a classically activated profile is the LPS, present in the cell wall of gram-negative bacteria. Macrophages respond to LPS through Toll-like receptors 4 (TLR4) (Poltorak et al., 1998; Lee et al., 2015) that induce the production of proinflammatory molecules as TNF-α and IL-6 in a IFN-γ and NF-κB dependent way (Koerner et al., 1987; Lee et al., 2015). GM-CSF is a cytokine produced by many cells, including T cells, mast cells, NK cells and play a major role in macrophage activation (Hamilton et al., 2014). GM-CSF is recognized by the GM-CSF receptor that recruits JAK2, STAT3, and STAT5 that are translocated to the nucleus and transcribe genes that change macrophages to a proinflammatory profile. In general,

these cytokines induce an M1 classically activated profile in macrophages, increasing their microbicidal capacity by enhancing phagocytosis, as well as the production of ROS, NO, and IRGs.

2.1. PHAGOCYTOSIS

Macrophages are key players in the immune system linking the innate and the adaptive immunity. Among the many central functions that macrophages are able to assume, their phagocytic ability plays a major role in homeostasis by eliminating apoptotic (Arandjelovic and Ravichandran, 2015), senescent (Arandjelovic and Ravichandran, 2015) and necrotic cells. Phagocytosis was first described in 1777 with the addition of other reports up to the late eighteen hundreds, when a broad and central definition was described by Elie Metchnikoff in 1884 (Stossel, 1999). Phagocytosis is a general process involved in tissue digestion during embryonic development crucial for host defense (Metchnikoff, 1884; Stossel, 1999). Because of that, in 1908 Metchnikoff was awarded the Nobel Prize in Physiology or Medicine for his huge contribution in the cell biology understanding of immunity. Phagocytosis is a physiological phenomenon that is highly performed by "professional phagocytes", which are myeloid cells that accumulate in sites of inflammation and infection (Rabinovitch, 1995). These important cells include neutrophils, DCs, monocytes and macrophages (Rabinovitch, 1995).

Macrophages have an important role in eliminating invading parasites through phagocytosis. Phagocytosis can be defined as the engulfment of large particles (>0.5 μm) that are recognized by cell surface receptors of professional phagocytes and internalized into a plasma-membrane vacuole, called phagosome. The activation of phagocytosis is initiated by receptors that are expressed at the cell surface of the macrophage and mediates target selection, distinguishing self from non-self-particles (Griffin et al., 1975). The recognition of pathogen associated molecular patterns by pattern recognition receptors (PRRs) on the phagocyte's surface activates the process of phagocytosis that consists of a dynamic membrane interaction in an actin-dependent internalization process, forming the phagosome (Swanson, 2008). The acidification of the phagosome is essential to degrades the engulfed particle and occurs by the interaction with the endocytic system in two major steps: first, an initial acidification results in a small reduction of pH inside the phagosome (Hackam et al., 1997);

sequentially, V-type ATPases are translocated to the phagosome and progressively acidify the phagosome that fuse with lysosomes, forming the phagolysosome, and the phagocytosed particle is degraded into small peptides by the action of the lysosomal enzymes (Lennon-Dumenil et al., 2002). These peptides end up in MHC-II molecules on cell surface, a phenomenon known as antigen-presentation. Once presented by MHC-II, peptide complexes are recognized by CD4+ and CD8+ T lymphocytes that become activated and induce an immune response (Wubbolts et al., 1996).

T. gondii can be phagocytosed by host cells. Jones et al. (1972) described that this parasite is internalized by phagocytosis in mice peritoneal macrophages, fibroblasts (L929) and HeLa. The evidences for phagocytosis came from transmission electron microscopy and time-lapse microscopy (known at the time as microcinematography) showing the internalization without parasite direction (apical pole of the parasite is not the first to enter), the clear presence of long pseudopods around the parasite, and a process that takes 5 minutes. In addition, the phagocytic process of pure *T. gondii* was similar to opsonized parasites. Curiously, about half of the internalized population survived and multiplied while the other half was digested after lysosomes fused to the PV (Jones and Hirsch, 1972).

If the parasites are coated with antibody they are all phagocytosed, lysosomes fuse to the PV, and *T. gondii* is killed (Jones and Hirsch, 1972; Anderson et al., 1976; Sibley et al., 1985). In addition, antibody opsonization induces ROS production that may help to kill the parasite (Wilson et al., 1980). Therefore, phagocytosis is a cellular process that controls *T. gondii* multiplication.

2.2. REACTIVE OXYGEN SPECIES

After the discovery of phagocytosis in macrophages, generation of the phagosome and phagolysosome to kill microorganisms, other antimicrobial mechanisms were described and ROS was one of the first (Nathan, 1983). ROS are the product of NADPH oxidase. This molecular complex enzyme is located in biological membranes, transfer electron from cytoplasmic NADPH to molecular oxygen on the other side of the membrane generating superoxide ions (O_2^-) that spontaneously, or by the action of superoxide dismutase, turn into hydrogen peroxide (H_2O_2); both radicals can react and produce hydroxyl radicals (OH^-, $OH^\bullet$) (Cross and Segal, 2004; Leto et al., 2009). These

molecules are extremely toxic and were intimately related to the microbicidal activity of macrophages against distinct organisms, mainly by the studies with cells from humans with chronic granulomatous diseases (genetic disorder) that result in no functioning or lack of NADPH oxidase (Cross and Segal, 2004).

ROS has been implicated in the control of *T. gondii* by classical work showing that incubation of macrophages with catalase and superoxide dismutase (scavenges of ROS) reverted the efficiency of H_2O_2 on *in vitro* parasite killing (Murray and Cohn, 1979). In a subsequent work, ROS involvement was further correlated to *T. gondii* killing where three distinct peritoneal mouse populations obtained from mice (infected and immunized, infected only, or naïve) were distinctly capable of controlling parasite growth, with the first being more microbicidal and capable of producing more ROS (Murray et al., 1979). At this time, NO and IRGs were not discovered and ROS became the sole mechanism responsible for *T. gondii* control by activated peritoneal macrophages.

Another work showing that ROS is important to control *T. gondii* growth was performed with DCs *in vitro* (Aline et al., 2002). Activated DCs were able to control *T. gondii* growth with a positive correlation to the used concentration of IFN-γ. ROS scavengers were capable of restoring *T. gondii* growth showing the importance of this microbicidal system in this host cell (Aline et al., 2002). ROS was also shown to be involved in *T. gondii* control in a study involving activation of macrophages by extracellular ATP (Corrêa et al., 2010). Extracellular ATP is recognized by purinergic receptor present in many cells translating as a danger signal. Macrophages from wild-type mice treated with ATP control *T. gondii* growth. In addition, macrophages from $P2X_7$ receptor knockout mice, the most involved purinergic receptor in inflammation and immunological response, could not control *T. gondii* growth after ATP treatment. ROS was produced in wild-type macrophages and implicated in this control (Corrêa et al., 2010).

2.3. INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) AND NITRIC OXIDE (NO)

Nitric oxide synthases (NOS) were first described in early 1990s, with the isolation of the neuronal isoform (nNOS or NOS1) (Bredt and Snyder, 1990). The other two isoforms, inducible nitric oxide synthase (iNOS or NOS2) (Stuehr et al., 1991) and

endothelial nitric oxide synthase (eNOS or NOS3) were described latter and since then great attention to the field has been given. Since NOS discovery, more than 73000 papers have been published showing the interested to NOSs. Formal recognition came in 1998 by the Nobel Prize in Physiology or Medicine award to Robert F. Furchgott, Louis J. Ignarro and Ferid Murad for the discovery of NO as a biologic mediator in the cardiovascular system. Since then, publications about the NOS structure, function, regulation and inhibition have been published.

NOSs are homodimers with molecular weight that vary from 110 kDa to 160 kDa, depending on the isoform and share similar genomic and molecular structure, suggesting a common ancestral NOS gene (Janssens et al., 1992; Nakane et al., 1993; Geller et al., 1993). The NOS isoforms share an N-terminal oxygenase domain and a C-terminal reductase domain that are linked by a calmodulin binding site (Daff, 2010). The N- terminal oxygenase domain has a binding site for haem, BH_4 and L-arginine and the C-terminal reductase domain has binding sites for FAD, FMN and NADPH (McMillan and Masters, 1995; Ghosh and Stuehr, 1995; Forstermann and Sessa, 2012). The patterns of expression and characteristics of NOSs isoforms are used to define their nomenclature (Forstermann and Sessa, 2012). nNOS is expressed in neurons and is calcium dependent (Huang et al., 2012); iNOS is induced by cellular activation and is calcium independent (Forstermann and Sessa, 2012); eNOS is expressed in the endothelium and is calcium dependent (Forstermann and Münzel, 2006).

Immunologic cells as macrophages and neutrophils have high levels of iNOS expression and can be found in many subcellular localization as cytosol (Choi et al., 2012), small vesicles (Vodovotz et al., 1995), phagosome (Jyoti et al., 2014), actin cytoskeleton (Webb et al., 2001), and mitochondria (Zaobornyj and Ghafourifar, 2012). The nuclear expression of iNOS has also been reported but the biological role of nuclear expression remains unclear (Jyoti et al., 2014).

Macrophages activated with proinflammatory cytokine such as IFN-γ and microbial product as LPS have high expression of iNOS, producing large amount of NO that is rapidly converted to nitrite ($NO3^-$) and nitrate ($NO2^-$) (Lowenstein and Padalko, 2004). NOS catalyze the conversion of L-arginine and molecular oxygen in L-citrulline and NO in two steps. In the first step, NOS hydrolyses L-Arginine to N-hydroxy-L-Arginine and after that, NOS oxidizes N-hydroxy-L-Arginine to L-Citrulline and NO

(Stuehr et al., 2001). The first discovery of NO as a molecule with biological functions was reported by Furchgott and Zawadzki in 1980, which showed the role of NO as a meditator of vasodilatation (Furchgott and Zawadzki, 1980). Once produced, NO is able to phosphorylate several proteins that leads to smooth muscle relaxation (Furchgott and Zawadzki, 1980).

NO was first described by Joseph Priestly as a gas in 1772, consisting of a molecule with just one atom of oxygen and one atom of nitrogen (Priestley, 1774). NO has a low molecular weight, is soluble in aqueous and hydrophobic environments and can diffuse freely through biological membranes (Thomas et al., 2008), being involved in many homeostatic and immunological functions as vasodilatation (Miyahara et al., 1994; Nowicki et al., 2007), neurotransmission (Taqatqeh et al., 2009), inflammation (Maeda and Akaike, 1998), and microbicidal activity against pathogens (Nathan and Hibbs, 1991; Hoffman et al., 1997; Bogdan, 2001; Hunter and Sibley, 2012).

In the last twenty years, the number of studies showing the major role of NO production by activated macrophages against protozoan parasites has increased dramatically (Nathan and Hibbs, 1991; Hoffman et al., 1997; Hunter and Sibley, 2012). The precise mechanisms of action of NO against pathogens is not fully understood, but several hypothesis have been proposed: a) binding of NO to iron-sulfur centers forming iron–sulfur–nitrosyl derivatives of key enzymes of protozoan parasites; b) disruption of protozoan parasite membranes by oxidizing agents as peroxynitrite formed by the combination of NO with superoxide anions; c) mutations and strand breaks of DNA bases by deamination.

Several studies have contributed with the understanding of the role of NO in *T. gondii* control (Khan et al., 1997; Hunter and Sibley, 2012). NO has a dichotomous role in *T. gondii* control that depends on the stage of infection, mediating intestinal pathology during the acute phase of infection or being benefic during the chronic phase of infection at the central nervous system (Scharton-Kersten et al., 1997). Knockout mice for iNOS gene and their genetically matched parental controls orally infected with different parasite burden cysts have distinct capacities to survive to *T. gondii* infections (Khan et al., 1997). Surprisingly, knockout mice survived the acute infection and do not present any clinical evidence of illness if compared with the parental controls that died in the acute phase of infection showing stronger liver and gut pathological changes

(Khan et al., 1997). Moreover, orally infected parental controls with *T. gondii* cysts treated with aminoguanidine, an iNOS inhibitor, have prolonged time to death, similar as iNOS knockout mice. This study suggests that NO has a dichotomous role in infected mice, with a major role in controlling *T. gondii* replication and a detrimental histopathologic disorder in the acute phase of infection (Khan et al., 1997).

The importance of the neuroprotective role of NO during the chronic phase of *T. gondii* infection has also been reported by others (Bohne et al., 1994; Dincel and Atmaca, 2015). Recent studies have shown that NO has immunoprotective and immunomodulatory roles by preventing tissue cysts reactivation in mice, with high expression levels of eNOS, iNOS and nNOS (Dincel and Atmaca, 2015). In immune compromised patients, the reactivation of tissue cysts can cause toxoplasma encephalitis, a severe condition that is usually fatal and is directly related to the reduction of iNOS expression (Gazzinelli et al., 1993; Khan et al., 1997). However, overproduction of NO has neurotoxic effects with neuronal degeneration and necrosis, also inducing cysts reactivation (Giovannoni et al., 1998; Calabrese et al., 2007; Dincel and Atmaca, 2015).

Despite numerous functions of NO against pathogens, a balance between anti-parasitic activity that leads to parasite elimination and the cytotoxic effects, that leads to host tissue damage must be achieved for host benefit (Khan et al., 1997). That way, NO production has to be tightly regulated to avoid damage to the host. If the NO production is exacerbated, host can suffer morbidity and if the host does not produce sufficient amounts of NO to control the infection, the host can die by high parasite burden (Khan et al., 1997).

2.4. INTERFERON-INDUCIBLE GTPASES: IMMUNITY RELATED GTPASES (IRGS)

IFN-γ is a cytokine critical for innate and adaptive immunity and is secreted by T helper cells, cytotoxic T cells and natural killer cells (Schoenborn and Wilson, 2007). Activation of macrophages by IFN-γ upregulates gene expression of thousands of molecules, including IFN-inducible GTPases, a group of proteins that includes p65 guanylate-binding proteins (p65 GBPs) (Cheng et al., 1983), myxovirus resistance proteins (Mx) (Horisberger et al., 1983), very large IFN-inducible GTPases (VLIGs) (Klamp et al., 2003) and immunity related GTPases p47 (IRGs) (Gilly and Wall, 1992).

This resistance program provides protection to a variety of pathogens, through: rupture of PV containing bacteria, viruses or protozoan parasites (Khaminets et al., 2010); autophagy (Singh et al., 2006); activation of inflammasome (Shenoy et al., 2012); and manipulation of vesicle trafficking (Williams and Kim, 2014).

Mouse Mx and human GBP was the first IFN-inducible GTPases to be discovered (Cheng et al., 1983; Horisberger et al., 1983). The Mx gene encodes the Mx protein, a 72 kDa protein responsible for inhibiting the influenza virus replication (Staeheli et al., 1988). Studies with inbred mice lacking functional Mx proteins showed a high susceptibility to viral pathogens (Staeheli et al., 1988). p65 GBPs are a family of 65-73 kDa proteins highly expressed in mice and human cells after IFN-γ signalization (Gupta et al., 1979). Studies with GBPs-deficient mice and macrophages revealed loss of *Mycobacterium bovis* and *Listeria monocytogenes* control (Kim et al., 2011) and susceptibility to *T. gondii* infection (Yamamoto et al., 2012).

VLIGs are the largest IFN-inducible GTPases, with 280 kDa of molecular weight, being induced in mice and zebrafish (Klamp et al., 2003). Despite all efforts, no function has been reported so far for these proteins.

p47 GTPase are a family of proteins that was first described in the 1990s as the smallest GTPase group with 47kDa (Gilly and Wall, 1992; Taylor et al., 1996) and the most numerous, being expressed in tissues and culture cells as astrocytes, fibroblasts and macrophages (Zerrahn et al., 2002; Hunn et al., 2008; Khaminets et al., 2010). These proteins can be found in many intracellular compartments as Golgi apparatus (Martens et al., 2004), endoplasmic reticulum (Martens et al., 2004) and endolysosomal compartments (Zhao et al., 2010). p47 GTPase has been grouped in two subfamilies according to differences in its structure and function: a) GKS effectors proteins as Irga, Irgb, Irgc, and Irgd that have a canonical GKS motif (GX_4GKS) in the GTP-binding site (Hunn et al., 2008). GKS proteins are found transiently inactivated in a GDP-bound form at the cytosol, but when effector cells are infected with a pathogen, like *T. gondii*, GKS proteins are activated in a GTP-binding form and associated to the PV (Haldar et al., 2013); b) GMS regulatory proteins as Irgm1, Irgm2 and Irgm3 that have an atypical nucleotide binding site (GX_4GMS) and is responsible for correct activation and intracellular positioning of the GKS subfamily members (Bekpen et al., 2005).

The initial findings of the roles of p47 GTPases *in vivo* were first described by

three independent research groups that analyzed the response of IRG-deficient mice to pathogens (Taylor et al., 2000; Halonen et al., 2001; Collazo et al., 2001). A critical role of p47 GTPase were described for resistance against a wide variety of pathogens, including *Mycobacterium tuberculosis* (Feng et al., 2004), *Trypanosoma cruzi* (Santiago et al., 2005), *T. gondii* and others (Howard et al., 2011). The complete molecular mechanisms of action of p47 GTPases are not well understood, but three models have been proposed: a) accumulation of 7 IRGs proteins, effectors and regulators, in the pathogen containing vacuole, that lead to the rupture of the PV membrane, exposure of the parasite and necrotic host cell death (Khaminets et al., 2010); b) induction of autophagy and autolysosomal organelles (Singh et al., 2006); c) phagosome maturation (MacMicking et al., 2003).

The role of p47 GTPases in the control of *T. gondii* infected macrophages has been described by several groups (Butcher et al., 2005; Ling et al., 2006; Zhao et al., 2009b). It appears to be dependent on at least 7 IRGs proteins that act in a hierarchical and collaborative way to control *T. gondii* (Khaminets et al., 2010). The coating of the PV containing *T. gondii* occurs a few minutes after host cell entry and effectors proteins Irgb6 and Irgb10 seem to be the pioneers to coat the PV (Zhao et al., 2009a; Khaminets et al., 2010). Regulatory p47 GTPases as Irgm1 also appear to be extremely important for the action of effectors p47 GTPases, acting as a negative regulator (Henry et al., 2010). Irgm1 prevents the premature activation of effectors p47 GTPases and, consequently, loss of coating of the PV containing *T. gondii* (Henry et al., 2009; Khaminets et al., 2010). Despite all efforts to identify the precise mechanism of *T. gondii* death by p47 GTPases, some issues still remain unclear and some studies suggest the following hypothesis: a) after membrane disruption by effector p47 GTPases and *T. gondii* exposure, autophagosomal formation containing the parasite occurs that fuse with lysosomes and degradation takes place (Ling et al., 2006); b) *In vitro* studies have shown that with *T. gondii* exposure and death, host cells also die in a process similar to necrosis, but it is still unclear if this mechanism occurs *in vivo* (Zhao et al., 2009b); c) production of mechanical and/or chemical forces by GKS proteins through GTP hydrolysis on the target PV causes parasite death (Williams and Kim, 2014); d) GKS proteins working cooperatively with p65 GBPs and ubiquitin E3 ligases promote the lysis of the PV (Yamamoto et al., 2012). All these mechanisms are elegantly proposes

explaining how p47 GTPases control *T. gondii*.

3. EVASION MECHANISMS

Apicomplexan phylum is composed by more than 5000 species of Protozoan parasites that have a worldwide distribution, as *T. gondii*. This parasite is one of the most successful pathogen and infects all warm-blooded vertebrates (Tenter et al., 2000), being one of the most abundant Apicomplexan parasite. The selective pressure between the host and *T. gondii* generates reciprocal adaptive genetic changes that ensure the capacity of the host to control the parasite and, at the same time, the ability of the parasite to subvert cell autonomous immunity and the host immune system. As discussed above, macrophages have an arsenal of effector mechanisms to control *T. gondii*, being an important cell in the parasite control (Adams et al., 1990; Khaminets et al., 2010; Raetz et al., 2013). However, through the evolutive process, *T. gondii* developed many evasion mechanisms to subvert the host immune system as inhibition of lysosomal fusion, inhibition of ROS and NO production, inhibition of IRGs and induction of an alternative activation profile state in the macrophage (Table 1), among other strategies. Some of these evasion mechanisms will be discussed in details in the following topics.

3.1. INHIBITION OF LYSOSOMAL FUSION

The first evasion mechanism described for *T. gondii* was the inhibition of the fusion of host cell lysosomes with the PV containing-parasite (Jones and Hirsch, 1972) (Table 1). However, the capacity of inhibition of lysosomal fusion is dependent on the virulence of the parasite and the route of the host cell entry. How *T. gondii* enter host cells was a biological question for some time (Jones et al., 1972). The first work describing that the entrance of this parasite into the host cell is an active process and that the parasite ends up surrounded by a vacuole, was done by Guimarães and Meyer in 1942 (Lund et al., 1961). Guimarães and Meyer (1942) also described a method for intracellular cultivation of this parasite allowing its broad experimentation in the world (Jacobs, 1956; Souza et al., 2009). Filming the parasite interacting with host cells was revealing. After cell rupture and release of the parasite, neighbor cells were rapidly invaded, faster than in the phagocytic process (Lund et al., 1961). Another entrance feature indicating active invasion was the constriction of the parasite in the site of

entrance at the plasma membrane of the host cell that is not observed during the phagocytic process (Hirai et al., 1966). During this fast entrance into host cells, parasites were always oriented with the apical end first (Werk, 1985). In addition, *T. gondii* enters virtually all cell types, including professional and non-professional phagocytes, thus, it must have a proper general mechanism of entry (Black and Boothroyd, 2000).

It was shown that in professional phagocytes two forms of entry of *T. gondii* existed: active invasion and phagocytosis (Morisaki et al., 1995). Active invasion occurred rapidly resulting in a tight-fitting PV formed by the invagination of the host cell plasma membrane; phagocytosis took longer, resulted in parasites in loose-fitting PV and involved the reorganization of the cell host cytoskeleton and phosphorylation of their proteins. In addition, it was reported that some parasites were phagocytosed, but could actively invade the phagosome forming a secondary tight-fitting PV. These results indicated that the entrance mechanism determines the fate of the parasite. Active invasion of the parasite resulted in a PV that does not acidify nor fuse to lysosomes; phagocytosis resulted in the death of the parasite caused by lysosomal fusion to the phagosome and the consequent digestion (Morisaki et al., 1995).

The host cell invasion by strains of *T. gondii* is a dynamic and active process that is powered by "glideosomo" (Dobrowski and Sibley, 1996). The glideosomo is an actin-myosin-based machinery of the parasite that is located between the plasma membrane and the inner membrane complex, and is responsible for powering the gliding, host cell invasion and egress, permitting the parasite to cross nonpermissive biological barriers, as the host cell membrane (Opitz and Soldati, 2002). Furthermore, virulent strains of *T. gondii* actively exclude proteins from the host that are involved in the phagosome-lysosome fusion, avoiding the PV destruction by lysosomes. During invasion, the MJ controls the internalization of membrane lipids from the host cell, as glycosphingolipid G_{M1} and Glycosylphosphatidylinositol-anchored surface proteins and in contrast, transmembrane proteins as Na^+/K^+ ATPase, and β1-integrin are excluded from the PV (Mordue et al., 1999). The major role of β1-integrin in phagosome maturation in bacterial infection has been shown (Wang et al., 2008). Probably, the same strategy is adopted by *T. gondii* to evade from the lysosomal fusion (Mordue et al., 1999).

Recently, interesting results showed that avirulent strains of *T. gondii* use a noncanoniocal pathway to enter the host cell through a phagosome to vacuole invasion pathway (Zhao et al., 2014). This model proposes a dual route of infection in macrophages: initially, avirulent strains of *T. gondii* are phagocytosed by macrophages and subsequently, form a PV from the phagosomal compartment. Furthermore, as the virulent strains, the phagocytosed avirulent strains also form a MJ and a PV. However, this process is initiated from the phagosome membrane rather than on the host cell surface, and also appears to be dependent on phagosome maturation, as shown by transient inhibition of phagosomal acidification with Baf A1 (Zhao et al., 2014). Additionally, this study proposes the recognition of avirulent strains ligands by PRRs on host cell surface, although, the main determinants involved still needs to be defined. Thus, this is a parasite strategy as the Trojan horse strategy that leads to an enhancement of parasite dissemination and chronic infection establishment (Zhao et al., 2014).

3.2. EVASION FROM ROS

Although ROS has been implicated in the control of *T. gondii* by macrophages (Murray et al., 1979), the same group and other showed right away that this parasite was able to evade from this microbicidal system (Wilson et al., 1980; Murray and Cohn, 1980) (Table 1). This became the second described evasion mechanism of this parasite, and opened a new and productive area of research on how this parasite escapes microbicidal action of host cells. It was shown that ROS was not detected in the contact sites between the parasite and the macrophage, a result that was further confirmed with different preparations of *T. gondii* (dead x alive; opsonized x non-opsonized), after or before infection of macrophages with *Candida albicans* (a fungus that induces high levels of ROS) and treatments of the macrophages with recombinant IFN-γ or phorbol myristate acetate (an induction of ROS production) (Chang and Pechère, 1989). The capacity to inhibit ROS production at the contact sites of the parasite and the host cell was also demonstrated using chicken macrophage as host cells (Guillermo and DaMatta, 2004) indicating similar mechanisms in birds. Further evidence that ROS are not important to control *T. gondii* came from *in vivo* studies with p47 *phox* knockout mice where the parasite develops similarly as wild-type mice (Alexander et al., 1997) and from a flow cytometry study that analyzed the infected peritoneal population with

ROS markers (Shrestha et al., 2006). These results can be explained by the elaborated antioxidant enzyme arsenal *T. gondii* has (Sibley et al., 1986; Ding et al., 2004). However, depending on the host cell (DCs, Aline et al., 2002) and special activation (ATP, Corrêa et al., 2010), ROS may be important in the control of *T. gondii*.

3.3. iNOS DEGRADATION AND NITRIC OXIDE INHIBITION

With host cell invasion and PV formation, *T. gondii* is able to capture host nutrients and subvert the immune system (Lang et al., 2007; Nelson et al., 2008; Kemp et al., 2013). One of the parasite's strategies is the modulation of host signaling pathways involved in Th1 response, the proinflammatory pathway responsible for the control of intracellular parasite replication (Lang et al., 2007). During macrophage infection, *T. gondii* actively suppresses host IL-12 and TNF-α productions (Butcher and Denkers, 2002; Butcher et al., 2011) and, at the same time, induces secretion of active TGF-β (Seabra et al., 2004). The autocrine TGF-β signaling in infected macrophages was achieved by *T. gondii* exposure to phosphatidylserine (PS), an inner membrane phospholipid. TGF-β signaling in infected macrophages was confirmed by translocation of Smad 2 and Smad 3 to the host cell nucleus, leading to iNOS degradation, actin filament depolymerization and removal of NF-κB from the host cell nucleus (Seabra et al., 2004) (Table 1). Treatment of macrophages with an anti-TGF-β1 neutralizing antibody reverted these features, confirming the major role of PS exposure and TGF-β to evade microbicidal mechanisms of macrophages (Seabra et al., 2004).

Apoptotic cells expose PS that induces TGF-β secretion by macrophages, a potent macrophage deactivator that acts in a paracrine and autocrine signaling way (Ashcroft, 1999). Studies have shown that tachyzoites of *T. gondii* has two subpopulations, one that exposes PS (PS+) and another that does not (PS-) (Santos et al., 2011). With PS exposure, *T. gondii* mimicry an apoptotic cell and is able to actively invade macrophages (Santos et al., 2011, see also chapter 4), subverting the immune system of these cells, inducing inhibition of NO production (Seabra et al., 2002; 2004). Only the PS+ and the total population of *T. gondii* were able to inhibit NO production of activated macrophages (Santos et al., 2011). Similar results were also observed for *T. gondii* growth in resident macrophages. Growth in resident macrophages was higher for PS+ subpopulation and the total population of *T. gondii*, but lower for the PS-

subpopulation. These findings are directly related to the parasite entry mechanism that dictates the survival in the host cell. PS+ subpopulation invades macrophages by active penetration and is capable of forming a tight-fitting vacuole able to control and subvert the host's immune system. The PS- subpopulation enters macrophages by phagocytosis, and forms a loose-fitting vacuole. Infection of C57BL/6 mice with the isolated *T. gondii* PS subpopulation caused an early death of mice when compared with the total parasite population, showing that both PS subpopulations are required for parasite and host survival (Santos et al., 2011).

With PS exposure, *T. gondii* induces a TGF-β autocrine signalization on macrophages, leading to an anti-inflammatory profile, inducing downstream inhibition of NO production (Seabra et al., 2004) because iNOS is degraded (Seabra et al., 2002; 2004; Guillermo and DaMatta, 2004; Padrão et al., 2014) (Table 1). iNOS can be regulated by transcriptional, translational and posttranslational control (Kone et al., 2003). Physiologically, iNOS can be regulated by three degradation pathways: proteasome (Musial and Eissa, 2001; Kone et al., 2003), lysosomes (Musial and Eissa, 2001) and calpain (Walker et al., 1997). Recently, the iNOS degradation pathway in *T. gondii* infected macrophages have been reported (Padrão et al., 2014). After infection of activated J774-A1 macrophages with *T. gondii*, the NO production is inhibited and iNOS is degraded by the proteasome pathway, a phenomenon that occurs after 2 and 6 hours after parasite entry (Padrão et al., 2014). However, the pretreatment of these cells with lactacystin, a specific proteasome inhibitor, blocked iNOS degradation and NO levels were higher in these cells when compared to untreated infected macrophages, showing that the proteasome is the main iNOS degradation pathway in macrophages infected with *T. gondii* (Padrão et al., 2014). Despite the increase in NO production by J774-A1, infected macrophages with *T. gondii* treated with lactacystin were not able to fully resettle NO production, probably because of the induction of an iNOS aggresome after lactacystin treatment (Padrão et al., 2014). Aggresomes are cytoplasmic inclusion bodies with a perinuclear localization that work as a physiologic mechanism to regulate certain proteins, as iNOS (Sha et al., 2009). iNOS aggresome formation in macrophages treated with lactacystin and infected with *T. gondii* probably explaining the lower NO levels of these cells when compared with uninfected macrophages (Padrão et al., 2014). The aggresome formation indicated that iNOS was ubiquitinated, but failed to be

degraded due to lactocystin treatment. However, ubiquitination was not blocked and iNOS concentrated in the aggresome. The lysosomal and calpain pathways were also evaluated by pretreatment of J774-A1 macrophages with pharmacological inhibitors of both pathways, but were not able to revert iNOS degradation and NO levels continued to be inhibited after macrophage infection (Padrão et al., 2014) (Table 1).

3.4. INACTIVATION OF IRGS

T. gondii strains isolated in North America and Europe are clonal and genetically classified into three major types: highly virulent, type I strain; intermediate virulent, type II strain; and avirulent, type III strain (Hoffman et al., 1995) and cause distinct phenotype in mice (Saeij et al., 2005). The classification of virulence of *T. gondii* strains is based on morbidity and mortality in the laboratory mice. For *T. gondii*, the virulence classification is directly related to the virulence factors of each parasite strain, and consequently the ability to evade microbicidal mechanisms of host cells, including macrophages.

To determine the virulence factors responsible for phenotypic differences in *T. gondii* infected mice, genetic cross between highly virulent type I, GT-1 strain, with avirulent type III, CTG strain, were realized in cats and the progeny evaluated (Su et al., 2002). Genomic markers of the recombinant progeny clonal lineages of *T. gondii* reveled 1-2% difference in DNA sequence (Sibley and Ajioka, 2008). Therefore, phenotypic differences between *T. gondii* strains of North America and Europe rely on a small number of genes (Howe and Sibley, 1995), mostly encoded by ropthry proteins (Taylor et al., 2006; Lilue et al., 2013).

Tachyzoites usually have 8 to 12 individual rhoptries, each about 3 μm in length with two regions (Dubey et al., 1998) containing RON and ROP proteins, respectively. During host cell invasion, *T. gondii* secretes its rhoptry content that is addressed to distinct destinations of the host cell (Alexander et al., 2005). Recently, the pseudokinase ROP 5 and the serine-threonine kinase ROP 18 were identified as important virulence factors that subvert the immune system (Etheridge et al., 2014). The ROP 18 and the ROP 5 are the major mice virulence factors explaining differences between *T. gondii* strains by distinct expression levels and polymorphism (Taylor et al., 2006; Behnke et al., 2011). ROP 18 is highly expressed in type I and type II strains and lowly expressed

in type III strains (Saeij et al., 2006); the expression levels vary more than 1,000-fold between type I and type III parasites (Taylor et al., 2006). Studies analyzing the evolutionary history and the acting forces on ROP 18 among *T. gondii* strains identified variation in three alleles responsible for ROP 18 expression among natural isolates (Khan et al., 2009). Despite the fact that *T. gondii* type III strains are older, type I and type II strains showed evidences of strong selective pressure, resulting in enhanced virulence (Khan et al., 2009). Further analysis revealed that expression of ROP 18 alleles of type I and II strains in type III background parasites enhanced the virulence in mice, promoting subversion of the immune system, growth and parasite survival (Taylor et al., 2006; Saeij et al., 2006). Moreover, parasites with a point mutation in a specific key catalytic residue for the kinase activity in ROP 18 becomes avirulent (Taylor et al., 2006).

Forward genetics analysis between type II x III strains also identified a major role of ROP 5 in the evasion of the IRG system (Behnke et al., 2011). ROP 5 is composed of a cluster of genes that is shared by type I and type III strains, but not by type II strains that have a distinct cluster of ROP 5 alleles responsible for their lower virulence (Behnke et al., 2011). The complete mechanism through which ROP proteins evade from the IRGs system is not completely understood. However, after secreted in the host cell during *T. gondii* invasion, ROP 18 from type I strains decorates the cytoplasmic side of the PV membrane of mice cells activated by IFN-γ, phosphorylating Irga6, Irgb6, Irgb10, inhibiting them (Fentress et al., 2010; Steinfeldt et al, 2010). Moreover, recent results have shown that ROP 5 from type I strains is able to bind to Irga6 and to enhances ROP 18 activity. Although parasites from type II strains express high levels of ROP 18, their ROP 5 does not enhance ROP 18 activity, making this strain more susceptible to IRGs. The same susceptibility to IRGs is observed for type III strains that have low levels of ROP 18. The differential expression levels and polymorphisms of ROP 5 and ROP 18 from different *T. gondii* strains dictates the capacity of the distinct strains to evade the IRG system in mice (Niedelman et al., 2012; Hunter and Sibley, 2012) (Table 1).

3.5. MODULATION OF ACTIVATION PROFILE OF MACROPHAGES: SWITCH OF M1X M2 ACTIVATION STATE BY *T. GONDII* INFECTION

The plasticity of macrophages to acquire distinct activation profiles evokes distinct phenotypic responses in the two basic states of macrophages activation, classically activated (M1) characterized by a proinflammatory response, and alternatively activated (M2), being characterized by an anti-inflammatory and tissue repair response (Mills et al., 2000; Mills, 2012; Martinez and Gordon, 2014; Murray et al., 2014). The activation of macrophages in a M1 or M2 profile is controlled by cytokines in the microenvironment of the cell and signaling pathways that controls the proinflammatory or anti-inflammatory response (Martinez et al., 2008). The M1 phenotype is triggered by IFN-γ and LPS and controlled by the translocation of STAT1 and NF-κB to the cell nucleus. This phenotype is characterized by expression of MHC II, production of proinflammatory cytokines (as IL-12 and TNF-α), chemokines as CCL15 and CCL20, and high expression of iNOS (Martinez et al., 2008; Mills, 2012). M1 macrophages are extremely microbicidal and capable of controlling many intracellular pathogens, as *T. gondii* (Adams et al., 1990). However, the M2 profile is triggered by anti-inflammatory cytokines as IL-4, IL-10 and TGF-β and is characterized by expression of mannose receptor and ARG1 expression (Martinez et al., 2009). M2 macrophages are typically associated with wound healing, production of growth factors and extracellular matrix proteins (Mills, 2012). The modulation of proinflammatory and anti-inflammatory profiles by pathogens have been studied, including *T. gondii* infections (Khan et al., 1995; Butcher and Denkers, 2002, Butcher et al., 2011).

Host cell invasion by *T. gondii* is a multistep complex process that is tightly regulated by the parasite and is able to result in the evasion of microbicidal mechanisms and modulation of the host cells (Nelson et al., 2008; Kemp et al., 2013). One of the parasite strategies is the inhibition of host proinflammatory profile and an induction of an anti-inflammatory profile that deactivates the host cell (Khan et al., 1995; Butcher and Denkers, 2002; Woods et al., 2013). To subvert the IFN-γ signaling in macrophages, *T. gondii* upregulates the expression of suppressor of cytokine signaling (SOCS) proteins, a family of proteins that blocks the activity of JAK and the recruitment of STAT1 (Murray, 2007). Thus, *T. gondii* blocks the IFNγ-dependent signaling in macrophages, causing an inhibition on NO production and consequently

reducing the macrophage capacity to control parasite replication (Zimmermann et al., 2006). Another parasite strategy is to block the NF-κB translocation to the host nucleus, but it appears to depend on the host cell type (Butcher et al., 2001; Molestina et al., 2003; Seabra et al., 2004). In mice macrophages, *T. gondii* blocks the translocation of NF-κB to the host nucleus, resulting in the reduction of the proinflammatory cytokines response such as TNF-α and IL-12 (Shapira et al., 2005) and deactivation of macrophages, causing iNOS degradation and consequently NO inhibition (Seabra et al., 2004; Padrão et al., 2014). The enhanced susceptibility in both acute and chronic infections with *T. gondii* caused by the blocking of NF-κB to host cell nucleus has also been reported (Caamano et al., 1999). Controversially, in murine fibroblast, *T. gondii* induces the translocation of NF-κB to the host nucleus, resulting in expression of anti-apoptotic genes (Molestina and Sinai, 2005). The translocation or not of NF-κB to the host nucleus also depends not only on the host cell type but on the *T. gondii* strain. It has been shown that type II but not type I strains of *T. gondii* induce the translocation of NF-κB to the host nucleus in bone marrow-derived macrophages (Robben et al., 2004) and the levels of genes regulated by NF-κB is higher in the infected host if compared with uninfected cells (Blader et al., 2001). Moreover, *T. gondii* infected mice has increased levels of IL-10 at day 7 post-infection and reduced levels of IFN-γ mRNA and soluble IFN-γ in the culture supernatant of splenocytes, showing that IL-10 is a potent inhibitor of IFN-γ and mediator of immunosuppression in the infected host (Khan et al., 1995).

Many studies have shown that the modulation of mice macrophages activation profile is dependent on the *T. gondii* strain (Saeij et al., 2006; Butcher et al., 2011). During host cell invasion *T. gondii* from type I and type III strains activate STAT3 and STAT6 and polarize macrophages towards an M2 activation profile (Butcher et al., 2011; Jensen et al., 2011), while type II parasites activates NF-κB and polarizes macrophages towards an M1 activation profile (Butcher et al., 2011; Jensen et al., 2011; Murray, 2011). This difference is due to polymorphisms among *T. gondii* virulence factors that dictates the gene expression program that is activated in the host cell (Jensen et al., 2011). ROP16 from type I and III parasites ($ROP16_{I/III}$) phosphorylate and activate STAT3 in macrophages activating a gene expression program related to an alternative activation profile, especially the IL-10-dependent anti-inflammatory

response. Moreover, dense granules proteins from type I and III parasites ($GRA15_{I/III}$), phosphorylate and activate STAT6 that is translocated to host nucleus and upregulates the expression of ARG1, benefitting *T. gondii* (Murray, 2011). This phenomenon is characterized as an evasion mechanism of the parasite because ARG1 produces polyamines that benefits the parasite growth (Morris, 1992)and, at the same time, ARG1 competes with iNOS for the substrate L-Arginine, down modulating the NO production in classically activated macrophages (El-Gayar et al., 2003). However, the benefits of ARG1 expression in *T. gondii* infected macrophages still remains controversial. Studies showed that deletion of ARG1 in macrophages infected with *T. gondii* type II strains favored host survival (El Kasmi *et a*l., 2008). *T. gondii* type II strains polarize macrophages towards an M1 activation profile, because a single amino acid substitution in the kinase domain of $ROP16_{II}$ impairs the activation of STAT3 in infected macrophages and also by activation of NF-κB by $GRA15_{II}$, which is translocated to the host nucleus and upregulates the expression of proinflammatory cytokines (Butcher et al., 2011; Jensen et al., 2011; Murray, 2011).

The major role of MAP kinase phosphatase-2 (MKP-2) in parasitic infections with *T. gondii* has also been reported (Woods et al., 2013). MKP-2 is a phosphatase that is involved in multiple pathways responsible for regulating the body homeostasis and it has also recently been demonstrated to down modulate the ARG1 expression and, at the same time, to up regulate iNOS expression (Al-Mutairi et al., 2010), playing a significant role in *T. gondii*-macrophage interactions. Recent studies have shown that C57BL/6 MKP-$2^{-/-}$ has increased susceptibility to *T. gondii* of type II strains with increased parasite multiplication, leading to mortality of mice with 12 days post-infections and increased parasite burdens in the brain (Woods et al., 2013). The capacity of C57BL/6 MKP-$2^{+/+}$ to control *T. gondii* replication was dependent on NO, as demonstrated by increased parasite burden in this mouse, following treatment with the iNOS inhibitor L-NAME and mortality with 10 days post infections (Woods et al., 2013). Inhibition of ARG1 in MKP-$2^{-/-}$mice treated with nor-NOHA (ARG1 inhibitor) increased parasite burdens indicating that ARG1 has a protective role (Woods et al., 2013). Thus, MKP-2 has a major role in controlling *T. gondii* replication because it up regulates iNOS expression and consequently enhances the NO levels that play a protective role for the host and at the same time down modulates ARG1 expression, an

enzyme that was demonstrated to compete and modulate iNOS activity (El-Gayar et al., 2003).

CONCLUSION

Host-parasite coevolution can be defined as reciprocal selective pressures among different species of hosts and parasites, generating reciprocal adaptive genetic change. Therefore, microbicidal mechanism developed by the host is eventually overcome by the selection of adapted parasites. All these evasion mechanisms developed by *T. gondii* over the coevolution process with the host, made this parasite one of the most successful in the world. Moreover, *T. gondii* has become a model organism for the study of the Apicomplexan phylum, to study the cell biology of the host-parasite interaction, and the understanding of these evasion mechanisms may open new therapeutic venues.

Table 1.Subversion of the host microbicidal mechanisms by *Toxoplasma gondii* evasion strategies.

Microbicidal mechanisms	Evasion strategies	References
Phagocytosis	Inhibition of lysosomal fusion by active invasion	Jones and Hirsch (1972), Morisaki et al. (1995)
ROS[1]	Inhibition of ROS	Wilson et al. (1980), Murray and Cohn (1980)
Nitric oxide	TGF-β secretion and degradation of iNOS[1] by the proteasome	Seabra et al. (2004), Padrão et al. (2014)
IRGs[1]	Phosphorylation and inactivation by ROPs[1]	Zhao et al. (2009b), Fentress et al. (2010), Steinfeldt et al. (2010), Behnke et al. (2011), Niedelman et al. (2012)
Proinflamatory (M1) macrophage profile	Induction of M2 activation profile by ROP16 and GRA15[1]	Butcher et al. (2001), Caamano et al. (1999), Woods

1. Abbreviation: ROS: reactive oxygen species, iNOS: inducible nitric oxide synthase, IRG: immune related GTPases, ROP: proteins of the bulb of the rhoptries, GRA: proteins from dense granules.

ACKNOWLEDGMENTS

The authors thank Andrèa Carvalho César for proofing the manuscript. This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and Fundação de Coordenação de Pessoal de Nível Superior (CAPES).

REFERENCES

Abu-madi, M. A., Al-Molawi, N., Behnke, J. M. (2008) Seroprevalence and epidemiological correlates of *Toxoplasma gondii* infections among patients referred for hospital-based serological testing in Doha, Qatar. Parasitol Vectors. 39, 1-9.

Adams, L. B., Hibbs, J. B., Taintor, R. R., Krahenbuhl, J. S. (1990) Microbiostatic effect of murine activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. J Immunol. 144, 2725-2729.

Alexander, D. L., Mital, J., Ward, G. E., Bradley, P. Boothroyd, J. C. (2005) Identification of the moving junction complex of *Toxoplasma gondii*: a collaboration between distinct secretory organelles. PLoS Pathog. 1, e17.

Alexander, J., Scharton-Kersten, T. M., Yap, G., Roberts, C. W., Liew, F. Y., Sher, A. (1997) Mechanisms of innate resistance to *Toxoplasma gondii* infection. Philos Trans R Soc Lond B Biol Sci. 352, 1355-1359.

Aline, F., Bout, D., Dimier-Poisson, I. (2002) Dendritic cells as effector cells: gamma interferon activation of murine dendritic cells triggers oxygen-dependent inhibition of *Toxoplasma gondii* replication. Infect Immun. 70, 2368-2374.

Anderson, S. E. Jr, Bautista, S. C., Remington, J. S. (1976) Specific antibody-dependent killing of *Toxoplasma gondii* by normal macrophages. Clin Exp Immunol. 26, 375-380.

Arandjelovic, S., Ravichandran, K. S. (2015) Phagocytosis of apoptotic cells in homeostasis. Nat Immunol. 16, 907-917.

Ashcroft, G. S. (1999) Bidirectional regulation of macrophage function by TGF-beta. Microbes Infect. 1, 1275-1282.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., Geissmann F. (2007) Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science. 317, 666-670.

Behnke, M. S., Fentress, S. J, Mashayekhi, M., Li, L. X., Taylor, G. A., Sibley, L. D. (2012) The polymorphic pseudokinase ROP5 controls virulence in *Toxoplasma gondii* by regulating the active kinase ROP18. PLoS Pathog. 8, e1002992.

Bekpen, C., Hunn, J.P., Rohde, C., Parvanova, I., Guethlein, L., Dunn, D. M., Glowalla, E., Leptin, M., Howard, J. C. (2005) The interferon-inducible p47 (IRG) GTPases in vertebrates: loss of the cell autonomous resistance mechanism in the human lineage. Genome Biol. 6, R92.

Benard, A., Petersen, E., Salamon, R., Chene, G., Gilbert, R., Salmi, L. R. (2008) Survey of European programmes for the epidemiological surveillance of congenital toxoplasmosis. European Toxo Prevention Study Group (EUROTOXO). Euro Surveill.13, 257-263.

Bigley, V., Haniffa, M., Doulatov, S., Wang, X. N., Dickinson, R., McGovern, N., Jardine, L., Pagan, S., Dimmick, I., Chua, I., Wallis, J., Lordan, J., Morgan, C., Kumararatne, D. S., Doffinger, R., Van Der Burg, M., Van Dongen J., Cant, A., Dick, J. E., Hambleton, S., Collin, M. T (2011) The human syndrome of dendritic cell, monocyte, B and NK T lymphoid deficiency. J Exp Med. 208, 227-234.

Black, M. W., Boothroyd, J. C. (2000) Lytic cycle of *Toxoplasma gondii*. Microbiol Mol Biol Rev. 64, 607-623.

Blader, I. J., Manger, I. D., Boothroyd, J. C. (2001) Micro-array analysis reveals previously unknown changes in *Toxoplasma gondii*-infected human cells. J Biol Chem. 276, 24223-24231.

Blanden, R. V., Lefford, M. J., Mackaness, G. B. (1969) The host response to Calmette-Guerin bacillus infection in mice. J exp Med. 129,1079-1107.

Bogdan, C. (2001) Nitric oxide and the immune response. Nat. Immunol. 2, 907-916.

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, 1761-1767.

Boothroyd, J. C., Dubremetz, J. F. (2008) Kiss and spit: the dual roles of Toxoplasma rhoptries. Nat Rev Microbiol. 6, 79-88.

Braun, L., Brenier-Pinchart, M. P., Yogavel, M., Curt-Varesano, A., Curt-Bertini, R. L., Hussain, T., Kieffer-Jaquinod, S., Coute, Y., Pelloux, H., Tardieux, I., Sharma, A., Belrhali, H., Bougdour, A., Hakimi, M. A. (2013) Toxoplasma dense granule protein, GRA24, modulates the early immune response to infection by promoting direct and sustained host p38 MAPK activation. J Exp Med. 210, 2071-2086.

Bredt, D. S., Snyder, S. H. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc Natl Acad Sci. 87, 682-685.

Brown, G. C., Vilalta, A., Fricker, M. (2015) Phagoptosis - Cell Death By Phagocytosis - Plays Central Roles in Physiology, Host Defense and Pathology. Curr Mol Med. 15, 842-851.

Butcher, B. A., Fox, B. A., Rommereim, L. M., Kim, S. G., Maurer, K. J., Yarovinsky, F., Herbert, D. R., Bzik, D. J., Denkers, E. Y. (2011) *Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. Plos Pathogens. 7, 1-16.

Butcher, B. A., Denkers, E. Y. (2002) Mechanism of entry determines ability of *Toxoplasma gondii* to inhibit macrophage proinflammatory cytokine production. Infect Immun. 70, 5216-5224.

Butcher, B. A., Greene, R. I., Henry, S. C., Annecharico, K. L., Weinberg, J. B., Denkers, E. Y., Sher, A., Taylor, G. A. (2005) p47 GTPases regulate *Toxoplasma gondii* survival in activated macrophages. Infect Immun. 73, 3278-3286.

Butcher, B. A., Kim, L., Johnson, P. F., Denkers, E. Y. (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.

Caamano J, Alexander J, Craig L, Bravo R, Hunter, C. A. (1999) The NF-kappa B family member RelB is required for innate and adaptive immunity to *Toxoplasma gondii*. J Immunol. 163, 4453-4461.

Cabral, A. S. G., Balbino, F., Abdalla, L. F., Costa, S. S. S., Vaz Jar, Fonseca, S. F. (2008) Seroprevalence of toxoplasmosis in women of reproductive age in the city of Brasilia, capital of Brazil. International Congress of Infectious Diseases. Kuala Lumpur, Malaysia. Abstract 179.

Calabrese, V., Mancuso, C., Calvani, M., Rizzarelli, E., Butterfield, D.A., Stella, A. M. (2007) Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nat Rev Neurosci. 8, 766-775.

Carruthers, V. B., Sibley, L. D. (1997) Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. Eur J Cell Biol. 73, 114-123.

Carruthers, V. B., Sibley, L. D. (1999) Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. Mol Microbiol. 31, 421-428.

Cesbron-Delauw, M.F. (1994) Dense granule organelles of *Toxoplasma gondii*: Their role in the host-parasite relationship. Parasitol Today. 10, 293-296.

Chang, H. R., Pechère, J. C. (1989) Macrophage oxidative metabolism and intracellular *Toxoplasma gondii*. Microb Pathog. 7, 37-44.

Cheng, YS., Colonno, R. J., Yin, F. H. (1983) Interferon induction of fibroblast proteins with guanylate binding activity. J Biol Chem. 258, 7746-7750.

Chobotar, B., Scholtyseck, E. (1982) Ultraestructure. In The biology of coccidia. (P.L. Long, ed.), Univ. Park Press, Baltimore. pp. 101–165.

Choi, J. Y., Nam, S. A., Jin, D. C., Kim, J., Cha, J. H. (2012) Expression and cellular localization of inducible nitric oxide synthase in lipopolysaccharide-treated rat kidneys. J Histochem Cytochem. 60, 301-315.

Collazo, C. M., Yap, G. S., Sempowski, G. D., Lusby, K. C., Tessarollo, L. Vande Woude, G. F., Sher, A., Taylor, G. A. (2001) Inactivation of LRG-47 and IRG-47 reveals a family of interferon gamma-inducible genes with essential, pathogen specific roles in resistance to infection. J Exp Med. 194,181-188.

Coppens, I., Dunn, J. D., Romano, J. D., Pypaert, M., Zhang, H., Boothroyd, J. C., Joiner, K. A. (2006) *Toxoplasma gondii* sequesters lysosomes from mammalian hosts in the vacuolar space. Cell. 125, 261-274.

Coppens, I., Sinai, A. P., Joiner, K. A. (2000) *Toxoplasma gondii* exploits host low density lipoprotein receptor mediated endocytosis for cholesterol acquisition. J Cell Biol. 149,167-180.

Corrêa, G., Marques da Silva, C., de Abreu Moreira-Souza, A. C., Vommaro, R. C., Coutinho-Silva, R. (2010) Activation of the P2X(7) receptor triggers the elimination of *Toxoplasma gondii* tachyzoites from infected macrophages. Microbes Infect. 12, 497-504.

Cross, A. R., Segal, A. W. (2004) The NADPH oxidase of professional phagocytes--prototype of the NOX electron transport chain systems. Biochim Biophys Acta. 1657, 1-22.

Daff, S. (2010) NO synthase: structures and mechanisms. Nitric Oxide. 23,1-11.

Dalod, M., Chelbi, R., Malissen, B., Lawrence, T. (2014) Dendritic cell maturation: functional specialization through signaling specificity and transcriptional

programming. EMBO J. 33, 1104-1116.
Dincel, G. C., Atmaca, H. T. (2015) Nitric oxide production increases during *Toxoplasma gondii* encephalitis in mice. Exp Parasitol. 156, 104-112.
Ding, M., Kwok, L. Y., Schlüter, D., Clayton, C., Soldati, D. (2004) The antioxidant systems in *Toxoplasma gondii* and the role of cytosolic catalase in defense against oxidative injury. Mol Microbiol. 51, 47-61.
Dobrowski, J. M., Sibley, L. D. (1996) Toxoplasma invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. Cell. 84, 933-939.
Dubey, J. P. (2004) Toxoplasmosis – a waterborne zoonosis. Vet Parasitol. 126, 57-72.
Dubey, J. P., Lindsay, D. S. Speer, C. A. (1998) Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin Microbiol Rev. 11, 267-299.
Dunay, I. R., Damatta, R. A., Fux, B., Presti, R., Greco, S., Colonna, M., Sibley, L. D. (2008) Gr1(+) inflammatory monocytes are required for mucosal resistance to the pathogen *Toxoplasma gondii*. Immunity 29, 306-317.
El Kasmi, K. C., Qualls, J. E., Pesce, J. T., Smith, A. M., Thompson, R. W., Henao-Tamayo, M., Basaraba, R. J., König, T., Schleicher, U., Koo, M. S., Kaplan, G., Fitzgerald, K. A., Tuomanen, E. I., Orme, I. M., Kanneganti, T. D., Bogdan, C., Wynn, T. A., Murray, P. J. (2008) Toll-like receptor–induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol. 9, 1399-1406.
El-Gayar, S., Thuring-Nahler, H., Pfeilschifter, J., Rollinghoff, M., Bogdan, C. (2003) Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. J Immunol. 171, 4561-4568.
Erwig, L. P., Henson, P. M. (2008) Clearance of apoptotic cells by phagocytes. Cell Death Differ. 15, 243-250.
Etheridge, R. D., Alagan, A., Tang, K., Turk, B. E., Sibley, L. D. (2014) The Toxoplasma pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice. Cell Host Microbe. 15, 537-550.
Fayer, R. (1972) Penetration of cultured cells by *Eimeria meleagrimitis* and *E. tenella* sporozoites. J Parasitol. 58, 921-927.
Feng, C. G., Collazo-Custodio, C. M., Eckhaus, M., Hieny, S., Belkaid., Elkins, K., Jankovic, D., Taylor, G. A., Sher, A. (2004) Mice deficient in LRG-47 display increased susceptibility to mycobacterial infection associated with the induction of lymphopenia. J Immunol. 172, 1163-1168.
Fentress, S. J., Behnke, M. S., Dunay, I. R., Mashayekhi, M., Rommereim, L. M., Fox, B. A., Bzik, D. J., Taylor, G. A., Turk, B. E., Lichti, C. F., Townsend, R. R., Qiu, W., Hui R, Beatty, W. L., Sibley, L. D. (2010) Phosphorylation of immunity-related GTPases by a *Toxoplasma gondii*-secreted kinase promotes macrophage survival and virulence. Cell Host Microbe. 16, 484-495.
Flannagan, R. S., Jaumouille, V., Grinstein, S. (2012) The cell biology of phagocytosis. Annu Rev Pathol. 7,61-98.
Fleckenstein, M. C., Reese, M. L., Könen-Waisman, S., Boothroyd, J. C., Howard, J. C., Steinfeldt, T. A. (2012) *Toxoplasma gondii* pseudokinase inhibits host IRG resistance proteins. PLoS Biol. 10, e1001358.
Förstermann, U., Münzel, T. (2006) Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation. 113, 1708-1714.
Förstermann, U., Sessa, W. C. (2012) Nitric oxide synthases: regulation and function.

Eur Heart J. 33, 829-837.
Frenal, K., Polonais, V., Marq, J. B., Stratmann, R., Limenitakis, J., Soldati-Favre, D. (2010) Functional dissection of the apicomplexan glideosome molecular architecture. Cell Host Microbe. 8, 343-357.
Furchgott, R. F., Zawadzki, J. V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 288, 373-376.
Gaskins, E., Gilk, S., DeVore, N., Mann, T., Ward, G., and Beckers, C. (2004). Identification of the membrane receptor of a class XIV myosin in *Toxoplasma gondii*. J. Cell Biol. 165, 383-393.
Gazzinelli, R. T., Eltoum, I., Wynn, T. A., Sher, A. (1993) Acute cerebral toxoplasmosis is induced by *in vivo* neutralization and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. J. Immunol. 151, 3672-3681.
Gazzinelli, R. T., Wysocka, M., Hayashi, S., Denkers, E. Y., Hieny, S., Caspar, P., Trinchieri, G., Sher, A. (1994) Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with *Toxoplasma gondii*. J Immunol. 15, 2533-2543.
Geissmann, F., Gordon, S., Hume, D. A., Mowat, A. M., Randolph, G. J. (2010) Unravelling mononuclear phagocyte heterogeneity. Nat Rev Immunol. 10, 453-460.
Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H., Billiar, T. R. (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. Proc Natl Acad Sci. 90, 3491-3495.
Ghosh, D. K., Stuehr, D. J. (1995) Macrophage NO synthase: characterization of isolated oxygenase and reductase domains reveals a head-to-head subunit interaction. Biochemistry 34, 801-807.
Gilbert, L. A., Ravindran, S., Turetzky, J. M., Boothroyd, J. C., Bradley, P. J. (2007) *Toxoplasma gondii* targets a protein phosphatase 2C to the nuclei of infected host cells. Eukaryot Cell. 6, 73-83.
Gilly, M., Wall, R. (1992) The IRG-47 gene is IFN-gamma induced in B cells and encodes a protein with GTP-binding motifs. J Immunol. 148, 3275-3281.
Giovannoni, G., Heales, S. J., Land, J. M., Thompson, E. J., (1998) The potential role of nitric oxide in multiple sclerosis. Mult Scler. 4, 212-216.
Gordon, S., Plűddemann, A. (2013) Tissue macrophage heterogeneity: issues and prospects. Semin Immunopathol. 35, 533-540.
Gordon, S., Taylor, P. R. (2005) Monocyte and macrophage heterogeneity. Nat Rev Immunol. 5, 953-964.
Griffin, F. M., Griffin, J. A., Leider, J. E., Silverstein, S. C. (1975) Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma mambrane. J Exp Med. 142, 1263-1282.
Guillermo, L. V. C., DaMatta, R. A. (2004) Nitric oxide inhibition after *Toxoplasma gondii* infection of chicken macrophages cell lines. Poult Sci. 83, 776-782.
Guimarães, F. N., Meyer, H. (1942) Cultivo de "Toxoplasma" Nicolle & Manceaux, 1909, em cultura de tecidos. Rev Bras Biol. 2, 123-129.
Gupta, S. L., Rubin, B. Y., Holmes, S. L. (1979) Interferon action: induction of specific proteins in mouse and human cells by homologous interferons. Proc Natl Acad Sci. 76, 4817-4821.

Hackam, D. J., Rotstein, O.D., Zhang, W. J., Demaurex, N., Woodside, M., Tsai, O., Grinstein, S. (1997) Regulation of phagosomal acidification. Differential targeting ofNa+/H+ exchangers, Na+/K+-ATPases, and vacuolar-type H+-atpases. J Biol Chem. 272, 29810-29820.

Haldar, A. K., Saka, H. A., Piro, A. S., Dunn, J. D., Henry, S. C., Taylor, G. A., Frickel, E. M., Valdivia, R. H., Coers, J. (2013) IRG and GBP host resistance factors target aberrant, "non-self" vacuoles characterized by the missing of “self” IRGM proteins. PLoS Pathog. 9, e1003414.

Halonen, S. K., Taylor, G. A., Weiss, L. M., (2001) Gamma interferon-induced inhibition of *Toxoplasma gondii* in astrocytes is mediated by IGTP. Infect Immun. 69, 5573-5576.

Hamilton, T. A., Zhao, C., Pavicic, P. G. J. R., Datta, S. (2014) Myeloid colony stimulating factors as regulators of macrophage polarization. Front Immunol. 5, 1-6.

Henry, S. C., Daniell, X. G., Burroughs, A. R., Indaram, M., Howell, D. N., Coers, J., Starnbach, M. N., Hunn, J. P., Howard, J. C., Feng, C. G., Sher, A., Taylor, G. A. (2009) Balance of Irgm protein activities determines IFN-gamma-induced host defense. J Leukoc Biol. 85, 877-885.

Henry, S. C., Traver, M., Daniell, X., Indaram, M., Oliver T., Taylor, G. A. (2010) Regulation of macrophage motility by Irgm1. J Leukoc Biol. 87, 333-343.

Henson, P. M., Bratton, D. L. (2009) Recognition and removal of apoptotic cells. In Phagocyte-pathogen interactions: macrophages and the host response to infection, D.G. Russell and S. Gordon, eds. (ASM Press), pp. 341–365.

Hirai, K., Hirato, K., Yanagawa, R. (1966) A cinematographic study of the penetration of cultured cells by *Toxoplasma gondii*. Jpn J Vet Res. 14, 81-90.

Hoffman, S. L., Crutcher, J. M., Puri, S. K., Ansari, A. A., Villinger, F., Franke, E. D., Singh, P. P., Finkelman, F., Gately, M. K., Dutta, G. P., Howe, D. K., Sibley, L. D. (1995) *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis. 172, 1561-1566.

Hoffman. S. L., Crutcher, J. M., Puri, S. K., Ansari, A. A., Villinger, F., Franke, E. D., Singh, P. P., Finkelman, F., Gately, M. K., Dutta, G. P., Sedegah, M. (1997) Sterile protection of monkeys against malaria after administration of interleukin-12. Nat Med. 3, 80-83.

Horisberger, M. A., Staeheli, P. O. (1983) Haller, Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. Proc. Natl. Acad. Sci. 80, 1910-1914.

Howard, J. C., Hunn, J. P., Steinfeldt, T. (2011) The IRG protein-based resistance mechanism in mice and its relation to virulence in *Toxoplasma gondii*. Curr Opin Microbiol.14, 414-421.

Howe, D. K., Sibley, L. D. (1995) *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis. 172, 1561-1566.

Hu, X., Ivashkiv, L. B. (2009) Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. Immunity. 31, 539-550.

Huang, Z., Hoffmann, F. W., Fay, J. D., Hashimoto, A. C., Chapagain, M. L., Kaufusi, P. H., Hoffmann, P. R. (2012) Stimulation of unprimed macrophages with immune complexes triggers a low output of nitric oxide by calcium-dependent neuronal nitric-oxide synthase. J Biol Chem. 287, 4492-4502.

Hume, D. A. (2006) The mononuclear phagocyte system. Curr Opin Immunol. 18, 49-

53.
Hume, D. A. (2008) Macrophages as APC and the dendritic cell myth. J Immunol. 181, 5829-5835.
Hunn, J. P., Koenen-Waisman, S., Papic, N., Schroeder, N., Pawlowski, N., Lange, R., Kaiser, F., Zerrahn, J., Martens, S., Howard, J. C. (2008) Regulatory interactions between IRG resistance GTPases in the cellular response to *Toxoplasma gondii*. EMBO J. 27, 2495-2509.
Hunter, C. A., Sibley, L. D. (2012) Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. Nat Rev Microbiol. 11, 766-778.
Jacobs, L. (1956) Propagation, morphology, and biology of *Toxoplasma*. Ann N Y Acad Sci. 64, 154-179.
Janssens, S. P., Shimouchi, A., Quertermous, T., Bloch, D. B., Bloch, K. D. (1992) Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. J. Biol. Chem. 267, 14519-14522.
Jenkins, S.J., Hume, D.A. (2014) Homeostasis in the mononuclear phagocyte system. Trends Immunol. 35, 358-367.
Jensen, K.D., Wang, Y., Wojno, E.D., Shastri, A.J., Hu, K., Cornel, L., Boedec, E., Ong, Y.C, Chien, Y.H., Hunter, C.A., Boothroyd, J.C., Saeij, J.P. (2011) Toxoplasma polymorphic effectors determine macrophage polarization and intestinal inflammation. Cell Host Microbe. 16, 472-483.
Joiner, K. A., Fuhrman, S. A., Miettinen, H. M., Kasper, L. H., Mellman, I. (1990) *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. Science. 249, 641-646.
Jones, T. C., Hirsch, J. G. (1972) The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. J Exp Med. 1, 1173-1194.
Jones, T. C., Yeh S., Hirsch, J. G. (1972). The interaction between *Toxoplasma gondii* and mammalian cells. I. Mechanism of entry and intracellular fate of the parasite. J Exp Med. 136, 1157-1172.
Jyoti, A., Singh, A. K., Dubey, M., Kumar, S., Saluja, R., Keshari, R. S., Verma, A., Chandra, T., Kumar, A., Bajpai, V. K., Barthwal, M. K., Dikshit, M. (2014) Interaction of inducible nitric oxide synthase with rac2 regulates reactive oxygen and nitrogen species generation in the human neutrophil phagosomes: implication in microbial killing. Antioxid. Redox Signal. 20, 417-431.
Keller, R., Keist, R., Ivatt, R.J. (1974) Functional and biochemical parameters of activation related to macrophage cytostatic effects on tumor cells. Int J Cancer. 14, 675-683.
Kemp, L. E., Yamamoto, M., Soldati-Favre, D. (2013) Subversion of host cellular functions by the apicomplexan parasites. FEMS Microbiol Rev. 37, 607-631.
Kessler, H., Herm-Gotz, A., Hegge, S., Rauch, M., Soldati-Favre, D., Frischknecht, F., Meissner, M. (2008) Microneme protein 8: a new essential invasion factor in *Toxoplasma gondii*. J Cell Sci. 121, 947-956.
Khaminets, A., Hunn, J. P., Könen-Waisman, S., Zhao, Y. O., Preukschat, D., Coers, J., Boyle, J. P., Ong, Y. C., Boothroyd, J. C., Reichmann, G., Howard, J. C. (2010) Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. Cell Microbiol. 12, 939-961.
Khan, A., Taylor, S., Ajioka, J. W., Rosenthal, B. M., Sibley, L. D. (2009) Selection at a single locus leads to widespread expansion of *Toxoplasma gondii* lineages that are

virulent in mice. PLoS Genet. 5, e1000404.
Khan, I. A., Schwartzman, J. D., Matsuura, T., Kasper, L. H. (1997) A dichotomous role for nitric oxide during acute *Toxoplasma gondii* infection in mice. Proc Natl Acad Sci. 94, 13955-13960.
Khan, I., Matsuura, T., Kasper, L. H. (1995) IL-10 mediated immunosuppression following primary infection with *Toxoplasma gondii* in mice. Parasite Immunol 17, 185-195.
Kim, B. H., Shenoy, A. R., Kumar, P., Das, R., Tiwari, S., and MacMicking, J. D. (2011) A family of IFN-g-inducible 65-kD GTPases protects against bacterial infection. Science. 332, 717-721.
Klamp, T., Boehm, U., Schenk, D., Pfeffer, K., Howard, J. C., (2003) A giant GTPase, very large inducible GTPase-1, is inducible by IFNs. J. Immunol.171, 1255-1265.
Koerner, T. J., Adams, D. O., Hamilton, T. A. (1987) Regulation of tumor necrosis factor (TNF) expression: interferon-gamma enhances the accumulation of mRNA for TNF induced by lipopolysaccharide in murine peritoneal macrophages. Cell Immunol. 15, 437-443.
Kone, B. C., Kuncewicz, T., Zhang, W., Yu, Z. (2003) Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. Am J Physiol Renal Physiol. 285, 178-190.
Lang, C., Gross, U., Lüder, C. G. (2007) Subversion of innate and adaptive immune responses by *Toxoplasma gondii*. Parasitol Res. 2, 191-203.
Lee, A. J., Cho, K. J., Kim, J. H. (2015) MyD88-BLT2-dependent cascade contributes to LPS-induced interleukin-6 production in mouse macrophage. Exp Mol Med. 47, e156.
Lemgruber, L., Lupetti, P., De Souza W, Vommaro, R. C. (2011) New details on the fine structure of the rhoptry of *Toxoplasma gondii*. Microsc Res Tech. 74, 812-818.
Lennon-Duménil, A. M., Bakker, A. H., Maehr, R., Fiebiger, E., Overkleeft, H. S., Rosemblatt, M., Ploegh, H. L., Lagaudrière-Gesbert, C. (2002) Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. J Exp Med. 196, 529-540.
Leto, T. L., Morand, S., Hurt, D., Ueyama, T. (2009) Targeting and regulation of reactive oxygen species generation by Nox family NADPH oxidases. Antioxid Redox Signal. 11, 2607-2619
Lilue, J., Muller, U. B., Steinfeldt, T., Howard, J. C. (2013) Reciprocal virulence and resistance polymorphism in the relationship between *Toxoplasma gondii* and the house mouse. Elife2, e01298.
Ling, Y. M., Shaw, M. H., Ayala, C., Coppens, I., Taylor, G. A., Ferguson, D. J., Yap, G. S (2006) Vacuolar and plasma membrane stripping and autophagic elimination of *Toxoplasma gondii* in primed effector macrophages. J Exp Med. 203, 2063-2071.
Lowenstein, C. J., Padalko, E. (2004) iNOS (NOS2) at a glance. J Cell Sci. 117, 2865-2867.
Lund, E., Lycke, E., Sourander, P. (1961) A cinematographic study of *Toxoplasma gondii* in cell cultures. Br J Exp Pathol. 42, 357-362.
Mackaness, G. B. (1962) Cellular resistance to infection. J Exp Med. 116, 381-406.
Mackaness, G. B. (1970) The monocyte in cellular immunity. Semin Hematol. 7, 172-184.
MacMicking, J. D., Taylor, G. A., McKinney, J. D. (2003) Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science. 302, 654-659.

Maeda, H., Akaike, T. (1998) Nitric oxide and oxygen radicals in infection, inflammation, and cancer. Biochemistry. 63, 854-865.

Mantegazza, A. R., Magalhaes, J. G., Amigorena, S., Marks, M. S. (2013) Presentation of phagocytosed antigens by MHC class I and II. Traffic. 14, 135-152.

Martens, S., Sabel, K., Lange, R., Uthaiah, R., Wolf, E., Howard, J. C (2004) Mechanisms regulating the positioning of mouse p47 resistance GTPases LRG-47 and IIGP1 on cellular membranes: retargeting to plasma membrane induced by phagocytosis. J Immunol. 173, 2594-2606.

Martinez, F. O., Gordon, S. (2014) The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000 Prime Rep. 3, 6-13.

Martinez, F. O., Helming, L. M., Gordon, S. (2009) Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol. 27, 451-483.

Martinez, F. O., Sica, A., Mantovani, A., Locati, M. (2008) Macrophage activation and polarization. Front Biosci.13, 453-461.

Mauel, J. (1982) Macrophage activation and effector mechanisms against microb. 155, 675-686.

McMillan, K., Masters, B. S (1995) Prokaryotic expression of the heme- and flavin-binding domains of rat neuronal nitric oxide synthase as distinct polypeptides: identification of the heme-binding proximal thiolate ligand as cysteine-415. Biochemistry 34, 3686-3693.

Mercier, C., Adjogble, K. D., Daubener, W., Cesbron-Delauw, M. F. (2005) Dense granules: Are they key organelles to help understand the parasitophorous vacuole of all apicomplexa parasites? Int J Parasitol. 35, 829-849.

Mercier, C., Dubremetz, J. F., Rauscher, B., Lecoedier, L., Sibley, L. D., Cesbron-Delauw, M. F. (2002) Biogenesis of nanotubular network in *Toxoplasma* parasitophorous vacuole induced by parasite proteins. Mol Biol Cell. 13, 2397-2409.

Metchnikoff, E. (1884) Uber die Beziehung der Phagocyten zu Milzbrandbacillen. Arch Pathol Anat. 97, 502-526.

Miller, S. A., Thathy, V., Ajioka, J. W., Blackman, M. J., Kim, K. (2003) TgSUB2 is a *Toxoplasma gondii* rhoptry organelle processing proteinase. Mol Microbiol. 49, 883-894.

Mills, C. D. (2012) M1 and M2 macrophages: oracles of health and disease. Crit Rev Immunol. 32, 463-488.

Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J., Hill, A. M. (2000) M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol. 164, 6166-6173.

Mital, J., Meissner, M., Soldati, D., Ward, G. E. (2005) Conditional expression of *Toxoplasma gondii* apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion. Mol Biol Cell. 16, 4341-4349.

Mitchell, G. H., Thomas, A. W., Margos, G., Dluzewski, A. R., Bannister, L. H (2004) Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells. Infect Immun. 72, 154-158.

Miyahara, K., Kawamoto, T., Sase, K., Yui, Y., Toda, K., Yang, L. X., Hattori, R., Aoyama, T., Yamamoto, Y., Doi, Y., Ogosh, S., Hashimoto, K., Kawai, C., Sasayama, S., Shizuta, Y. (1994) Cloning and structural characterization of human endothelial nitric oxide synthase gene. Eur J Biochem. 223, 719-726.

Molestina, R. E., Payne, T. M., Coppens, I., Sinai, A. P. (2003) Activation of NF-

{kappa}B by *Toxoplasma gondii* correlates with increased expression of antiapoptotic genes and localization of phosphorylated I{kappa}B to the parasitophorous vacuole membrane. J Cell Sci. 116, 4359-4371.

Molestina, R. E., Sinai, A. P. (2005) Host and parasite-derived IKK activities direct distinct temporal phases of NF-kappaB activation and target gene expression following *Toxoplasma gondii* infection. J Cell Sci. 118, 5785-5796.

Mordue, D. G., Dessai, N., Dustin, M., Sibley, L. D (1999) Invasion by *Toxoplasma gondii* establishes a moving junction that selectively excludes host cell plasma membrane proteins on the basis of their membrane anchoring. J Exp Med. 190, 1783-1792.

Morisaki, J. H., Heuser, J. E., Sibley, L. D. (1995) Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell. J Cell Sci. 108, 2457-2464.

Morris, S. M. J. R. (1992) Regulation of enzymes of urea and arginine synthesis. Annu Rev Nutr. 12, 81-101.

Murray, H. W. , Juangbhanich, C. W., Nathan, C. F., Cohn, Z. A. (1979) Macrophage oxygen-dependent antimicrobial activity. II. The role of oxygen intermediates. J Exp Med. 150, 950-964.

Murray, H. W., Cohn, Z. A. (1979) Macrophage oxygen-dependent antimicrobial activity. I. Susceptibility of *Toxoplasma gondii* to oxygen intermediates. J Exp Med. 1, 938-949.

Murray, H. W., Cohn, Z. A. (1980) Macrophage oxygen-dependent antimicrobial activity. III. Enhanced oxidative metabolism as an expression of macrophage activation. J Exp Med. 152, 1596-1609.

Murray, P. J (2011) Macrophages as a battleground for toxoplasma pathogenesis. Cell Host Microbe. 9, 445-447.

Murray, P. J. (2007) The JAK-STAT signaling pathway: input and output integration. J Immunol. 178, 2623-2629.

Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., Gordon, S., Hamilton, J. A., Ivashkiv, L. B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F. O., Mege, J. L., Mosser, D.M, Natoli, G., Saeij, J. P., Schultze, J. L., Shirey, K. A., Sica, A., Suttles, J., Udalova, I., van Ginderachter, J. A., Vogel, S. N., Wynn, T. A (2014) Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 41, 14-20.

Musial, A., Eissa, T. (2001) Inducible nitric-oxide synthase is regulated by the proteasome degradation pathway. J Biol Chem. 26, 24268-24273.

Nabias, R., Ngouamizokou, A., Migot-Nabias, F., Mbou-Moutsimbi, R. A., Lansoudsoukate, J (1998) Serological investigation of toxoplasmosis in patients of the M.I.P. center of Franceville (Gabon). Bull Soc Pathol Exot. 91, 318-320.

Nakane, M., Schmidt, H. H., Pollock, J. S., Forstermann, U., Murad, F (1993) Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. FEBS Lett. 316, 175-180.

Nathan, C. F. (1983) Mechanisms of macrophage antimicrobial activity. Trans R Soc Trop Med Hyg. 77, 620-630.

Nathan, C. F., Hibbs, J. B. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr Opin Immunol. 3, 65-70

Nelson, M. M., Jones, A. R., Carmen, J. C., Sinai, A. P., Burchmore, M., Wastling, J. M (2008) Modulation of host cell proteome by the intracellular apicomplexan parasite *Toxoplasma gondii*. Infection and Immunity. 76, 828-844.

Nicolle, C., Manceaux, L. (1908) Sur une infection à corps de Leishman (ou organismes voisins) du gondi. C R Seances Acad Sci. 147, 763-766.

Niedelman, W., Gold, D. A., Rosowski, E. E., Sprokholt, J.K., Lim, D., Farid Arenas A, Melo, M.B., Spooner, E., Yaffe, M.B., Saeij, J.P (2012) The rhoptry proteins ROP18 and ROP5 mediate *Toxoplasma gondii* evasion of the murine, but not the human, interferon-gamma response. PLoS Pathog. 8, e1002784.

Nowicki, P. T., Caniano, D. A., Hammond, S., Giannone, P. J., Besner, G. E., Reber, K. M., Nankervis, C. A. (2007) Endothelial nitric oxide synthase in human intestine resected for necrotizing enterocolitis. J Pediatr. 150, 40-45.

Opitz, C., Soldati, D (2002) 'The glideosome': a dynamic complex powering gliding motion and host cell invasion by *Toxoplasma gondii*. Mol Microbiol. 45, 597–604.

Ottaviani, E., Malagoli, D., Grimaldi, A., Eguileor M. (2012) The case of the ''serfdom'' condition of phagocytic immune cells. Invert Surv J. 9, 134-138.

Padrão, Jda C., Cabral, G. R. A., da Silva Mde F., Seabra, S. H., DaMatta, R. A. (2014) *Toxoplasma gondii* infection of activated J774-A1 macrophages causes inducible nitric oxide synthase degradation by the proteasome pathway. Parasitol Int. 63, 659-663.

Pantalone, R., Page, R. C. (1977) Enzyme production and secretion by lymphokine-activated macrophages. J Reticuloendothel Soc. 21, 343-357.

Pappas, G., Roussos, N., Falagas, M. E. (2009) Toxoplasmosis snapshots: global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. Int J Parasitol. 12, 1385-1394.

Paredes-Santos, T. C., De Souza, W., Attias, M. (2012) Dynamics and 3D organization of secretory organelles of *Toxoplasma gondii*. J Struct Biol. 177, 420-430.

Pearson, G., Robinson, F., Beers-Gibson, T., Xu, B. E., Karandikar, M., Berman, K., Cobb, M. H. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocrine Reviews. 22, 153-183.

Plattner, F., Yarovinsky, F., Romero, S., Didry, D., Carlier, M. F., Sher, A., Soldati-Favre, D. (2008) Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. Cell Host Microbe. 14, 77-87.

Poltorak, A., Smirnova, I., He, X., Liu, M. Y., Van Huffel, C., McNally, O., Birdwell, D., Alejos, E., Silva, M., Du, X., Thompson, P., Chan, E. K., Ledesma, J., Roe, B., Clifton, S., Vogel, S. N., Beutler, B. (1998) Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region. Blood Cells Mol Dis. 24, 340-355.

Priestley, J. (1774) Experiments and Observations on Different Kinds of Air. London W. Bowyer and J. Nichols, 1774; —. Experiments and Observations on Different Kinds of Air. Vol. 2. London: Printed for J. Johnson, 1775;. Experiments and Observations on Different Kinds of Air. London: Printed for J. Johnson.

Rabinovitch, M. (1995) Professional and non-professional phagocytes: an introduction. Trends Cell Biol. 5, 85-87.

Raetz, M., Kibardin, A., Sturge, C. R., Pifer, R., Li, H., Burstein, E., Ozato, K., Larin, S., Yarovinsky, F. (2013) Cooperation of TLR12 and TLR11 in the IRF8-dependent IL-12 response to *Toxoplasma gondii* profilin. J Immunol. 1, 4818-4827.

Robben, P. M., Mordue, D. G., Truscott, S. M., Takeda, K., Akira, S., Sibley, L. D. (2004) Production of IL-12 by macrophages infected with *Toxoplasma gondii* depends on the parasite genotype. J Immunol. 172, 3686-3694.

Saeij, J. P., Boyle, J. P., Boothroyd, J. C. (2005) Differences among the three major

strains of *Toxoplasma gondii* and their specific interactions with the infected host. Trends Parasitol. 21, 476-481.

Saeij, J. P., Boyle, J. P., Coller, S., Taylor, S., Sibley, L. D., Brooke-Powell, E. T., Ajioka, J. W., Boothroyd, J. C. (2006) Polymorphic secreted kinases are key virulence factors in toxoplasmosis. Science. 314, 1780-1783.

Santiago, H. C., Feng, C. G., Bafica, A., Roffe, E. , Arantes, R. M., Cheever, A., Taylor, G., Vieira, L. Q., Aliberti, J., Gazzinelli, R.T, Sher, A. (2005) Mice deficient in LRG-47 display enhanced susceptibility to *Trypanosoma cruzi* infection associated with defective hemopoiesis and intracellular control of parasite growth. J Immunol 175, 8165-8172.

Santos, T. A., Portes, Jde A, Damasceno-Sá, J. C., Caldas, L. A., Souza, Wd., DaMatta, R. A., Seabra, S. H. (2011) Phosphatidylserine Exposure by *Toxoplasma gondii* Is Fundamental to Balance the Immune Response Granting Survival of the Parasite and of the Host. PLoS One. 6, e27867.

Scharton-kersten, T. M., 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, 1261-1273.

Schoenborn, JR., Wilson CB. (2007) Regulation of interferon-gamma during innate and adaptive immune responses. Adv Immunol. 96, 41-101.

Schulz, C., Gomez-Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S. E., Pollard, J. W., Frampton, J., Liu, K. J., Geissmann, F. A. (2012) lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science. 336, 86-90.

Seabra, S. H., De Souza, W., DaMatta, R. A. (2004) *Toxoplasma gondii* exposes phosphatidylserine inducing a TGF-ß1 autocrine effect orchestrating macrophages evasion. Biochem Biophys Res Commun. 324, 744-752.

Seabra, S. H., De Souza, W., DaMatta, R. A. (2002) *Toxoplasma gondii* partially inhibits nitric oxide production of activated murine macrophages. Exp Parasitol. 100, 62-70.

Sha, Y., Pandit, L., Zeng, S., Eissa, N. T. (2009) A Critical Role for CHIP in the Aggresome Pathway. molecular and cellular biology. 29, 116-128.

Shapira, S., Harb, O. S., Margarit, J., Matrajt, M., Han, J., Hoffmann, A., Freedman, B., May, M. J., Roos, D. S, Hunter, C. A. (2005) Initiation and termination of NF-kappaB signaling by the intracellular protozoan parasite *Toxoplasma gondii*. J Cell Sci. 118, 3501-3508.

Shenoy, A. R., Wellington, D. A., Kumar, P., Kassa, H., Booth, C. J., Cresswell, P., MacMicking, J. D. (2012) GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals. Science. 336, 481-485.

Shrestha, S. P., Tomita, T., Weiss, L. M., Orlofsky, A. (2006) Proliferation of *Toxoplasma gondii* in inflammatory macrophages *in vivo* is associated with diminished oxygen radical production in the host cell. Int J Parasitol. 36, 433-441.

Sibley, L. D. (2003) *Toxoplasma gondii*: perfecting an intracellular life style. Traffic. 4, 581-586.

Sibley, L. D., Ajioka, J. W. (2008) Population structure of *Toxoplasma gondii*: Clonal expansion driven by infrequent recombination and selective sweeps. Annu Rev Microbiol. 62, 329-351.

Sibley, L. D., Lawson, R., Weidner, E. (1986) Superoxide dismutase and catalase in *Toxoplasma gondii*. Mol Biochem Parasitol, 19, 83-87.

Sibley, L. D., Weidner, E., Krahenbuhl, J. L. (1985) Phagosome acidification blocked by intracellular *Toxoplasma gondii*. Nature. 315, 416-419.

Sinai, A. P. Joiner, K. A. (2001) The *Toxoplasma gondii* protein ROP2 mediates host organelle association with the parasitophorous vacuole membrane. J. Cell. Biol. 154, 95-108.

Singh, S. B., Davis, A. S., Taylor, G. A., Deretic, V. (2006) Human IRGM induces autophagy to eliminate intracellular mycobacteria. Science. 313, 1438-1441.

Souza, Wd., DaMatta, R. A., Attias, M. (2009) Brazilian contribution for a better knowledge on the biology of *Toxoplasma gondii*. Mem Inst Oswaldo Cruz. 104, 149-54.

Splendore, A. (1908) Um nuovo protozoa parassita de' conigli, 4. Incontrato nelle lesioni anatomiche d'une malattia che ricorda in molti punti il Kala-azar dell'uomo. Nota preliminare pel. Rev Soc Scien São Paulo. 3,109-112.

Staeheli, P., Grob, R., Meier, E., Sutcliffe, J. G., Haller, O. (1988) Influenza virus-susceptible mice carry mx genes with a large deletion or a nonsense mutation. Mol Cell Biol. 8, 4518-4523.

Steinfeldt, T., Könen-Waisman, S., Tong, L., Pawlowski, N., Lamkemeyer, T., Sibley, L. D, Hunn, J. P, Howard, J. C. (2010) Phosphorylation of mouse immunity-related GTPase (IRG) resistance proteins is an evasion strategy for virulent *Toxoplasma gondii*. PLoS Biol. 8, e1000576.

Stossel T. P. (1999) The early history of phagocytosis. Advances in Cellular and Molecular Biology of Membranes and Organelles. Elsevier, 5, 3–18.

Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F. and Nathan, C. F. (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. Proc Natl Acad Sci. 88, 7773-7777.

Stuehr, D., Pou, S., Rosen, G. M. (2001) Oxygen reduction by nitric-oxide synthases. J Biol Chem. 276, 14533-14536.

Su, C., Howe, D. K., Dubey, J. P., Ajioka, J. W., Sibley, L. D. (2002) Identification of quantitative trait loci controlling acute virulence in *Toxoplasma gondii*. Proc Natl Acad Sci. 99, 10753-10758.

Swanson, J. A. (2008) Shaping cups into phagosomes and macropinosomes. Nat Rev Mol Cell Biol. 9, 639-649.

Taylor, G. A, Collazo, C. M., Yap, G. S., Nguyen, K., Gregorio, T. A., Taylor, L. S., Eagleson, B., Secrest, L., Southon, E. A., Reid, S. W., Tessarollo, L., Bray, M., McVicar, D. W., Komschlies, K. L., Young, H. A., Biron, C. A., Sher, A., Vande Woude, G. F. (2000) Pathogen-specific loss of host resistance in mice lacking the IFN-gamma-inducible gene IGTP. Proc Natl Acad Sci. 97, 751-755.

Taylor, G. A., Jeffers, M., Largaespada, D. A., Jenkins, N. A., Copeland, N. G., Woude, G. F (1996) Identification of a novel GTPase, the inducibly expressed GTPase, that accumulates in response to IFNγ. J Biol Chem. 271, 20399-20405.

Taylor, S., Barragan, A., Su, C., Fux, B., Fentress, S. J., Tang, K., Beatty, W. L., Hajj, H. E., Jerome, M., Behnke, M. S., White, M., Wootton, J. C., Sibley, L. D. (2006) A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. Science. 15, 1776-1780.

Tenter, A. M., Heckeroth, A. R., Weiss, L. M. (2000) *Toxoplasma gondii*: from animal to humans. Int J Parasitol. 30, 1217-1258.

Thomas, D. D., Ridnour, L. A., Isenberg, J. S., Flores-Santana W., Switzer, C. H.,

Donzelli, S., Hussain, P., Vecoli, C., Paolocci, N., Ambs, S., Colton, C. A., Harris, C. C., Roberts, D. D., Wink, D. A. (2008) The chemical biology of nitric oxide: implications in cellular signaling. Free Radic Biol Med. 45, 18-31.

Tyler, J.S., Boothroyd, J. C. (2011) The C-terminus of Toxoplasma RON2 provides the crucial link between AMA1 and the host-associated invasion complex. PLoS Pathog. 7, 1-12.

Vodovotz, Y., Russell, D., Xie, Q. W., Bogdan, C., Nathan, C. (1995) Vesicle membrane association of nitric oxide synthase in primary mouse macrophages. J Immunol. 154, 2914-2925.

Walker, G., Pfeilschifter, J., Kunz, D. (1997) Mechanisms of suppression of inducible nitricoxide synthase (iNOS) expression in interferon (IFN)-gamma-stimulated RAW 264.7 cells by dexamethasone. Evidence for glucocorticoid-induced degradation of iNOS protein by calpain as a key step in post-transcriptional regulation. J Biol Chem. 272, 16679-16687.

Wang, Q. Q., Li, H., Oliver, T., Glogauer, M., Guo, J., He, Y. W. (2008) Integrin beta 1 regulates phagosome maturation in macrophages through Rac expression. J Immunol. 180, 2419-2428.

Watts, C. (1997) Capture and processing of exogenous antigens for presentation on MHC molecules. Annu Rev Immunol. 15, 821-850.

Webb, J. L., Harvey, M. W., Holden, D. W., Evans, T. J. (2001) Macrophage nitric oxide synthase associates with cortical actin but is not recruited to phagosomes. Infect Immun. 69, 6391-6400.

Werk, R. (1985) How does *Toxoplasma gondii* enter host cells? Rev Infect Dis, 7, 449-457.

Williams, M., Kim, K. (2014) From membranes to organelles: emerging roles for dynamin-like proteins in diverse cellular processes. Eur J Cell Biol. 93, 267-277.

Wilson, C. B., Tsai, V., and Remington, J. S. (1980) Failure to trigger the oxidative burst by normal macrophages. Possible mechanism for survival of intracellular pathogens. J Exp Med. 151, 328-246.

Woods, S., Schroeder, J., Mcgachy, H. A., Plevin, R., Roberts, C. W., Alexander, J. (2013) MAP kinase phosphatase-2 plays a key role in the control of infection with *Toxoplasma gondii* by modulating iNOS and arginase-1 activities in mice. PLoS Pathog. 9, e1003535.

Wubbolts, R., Fernandez-Borja, M., Oomen, L., Verwoerd, D., Janssen, H., Calafat, J., Tulp, A., Dusseljee, S., Neefjes, J. (1996) Direct vesicular transport of MHC class II molecules from lysosomal structures to the cell surface. J Cell Biol. 135, 611-622.

Yamamoto, M., Okuyama, M., Ma, J.S., Kimura, T., Kamiyama, N., Saiga, H., Ohshima, J., Sasai, M., Kayama, H., Okamoto, T., Huang, D. C., Soldati-Favre, D., Horie, K., Takeda, J., Takeda, K. (2012) A Cluster of interferon-g-Inducible p65 GTPases plays a critical role in host defense against *Toxoplasma gondii*. Immunity. 37, 302-313.

Zaobornyj, T., Ghafourifar, P. (2012) Strategic localization of heart mitochondrial NOS: a review of the evidence. Am J Physiol Heart Circ Physiol. 303, 1283-1293.

Zerrahn, J., Schaible, U. E., Brinkmann, V., Guhlich, U., Kaufmann, S. H. (2002) The IFN-inducible Golgi- and endoplasmic reticulum-associated 47-kDa GTPase IIGP is transiently expressed during listeriosis. J. Immunol.168, 3428-3436.

Etheridge Zhao, Y., Marple, A. H., Ferguson, D. J., Bzik, D. J., Yap, G. S. (2014) Avirulent strains of *Toxoplasma gondii* infect macrophages by active invasion from

the phagosome. Proc Natl Acad Sci. 29, 6437-6442.

Zhao, Y.O., Khaminets, A., Hunn, J.P., Howard, J.C. (2009a) Disruption of the *Toxoplasma gondii* parasitophorous vacuole by IFN gamma-inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death. PLoS Pathog. 5, e1000288.

Zhao, Y.O., Könen-Waisman, S., Taylor, G.A., Martens, S., Howard, J.C. (2010) Localisation and mislocalisation of the interferon-inducible immunity-related GTPase, Irgm1 (LRG-47) in mouse cells. Plos One. 5, e8648.

Zimmermann, S., Murray, P. J., Heeg, K., Dalpke, A. H. (2006) Induction of suppressor of cytokine signaling-1 by *Toxoplasma gondii* contributes to immune evasion in macrophages by blocking IFN-gamma signaling. J Immunol.176, 1840-1847.

Trabalho 2

Inhibition of Nitric Oxide Production in Activated Macrophages Caused by *Toxoplasma gondii* Infection Occurs by Distinct Mechanisms in Different Mouse Macrophage Cell Lines

Gabriel R. de Abreu Cabral[1,2], Zi T. Wang[1†], L. D. Sibley[1*] and Renato A. DaMatta[1,2*]

[1]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, United States

[2]Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Brazil

Front. Microbiol., 20 August 2018 | https://doi.org/10.3389/fmicb.2018.01936

frontiers in Microbiology

ORIGINAL RESEARCH
published: 20 August 2018
doi: 10.3389/fmicb.2018.01936

Inhibition of Nitric Oxide Production in Activated Macrophages Caused by *Toxoplasma gondii* Infection Occurs by Distinct Mechanisms in Different Mouse Macrophage Cell Lines

Gabriel R. de Abreu Cabral[1,2], Zi T. Wang[1†], L. D. Sibley[1] and Renato A. DaMatta[1,2]**

[1] *Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, United States,*
[2] *Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Brazil*

Toxoplasma gondii, the causative agent of toxoplasmosis, is a widespread intracellular parasite able to infect virtually any nucleated cell. *T. gondii* infection of activated macrophages inhibits nitric oxide (NO) production; however, parasite effectors responsible for this block have not been defined. Macrophage populations are extremely heterogeneous, responding differently to stimuli and to parasite infection. Here we evaluated the inhibition of NO production caused by *T. gondii* infection of J774-A1 and RAW 264.7 macrophages and assessed the role of several known parasite virulence factors in this phenotype. Infection of activated macrophages from both macrophage lines reduced NO production, however, the mechanism of this decrease was different. Consistent with previous reports, infected J774-A1 macrophages had reduced iNOS expression and lower number of iNOS positive cells. In contrast, *T. gondii* infection of RAW 264.7 macrophages did not alter iNOS expression or the number of iNOS positive cells, and yet it led to lower levels of NO production. Deletion of a number of previously defined virulence factors including ROP kinases that disrupt innate immune factors, TgIST which blocks STAT1 activation, as well as the secretory trafficking proteins ASP5 and MYR1, did not alter the phenotype of decreased NO production. Taken together our findings indicate that *T. gondii* infection inhibits NO production of activated macrophages by different mechanisms that involve reduction of iNOS expression vs. iNOS impairment, and suggest that a novel parasite effector is involved in modulating this important host defense pathway.

Keywords: *Toxoplasma gondii*, virulence factors, macrophages, inducible nitric oxide synthase, nitric oxide

Abbreviations: ASP5, aspartyl protease 5; GRA, dense granule proteins; iNOS, inducible nitric oxide synthase; IRGs, immunity related GTPases; MYR1, myc regulation 1; NO, nitric oxide; ROP, rhoptry proteins; TgIST, *Toxoplasma gondii* inhibitor of STAT1-dependent transcription.

OPEN ACCESS

Edited by:
Celio Geraldo Freire-de-Lima, Universidade Federal do Rio de Janeiro, Brazil

Reviewed by:
Shuai Wang, Xinxiang Medical University, China
Bellisa Freitas Barbosa, Federal University of Uberlândia, Brazil

****Correspondence:***
L. D. Sibley
sibley@wustl.edu
Renato A. DaMatta
renato@uenf.br

†Present address:
Zi T. Wang, Customer Education and Training, MilliporeSigma, St. Louis, MO, United States

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *08 June 2018*
Accepted: *31 July 2018*
Published: *20 August 2018*

Citation:
Cabral GRA, Wang ZT, Sibley LD and DaMatta RA (2018) Inhibition of Nitric Oxide Production in Activated Macrophages Caused by Toxoplasma gondii Infection Occurs by Distinct Mechanisms in Different Mouse Macrophage Cell Lines. Front. Microbiol. 9:1936. doi: 10.3389/fmicb.2018.01936

INTRODUCTION

Toxoplasmosis is a worldwide disease affecting about one-third of the human population (Tenter et al., 2000). *Toxoplasma gondii*, the causative agent of toxoplasmosis, is an obligate intracellular parasite that infects distinct host cells (Tenter et al., 2000). Macrophages are key players of the host immune system and are able to control *T. gondii* replication following activation by interferon gamma and a second signal provide by LPS or TNF-α (Adams et al., 1990; Sibley et al., 1991). One of the main components of antimicrobial activities of activated macrophages is the production of NO through the induction of iNOS (MacMicking et al., 1997). Generation of NO has been implicated in control of chronic toxoplasmosis (Chao et al., 1993; Khan et al., 1997; Scharton-Kersten et al., 1997; Roberts et al., 2000). However, *T. gondii* coevolved with its host and evasion mechanisms have emerged to thwart many of the effectors of activated macrophages. Included among these virulence factors are proteins released from rhoptries that block innate immunity (Hunter and Sibley, 2012) and dense granules that alter host cell transcription (Hakimi et al., 2017).

During host cell invasion, *T. gondii* secretes contents from specialized secretory organelles including rhoptries and dense granules that have a central role in parasitophorous vacuole formation and host immunity subversion (Carruthers and Sibley, 1997; Bougdour et al., 2013; Braun et al., 2013; Etheridge et al., 2014; Olias et al., 2016). For example, the ROP5-ROP17-ROP18 complex, which is secreted from rhoptries, blocks the assembly and function of vacuolar-targeted IRGs (Saeij et al., 2006; Taylor et al., 2006; Behnke et al., 2011; Reese et al., 2011; Etheridge et al., 2014). Recruitment of IRGs to the vacuole normally results in its destruction and death of the parasite (Zhao et al., 2009; Khaminets et al., 2010), but ROPs of virulent strains of the parasite are able to phosphorylate key IRG proteins, inhibiting their activity and assembly, protecting *T. gondii* (Fentress et al., 2010; Steinfeldt et al., 2010). Proteins from GRA are another important class of virulence factors secreted by *T. gondii* during and after host cell invasion that perform major roles in parasite survival and replication (Mercier et al., 2002). For example, GRA16 down-modulates host p53 expression changing the cell cycle (Bougdour et al., 2013), while GRA24 causes host p38α activation, leading to a strong proinflammatory response (Braun et al., 2013). Recently, another important *T. gondii* virulence factor known as inhibitor of STAT1-dependent transcription (IST) has been described (Gay et al., 2016; Olias et al., 2016). IST translocates to the host nucleus where it recruits a repressive complex of STAT1 promoters, blocking the IFN-γ dependent transcription, avoiding host cell activation (Olias et al., 2016).

Classically activated macrophages produce NO that control *T. gondii* replication (Adams et al., 1990; Bohne et al., 1994; Khan et al., 1997). NO is an important microbicidal molecule that is produced by iNOS (Stuehr et al., 1991; Xie et al., 1994; Lowenstein and Padalko, 2004). It is well known that *T. gondii* evades the cytotoxic effects of NO by inhibiting NO production of activated mice peritoneal macrophages (Dobbin et al., 2002; Seabra et al., 2002, 2004; Luder et al., 2003). Furthermore, in activated J774-A1 macrophages, infection causes iNOS degradation by the proteasome (Padrao Jda et al., 2014). Although TGF-β 1 is involved in the inhibition of NO production in infected activated macrophages (Seabra et al., 2004), the parasite effector responsible for iNOS degradation and NO inhibition still remains elusive. In addition, most of these studies have been done *in vitro* with a single cell type and without comparison of other macrophage cell lines.

Macrophages are an extremely heterogeneous cell population with many subpopulations that behave differently (Geissmann et al., 2010). Thus, *T. gondii* has to deal with many distinct macrophage subpopulations during host infection. To better understand how *T. gondii* copes with NO production of distinct activated macrophages lines, production of this microbicidal molecule and expression of iNOS were compared in two macrophage cell lines after infection. In addition, several previously described virulence factors were also analyzed as possible effectors responsible for NO inhibition of infected activated macrophages. This study reveals intrinsic differences between both macrophage cell lines in activation patterns and mechanisms by which *T. gondii* infection disrupted NO production. Furthermore, previously identified virulence effectors that were tested here did not alter the NO inhibition phenotype detected in both macrophages cell lines, indicating that a novel effector is responsible for the inhibition of this important host microbicidal molecule.

MATERIALS AND METHODS

Biosecurity and Institutional Safety Procedures

This study was carried out in accordance with the NIH standard biosecurity and institutional safety procedures of Washington University School of Medicine.

Toxoplasma gondii and Cell Culture

Knockout parasites used in this work were previously generated as reported: RHΔku80 (Fox et al., 2009); Δrop5 (Behnke et al., 2011); Δrop17, Δrop17/18, Δrop18 (Etheridge et al., 2014); ΔTgIST (Olias et al., 2016); (Δasp5) (Curt-Varesano et al., 2016); (Δmyr1) (Franco et al., 2016). Wild type *T. gondii* tachyzoites, deficient in Ku80 (RHΔku80) and knockout parasites, all of the RH strain, were maintained by serial passage in Human Foreskin Fibroblast (HFF) monolayers. Parasites were released from HFF using a cell scraper (TPP, Switzerland). The cell suspension was passed three times in a 10 ml syringe (BD, United States) with a 22-gauge needle (CML Supply, United States) and it was filtered on a 3 μm Whatman Nuclepore membrane (GE Healthcare Life Sciences, United States).

Human Foreskin Fibroblast cells were obtained from the Boothroyd laboratory at Stanford University. HFF cells and the mouse macrophage cell lines RAW 264.7 (ATCC TIB-71, United States, from BALB/c mice ascites after Abelson murine leukemia virus inoculation) and J774-A1 (ATCC TIB-67, United States, from BALB/c mice ascites after reticulum cell sarcoma inoculation)

were cultivated in Dulbecco's modified Eagle's medium (DMEM - Invitrogen, United States) supplemented with 10% HyClone Fetal Bovine Serum (FBS - GE Healthcare Life Sciences, United States), 10 mM glutamine (Thermo Fisher Scientific, United States) and 10 μg/ml gentamicin (Gibco, United States) in an incubator (Thermo Fisher Scientific, United States) at 37°C in 5% CO_2 atmosphere. Cultures were negative for *Mycoplasma* spp. contamination with the e-Myco plus PCR detection kit (Boca Scientific, United States).

Generation of Δrop16 Knockout Parasites

The knockout plasmid construct for *ROP16* was created using the three-fragment Gateway™ recombination system (Invitrogen, United States), joining two constructs homologous to the 5′ and 3′ flanks of *ROP16* with a central HXGPRT expression cassette, as described previously (Etheridge et al., 2014). This plasmid was transfected into RHΔhxgprtΔku80 parasites, and resistant pools were expanded under treatment with mycophenolic acid (15 μg/ml) acid and xanthine (50 μg/ml). After PCR (Applied Biosystems, United States) confirmation of construct integration in the pool, parasites were subcloned by limiting dilution in 96-well plates (TPP, Switzerland) containing confluent monolayers of HFF cells. Clones were identified by visual confirmation of single plaques, screened by PCR to confirm replacement of the coding region of ROP16 with the HXGPRT cassette, expanded by growth on HFF monolayers, and cryo-preserved.

Macrophage Activation and Infection

The RAW 264.7 and J774-A1 cells were seeded at the density of 5×10^4 cells per well in 96-well plates, activated with 200 U/ml of recombinant mouse IFN-γ (R&D Systems, United States) and 0.2 μg/ml of LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, United States) for 24 h prior of the *T. gondii* infection. After 24 h of activation, cells were washed twice with sterile PBS, DMEM supplemented with 3% FBS was added, cells were infected with a 5 *T. gondii* per macrophage (5:1) ratio and incubated at 37°C for 2 h. The *T. gondii*-macrophage ratio used was determined by previous experiments examining NO production and number of adhered macrophages after 24 h infection. After infection, cells were washed twice in sterile PBS, and DMEM supplemented with 10% FBS with IFN-γ and LPS or these activators and L-arginine (Sigma-Aldrich, United States) at different concentration, was added. Supernatants were collected at 2, 4, 6, and 24 h after infection for the nitrite assay.

Nitrite Oxide (NO) Production

The NO produced by macrophages was assayed by the Griess reagent (Green et al., 1982). Briefly, 50 μl of cell supernatant from each well were collected and distributed in 96-well plates and 50 μl of Griess reagent (1:1, 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich, United States) in distilled water and 1% sulfanilamide (Sigma-Aldrich, United States) in 5% phosphoric acid (Sigma-Aldrich, United States) were added. Plates were incubated at room temperature and nitrite was read in a plate reader (BioTek, United States) at 540 nm. The nitrite value was calculated from a calibrated standard curve using sodium nitrite ranging from 0 to 100 μM.

In-Cell-ELISA

Infection of parental and knockout parasites into RAW 264.7 and J774-A1 macrophages was evaluated after 2 h of challenge in cells seeded in 96-well plates, activated and infected as described. After infection, cells were fixed and permeabilized for 30 min in PBS containing 4% formaldehyde (Polysciences, Inc., United States), 0.05% Triton X-100 (Fisher BioReagents, United States), washed with PBS and blocked for 30 min with 5% FBS and Normal Goat Serum (Sigma-Aldrich, United States) in PBS (FBS-NGS). Cells were incubated for 1 h with anti-RH (SAG1) rabbit antibody diluted 1:2000 in FBS-NGS, washed twice and incubated for 1 h with anti-rabbit HRP conjugated antibody (Life Technology, United States) diluted 1:4000 in FBS-NGS. Cells were washed three times, incubated for 15 min with 100 μl of TMB substrate (BD OptEIA, Thermo Fisher Scientific, United States), reaction stopped with 100 μl of 1M H_2SO_4 and the absorbance was read in a plate reader (BioTek, United States) at 450 nm. Non-infected macrophages were used as negative control.

Immunofluorescence Assay

RAW 264.7 and J774-A1 macrophages were seeded at the density of 5×10^5 cells per well over coverslips (Fisherbrand, United States) in 24-well plates (TPP, Switzerland), activated and infected as described. Cells were fixed for 30 min with PBS containing 4% formaldehyde, permeabilized for 15 min in PBS containing 0.1% Triton X-100, incubated for 30 min with PBS containing 100 mM of NH_4Cl (Sigma-Aldrich, United States), and washed three times with PBS containing 1.5% Bovine Serum Albumin (PBS-BSA, Sigma-Aldrich, United States). Cells were incubated for 1 h with anti-iNOS mouse monoclonal antibody (sc-7271, Santa Cruz Biotechnology, United States) diluted 1:100 and anti-RH (SAG1) rabbit antibody diluted 1:2000 in PBS-BSA, washed once in PBS and twice in PBS-BSA and incubated with goat anti-mouse IgG monoclonal antibody conjugated to Alexa Fluor 488 (Thermo Fisher Scientific, United States) diluted 1:200 and goat anti-rabbit IgG monoclonal antibody conjugated to Alexa Fluor 594 (Thermo Fisher Scientific, United States) diluted 1:2000 in PBS-BSA. Coverslips containing cells were mounted with the Prolong Gold antifade reagent with DAPI (Life Technologies, United States) and visualized with a Zeiss Axioskop 2 MOT Plus epifluorescence microscope with a 63× Plan Apochromat lens (numerical aperture of 1.40; Carl Zeiss, Inc., Germany), equipped with an AxioCam MRm camera (Carl Zeiss, Inc., Germany). Images were acquired using Axiovision v4.6 (Carl Zeiss, Inc., Germany) and processed with Adobe Photoshop 6.0 (Adobe Systems Inc., United States).

Cell Analysis

To quantify the percentage of iNOS positive and iNOS negative cells in infected or non-infected cells, RAW 264.7 and J774-A1 macrophages were seeded in black 96-well cellstar microplates with clear bottom and condensation rings (BioTek,

United States), activated and infected as described. Cells were fixed with PBS containing 4% formaldehyde for 15 min, washed three times with PBS and incubated for 15 min with FBS-NGS containing 5 μg/ml of Wheat Germ Agglutinin conjugated to Alexa Fluor 633 (Thermo Fisher Scientific, United States) for total cell staining. Cells were washed twice in PBS and incubated for 1 h with anti-iNOS mouse monoclonal antibody (Santa Cruz Biotechnology, United States) diluted 1:100 in FBS-NGS and anti-RH (SAG1) rabbit antibody diluted 1:10000 in 5% FBS-NGS. Cells were washed twice with PBS and incubated with goat anti-mouse IgG conjugated to Alexa Fluor 488 (Thermo Fisher Scientific, United States) diluted 1:200 and goat anti-rabbit IgG conjugated to Alexa Fluor 594 (Thermo Fisher Scientific, United States) diluted 1:2000 in FBS-NGS. Cell analysis was performed with a Cytation 3 (BioTek, United States) multimode plate imager equipped with Gen5 software and 20× objective.

Western Blot and Densitometry Analysis

RAW 264.7 and J774-A1 macrophages were seeded at the density of 2×10^6 cells per well in 6-well plate (TPP, Switzerland), activated and infected as described. Cells were washed once in sterile PBS and lysed with 50 μl of lysing buffer containing 50 mM of Tris–HCL (Sigma-Aldrich, United States), 150 mM NaCl (Sigma-Aldrich, United States), 1% Triton X-100 (Sigma-Aldrich, United States) and protease inhibitor cocktail (Sigma-Aldrich, United States). Protein samples were frozen in liquid nitrogen three times, centrifuged (Eppendorf, Germany) at 5,000 *g*, for 3 min and protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, United States). Samples were diluted 4:1 in 5× Laemmli buffer containing 10 mM of dithiothreitol (Sigma-Aldrich, United States), boiled for 5 min, resolved in 8% polyacrylamide gels (Bio-Rad Laboratories, Inc., United States) by SDS–PAGE, and transferred to nitrocellulose membranes Amersham Protran 0.45 NC (GE Healthcare Life Sciences, United States) for 1 h. Membranes were blocked overnight at 4°C with 5% fat-free milk in PBS 0.1% Tween 20 (Sigma-Aldrich, United States), probed for 1 h with anti-iNOS mouse monoclonal antibody (Santa Cruz Biotechnology, United States) dilute 1:1000 and rabbit anti-β-actin (Cell Signaling Technology, United States) diluted 1:1000 in 5% fat-free milk in PBS 0.1% Tween 20. Membranes were washed with PBS 0.1% Tween 20 and labeled for 1 h with IR dye-conjugated secondary antibodies (LI-COR Biosciences, United States) against mouse and rabbit dilute 1:15000 and visualized on a LiCor Odyssey Imaging System (LI-COR Biosciences, United States). Western blots were quantified using the software ImageJ and intensity values were normalized to β-actin at each time interval.

Statistical Analysis

Statistical analyses were conducted with Prism 7 (GraphPad Software Inc., United States), and differences in the means were assessed by one-way or two-way ANOVA with Tukey's multiple comparison post-test, or unpaired Student's *t*-test. $P \leq 0.05$ was the cutoff considered minimum for significance.

RESULTS

T. gondii Inhibits NO Production of Activated Macrophages Independently of Substrate Availability and Cell Type

To evaluate the ability of the parasite to inhibit NO production in different macrophage cell lines, activated J774-A1 or RAW 264.7 cells were infected with *T. gondii* and nitrite production was evaluated in culture supernatant at different time points. Differences in the timing of inhibition of NO production were observed between the two cell lines. In J774-A1 macrophages, inhibition of NO production occurred by 2 h post-infection (**Figure 1A**) and was sustained up to 24 h (**Figure 1B**). In contrast, inhibition of NO production in RAW 264.7 macrophages occurred only after 6 h post-infection (**Figure 1C**) and was sustained up to 24 h (**Figure 1D**).

During host cell invasion *T. gondii* secretes important virulence factors including ROP16, which activates STAT 3 and STAT 6 in macrophages resulting in upregulation of arginase 1 (ARG1) (Butcher et al., 2011). Induction of ARG1 competes with iNOS for the substrate L-arginine (Butcher et al., 2011), hence this may be a mechanism of avoiding NO production. To determine if inhibition of NO production in activated macrophages was caused by substrate depletion, we supplemented the culture medium with increasing concentrations of L-arginine and evaluated NO production after 24 h of infection. Treatment of activated J774-A1 macrophages with increasing concentrations of L-arginine did not enhance NO production in control or infected cells, nor reverted the inhibition of NO production phenotype caused by *T. gondii* infection (**Figure 1E**). In contrast, incubation of activated RAW 264.7 macrophages with increasing concentrations of L-arginine enhanced NO production in control and infected cells, and yet it did not reverse the inhibition of NO production in infected cells (**Figure 1F**).

iNOS Expression in Infected Macrophages Is Dependent on Cell Line Type

To examine whether the inhibition of NO production in activated J774-A1 macrophage was caused by protein degradation, iNOS expression was evaluated by different methods. iNOS showed variable levels of expression in activated non-infected J774-A1 macrophages based on IFA staining (**Figure 2A**). Following infection with *T. gondii* there were more iNOS negative cells at 24 h post-infection (**Figure 2A**). This finding was confirmed by analyzing the proportion of activated cells that were positive for iNOS in non-infected cells (Control) and *T. gondii* challenged cells (Non-infected or Infected) as depicted in **Figure 2B**. Although there was a tendency to decrease the number of iNOS positive cells in infected macrophages at 2 h, this effect was significant at 24 h post-infection (**Figure 2B**). Western blot (**Figure 2C** and **Supplementary Figure 1**) and densitometry analysis (**Figure 2D**) also confirmed inhibition of iNOS expression in cells infected for 24 h. However, reduction

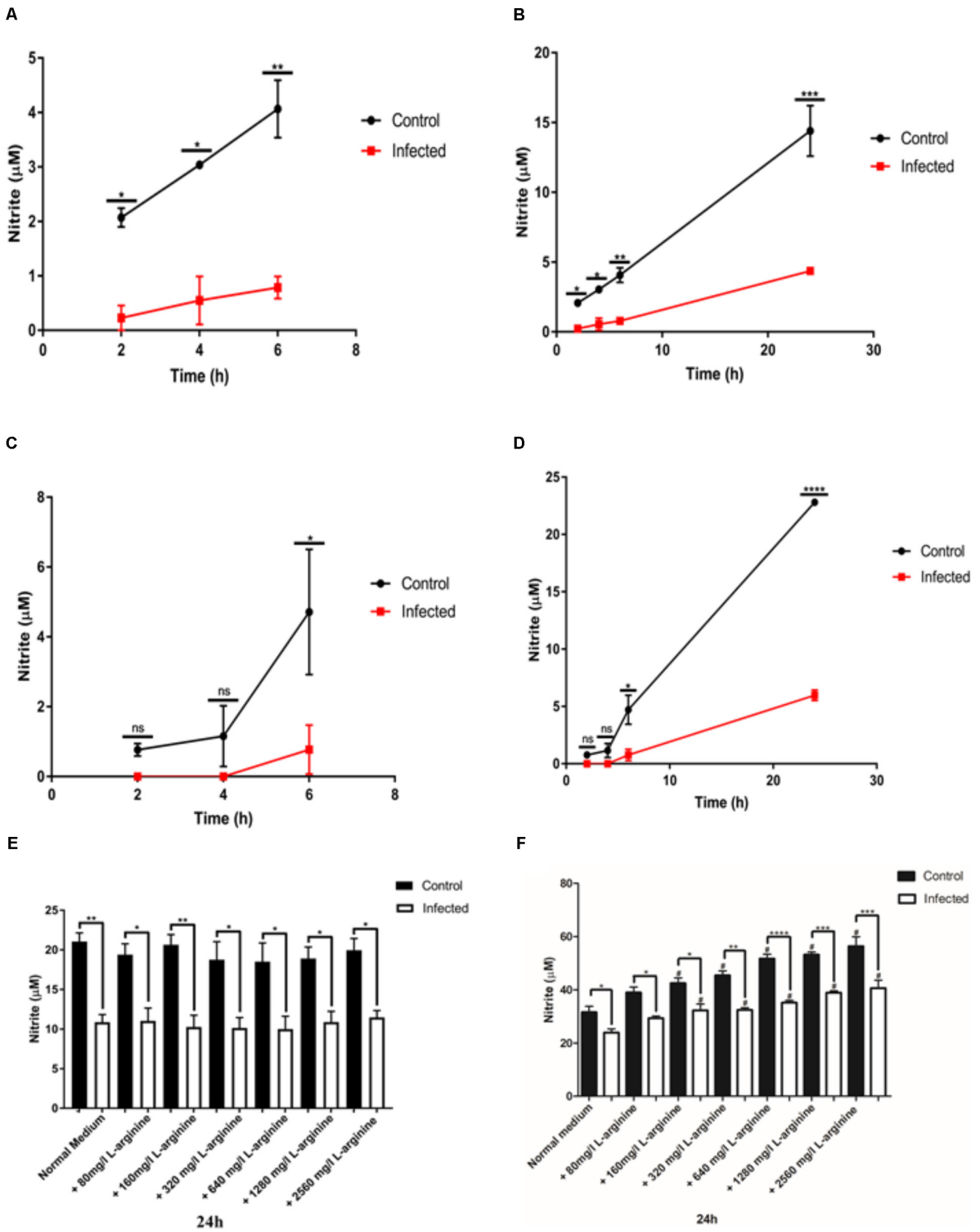


FIGURE 1 | Nitric oxide (NO) production (nitrite in μM) in activated J774-A1 and RAW 264.7 cells macrophages after *T. gondii* infection. **(A)** NO production of non-infected (Control) or *T. gondii* (RH) infected J774-A1cells at 6 h and **(B)** 24 h post-infection. Mean ± SEM (n = 3 experiments, each with 12 replicates). **(C)** NO production of non-infected (Control) or *T. gondii* (RH) infected RAW 264.7 cells at 6 h and **(D)** 24 h post-infection. Mean ± SEM (n = 3 experiments, each with 12 replicates). ns (not significant), $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, two-way ANOVA with Tukey post-test. **(E)** NO production of non-infected (Control) or *T. gondii* (RH) infected J774-A1 **(F)** or RAW 264.7 cells for 24 h with normal medium or supplemented with different levels of L-arginine. Mean ± SEM (n = 3 experiments, each with 6 replicates). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ one-way ANOVA with Tukey post-test, $^{\#}P \leq 0.05$ comparing the "Control" or "Infected" bar with the respective "Normal medium" bar.

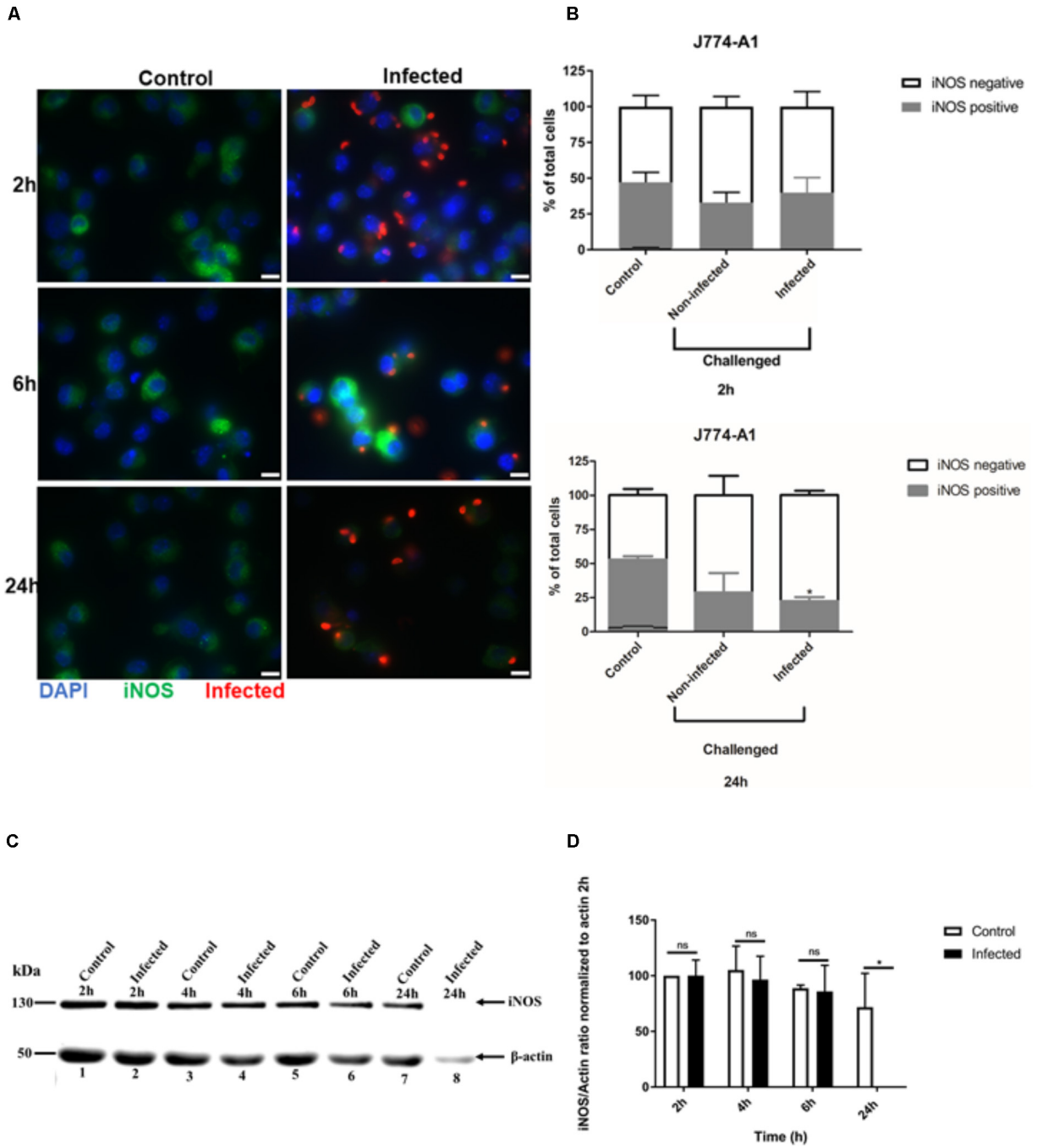


FIGURE 2 | Immunofluorescence detection of iNOS in activated J774-A1 macrophages infected with *T. gondii*. **(A)** Detection of iNOS (green) in non-infected (Control) and in *T. gondii* (red) infected cells (DAPI - blue) at 2, 6, and 24 h post-infection. Scale bar = 10 μm. **(B)** Analysis of the proportion of iNOS positive or negative macrophages in non-infected (Control) and *T. gondii* infected cells at 2 and 24 h post-infection. Mean ± SEM (n = 4 experiments, each with 8 replicates). **(C)** Western blot detection of iNOS expression in non-infected (Control) and *T. gondii* infected (Infected) cells. β-actin was used as loading control. **(D)** Densitometry of western blots normalized to β-actin at 2 h post-infection. Mean ± SD (n = 3 experiments, each with 1 replicate). *$P \leq 0.05$, two-way ANOVA with Tukey post-test, n.s (not significant).

in iNOS expression was not observed at earlier time points after infection (**Figure 2D**).

We also analyzed iNOS expression after *T. gondii* infection of RAW 264.7 macrophages using similar IFA and Western blot analyses. The signal intensity of iNOS expression in RAW 264.7 cells was much higher, with all uninfected cells being uniformly positive (**Figure 3A** and **Supplementary Figure 2**). No difference in iNOS expression by IFA was

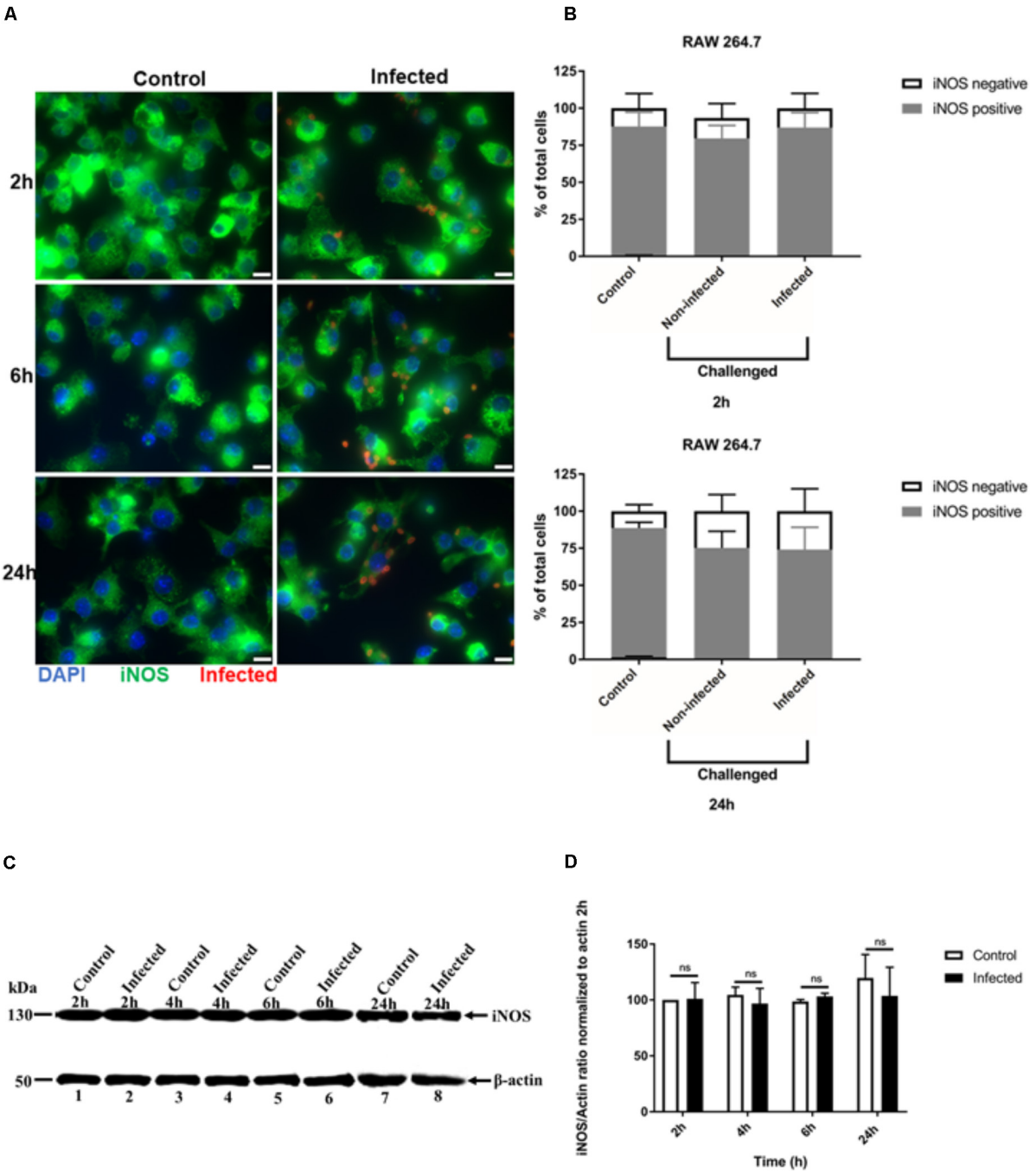


FIGURE 3 | Immunofluorescence detection of iNOS in activated RAW 264.7 macrophages infected with *T. gondii*. **(A)** Detection of iNOS (green) in non-infected (Control) and in *T. gondii* (red) infected cells (DAPI - blue) at 2, 6, and 24 h post-infection. Scale bar - 10 μm. **(B)** Analysis of the proportion of iNOS positive or negative macrophages in non-infected (Control) and *T. gondii* infected cells at 2 and 24 h post-infection. Mean ± SEM (n = 4 experiments, each with 8 replicates). **(C)** Western blot detection of iNOS expression in non-infected (Control) and *T. gondii* - infected (Infected) cells at different time intervals post-infection. β-actin was used as loading control. **(D)** Densitometry of western bolts normalized to β-actin at 2 h post-infection. Mean ± SD (n = 3 experiments, each with 1 replicate), n.s (not significant).

observed between non-infected and infected RAW 264.7 macrophages at 24 h post-infection (**Figure 3A**). This finding was confirmed by analyzing the proportion of cells that were positive for iNOS. In non-infected and *T. gondii* challenged RAW 264.7 macrophage populations, most of the cells remained iNOS positive up to 24 h post-infection (**Figure 3B**). Similarly, no difference in iNOS expression between control and infected cells was observed by Western blot (**Figure 3C**)

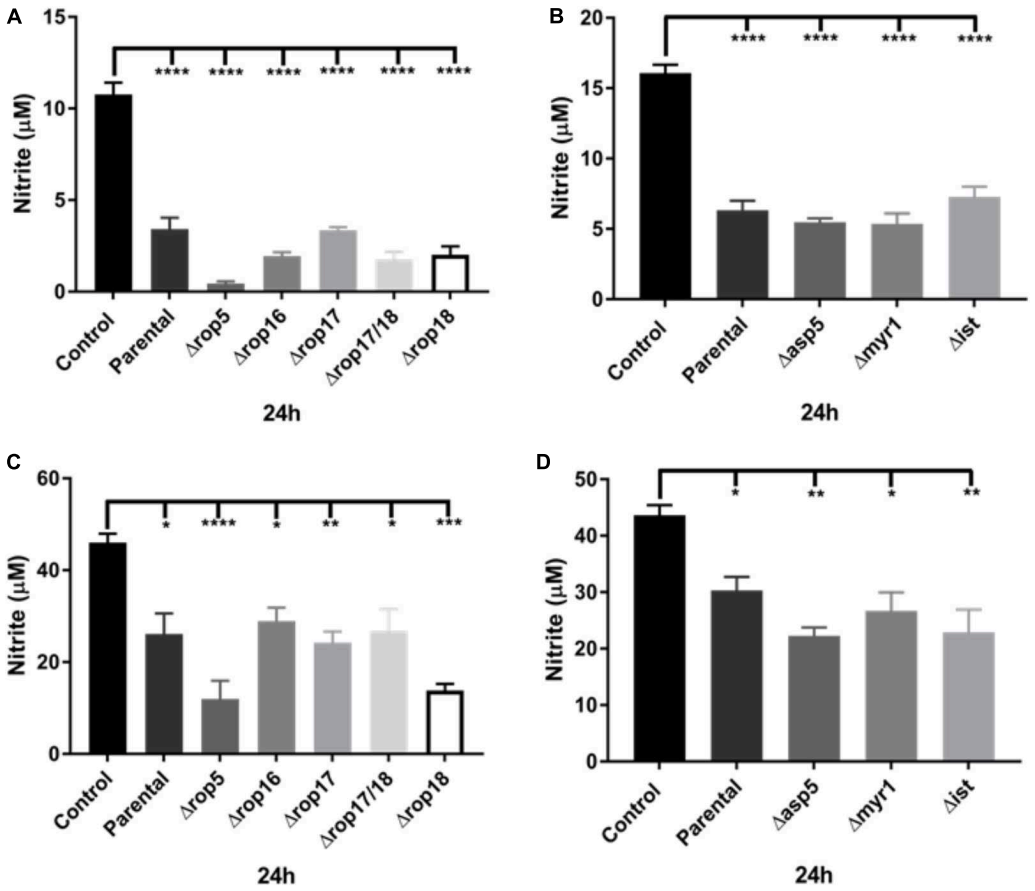


FIGURE 4 | Analysis of ROP and ASP5, MYR1, and IST deletion mutants on the ability of *T. gondii* to inhibit NO production (nitrite in μM) of activated macrophages. **(A)** NO production of non-infected (Control) or activated J774-A1 cells infected with parental (RHΔku80 strain) or various ROP deletion strains of *T. gondii* at 24 h post-infection. Mean ± SEM (n = 3 experiments, each with 12 replicates). ****$P \leq 0.0001$, one-way ANOVA with Tukey post-test. **(B)** NO production of non-infected (Control) or activated J774-A1 cells infected with parental (RHΔku80 strain) or Δasp5, Δmyr1, or Δist mutant strains of *T. gondii* at 24 h post-infection. Mean ± SEM (n = 3 experiments, each with 12 replicates). ****$P \leq 0.0001$, one-way ANOVA with Tukey post-test. **(C)** NO production of non-infected (Control) or activated RAW 264.7 cells infected with parental (RHΔku80 strain) or various ROP deletion strains of *T. gondii* at 24 h post-infection. Mean ± SEM (n = 3 experiments, each with 12 replicates). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$, one-way ANOVA with Tukey post-test. **(D)** NO production of non-infected (Control) or activated RAW 264.7 cells infected with parental (RHΔku80 strain) or Δasp5, Δmyr1, and Δist mutant strains of *T. gondii* at 24 h post-infection. Mean ± SEM (n = 3 experiments, each with 12 replicates). *$P \leq 0.05$, **$P \leq 0.01$, one-way ANOVA with Tukey post-test.

and densitometry analysis (**Figure 3D**) at different time points post-infection.

Evaluation of *T. gondii* Virulence Factors Do Not Influence Inhibition of NO Production

A number of previous virulence factors have been identified in *T. gondii* including a complex of ROP kinases consisting of ROP5, ROP17, and ROP18 that participates in defense of the parasitophorous vacuole by thwarting IRGs (Hunter and Sibley, 2012). To determine whether the inhibition of NO production in infected activated J774-A1 and RAW 264.7 macrophages was dependent on the ROP kinases, we examined the inhibition of NO production in activated macrophages infected with a series of mutants. Inhibition of NO production was similar in both macrophage cell lines at 24 h infection when comparing the parent RH line to a series of ROP deletion mutants (**Figures 4A,C**). We also examined the ability of a Δrop16 mutant to alter this phenotype, since this kinase has previously been shown to activate STAT3/STAT6 and hence activate ARG1 (Butcher et al., 2011). The rop16 mutant showed a similar capacity to block NO production in activated J774-A1 and RAW 264.7 cells (**Figures 4A,C**). The various knockout parasites presented no deficiency in entry in activated J774-A1 or RAW 264.7 macrophages (**Supplementary Figure 3**).

We also tested *T. gondii* mutants in the modulator IST that inhibits STAT1 transcription (Hakimi et al., 2017). These mutants had no effect on the NO inhibition phenotype (**Figures 4B,D**). Recently, the major role of the Golgi-associated protein, ASP5, and the parasitophorous vacuole (PV) associated protein, MYR1, in the cleavage and export of some dense granule effector proteins across the vacuole membrane into the host cell has been demonstrated (Curt-Varesano et al., 2016; Franco et al., 2016).

Therefore we examined the ability of mutants in these effectors to block the production of NO in activated macrophages. After infection, all knockout parasites were able to inhibit NO production of activated J774-A1 (**Figure 4B**) and RAW 264.7 macrophages (**Figure 4D**) similar to the parental parasite. The various knockout parasites presented no deficiency in entry in activated J774-A1 or RAW 264.7 macrophages (**Supplementary Figure 3**).

DISCUSSION

Toxoplasma gondii has many evasion mechanisms including the capacity to inhibit NO production of infected activated macrophages (Dobbin et al., 2002; Seabra et al., 2002, 2004; Luder et al., 2003; Padrao Jda et al., 2014). NO production inhibition, iNOS expression and the role of some *T. gondii* effectors were studied in parallel using two macrophage cell lines. Infection of both cell lines caused inhibition of NO production. However, only in J774-A1 macrophages was NO inhibition detected at early stages of infection (i.e., 2 h post-infection), while inhibition was seen starting at 6 h post-infection in both lines. Addition of extra L-arginine substrate to both macrophage lines did not change NO production inhibition, indicating that this result is not due to substrate limitation. Interestingly, reduction of iNOS expression after infection was only detected in J774-A1 cells, with RAW 264.7 presenting the same levels as non-infected cells. Finally, knockout parasites in known effectors were able to inhibit NO production similar to the parental strain. Our findings suggest that the inhibition of NO production of activated macrophages infected by *T. gondii* is a general phenomenon, but iNOS suppression varies depending on the macrophage cell line. In addition, it is likely that a novel parasite effector is responsible for this evasion mechanism.

Toxoplasma gondii infection causes ARG1 expression that competes with iNOS for L-arginine (El Kasmi et al., 2008; Butcher et al., 2011). However, extra L-arginine did not reverse NO production inhibition in either infected cell lines, indicating that the reduction in NO production is not due to substrate limitation. Following addition of L-arginine, J774-A1 produced the same amount of NO while RAW 264.7 macrophages responded to this addition by producing more NO. This finding is consistent with the differences in iNOS expression, and provides a further distinction in phenotypes between these two lines (Heming et al., 2001; Lindmark et al., 2004; El Aamri et al., 2015).

Previous report showed that NO production inhibition of *T. gondii* infected activated mice peritoneal macrophages was related to iNOS degradation (Seabra et al., 2002, 2004), which involves the proteasome in J774-A1 macrophages (Padrao Jda et al., 2014). Down modulation of iNOS expression was verified in infected J774-A1 but this was not observed in RAW 264.7 macrophages. These results suggest that the strategy adopted by *T. gondii* to inhibit NO production may be specific to the host cell, due to the intrinsic characteristics and origin of each macrophage cell line. The J774-A1 line was originally established from reticulum cell sarcoma (Hirst et al., 1971) while the RAW 264.7 line from a tumor induced by Abelson murine leukemia virus (Ralph and Nakoinz, 1977) both in BALB/c mice. Furthermore, exposure of both cell lines to *Streptococcus iniae* induces a higher respiratory burst response in RAW 264.7 than in J774-A1 macrophage (El Aamri et al., 2015). Moreover, RAW 264.7 produces 30-fold higher TNF-α mRNA than J774-A1 after LPS stimulation (Heming et al., 2001). These differences help to explain the higher expression of iNOS in RAW 264.7 compared to J774-A1 macrophages. In addition, a gene expression profile study (Lindmark et al., 2004) shows that J774-A1 is closer to peritoneal mice macrophages than RAW 264.7, despite the fact that both cell lines were derived from transformed cells obtained from ascites (Ralph and Nakoinz, 1977). Our findings reveal that *T. gondii* infection can down-regulate NO production in these different macrophage cell lines, albeit by different mechanisms.

During host cell infection, *T. gondii* secrets the content of rhoptries and dense granules ensuring the establishment of infection and hijacking host cell-autonomous immunity (Sibley, 2011). ROP and GRA proteins form complexes that protect *T. gondii* PV by avoiding recruitment of IRGs (Hunter and Sibley, 2012). GRA proteins are also exported across the PV altering important host functions (Hakimi et al., 2017). TgIST is a GRA effector that represses STAT1 transcription blocking gene expression induced by IFN-γ (Gay et al., 2016; Olias et al., 2016). In addition, the export and traffic of GRAs across the PV and into the host cell are dependent of ASP5 (Coffey et al., 2015; Curt-Varesano et al., 2016) and MYR1 (Franco et al., 2016). However, these virulence factors have not previously been evaluated for their ability to modulate other important host microbicidal systems such as NO production. Thus, knockout parasites were used to investigate whether some rhoptry proteins (ROP5, ROP16, ROP17, ROP18). The parasite ROP16 kinase has been implicated in modulating NO production in microglial cells and astrocytes (Butcher et al., 2011). However, the knockout in ROP16 did not affect the down modulation of NO production in either of the macrophage cells lines studied here. Additionally, the ROP5-ROP17-ROP18 complex, which has been implicated in blocking IRG-mediated clearance, did not affect the down modulation of NO production in *T. gondii* – infected cells. We also explored the roles of the GRA TgIST, or components of the PV membrane translocation system (ASP5, MYR1) for involvement in NO production control in infected macrophages. These GRA knockout parasites were able to inhibit NO production similar to the parental strain in J774-A1 and RAW 264.7 macrophages. These results indicate that the parasite effector that down modulates NO production is independent of TgIST, including other modulators that depend on the trafficking pathway based on ASP5 and MYR1.

Overall, our findings reveal that the strategy adopted by *T. gondii* to inhibit NO production in activated macrophages is independent of previously characterized virulence factors, such as the ROP5-ROP17-ROP18 complex, ROP16, GRA effectors, and the ASP5 and MYR1 export pathway. Thus, a possibly new parasite effector is involved in NO production inhibition in these cells. The mechanism causing NO production inhibition

of macrophages infected by *T. gondii* varies depending on the host cell background: it involves reduction of iNOS expression in J774-A1 and iNOS impairment in RAW 264.7. This may be relevant to *in vivo* infections where *T. gondii* infects and needs to cope with distinct macrophage populations.

AUTHOR CONTRIBUTIONS

GC performed the experiments. ZW produced the ROP16 knockout. GC, LS, and RD wrote the manuscript. LS and RD designed the experiments and revised the manuscript critically. All authors read and approved the final manuscript.

FUNDING

GC was supported by a fellowship from the Brazilian Federal Agency, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (88881.132450/2016-01) and partially by grants from the NIH (AI118426) obtained by LS; RD is supported by a CNPq fellowship (307014/2015-0).

ACKNOWLEDGMENTS

We are grateful to Jennifer Barks for support in cell culture and in-cell-ELISA experiments, Sumit Kumar for assistance in IFA and statistical analysis, Kevin Brown for total cell population analysis, Lisa Drewry for western blot assistance, and all of the LS' lab members for helpful advice. We would like to thank Andrèa Carvalho César for proof reading the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01936/full#supplementary-material

REFERENCES

Adams, L. B., Hibbs, J. B. Jr., Taintor, R. R., and Krahenbuhl, J. L. (1990). Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. *J. Immunol.* 144, 2725–2729.

Behnke, M. S., Khan, A., Wootton, J. C., Dubey, J. P., Tang, K., and Sibley, L. D. (2011). Virulence differences in *Toxoplasma* mediated by amplification of a family of polymorphic pseudokinases. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9631–9636. doi: 10.1073/pnas.1015338108

Bohne, W., Heesemann, J., and 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, 1761–1767.

Bougdour, A., Durandau, E., Brenier-Pinchart, M. P., Ortet, P., Barakat, M., Kieffer, S., et al. (2013). Host cell subversion by *Toxoplasma* GRA16, an exported dense granule protein that targets the host cell nucleus and alters gene expression. *Cell Host Microbe* 13, 489–500. doi: 10.1016/j.chom.2013.03.002

Braun, L., Brenier-Pinchart, M. P., Yogavel, M., Curt-Varesano, A., Curt-Bertini, R. L., Hussain, T., et al. (2013). A *Toxoplasma* dense granule protein, GRA24, modulates the early immune response to infection by promoting a direct and sustained host p38 MAPK activation. *J. Exp. Med.* 210, 2071–2086. doi: 10.1084/jem.20130103

Butcher, B. A., Fox, B. A., Rommereim, L. M., Kim, S. G., Maurer, K. J., Yarovinsky, F., et al. (2011). *Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. *PLoS Pathog.* 7:e1002236. doi: 10.1371/journal.ppat.1002236

Carruthers, V. B., and Sibley, L. D. (1997). Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *Eur. J. Cell Biol.* 73, 114–123.

Chao, C. C., Anderson, W. R., Hu, S., Gekker, G., Martella, A., and Peterson, P. K. (1993). Activated microglia inhibit multiplication of *Toxoplasma gondii* via a nitric oxide mechanism. *Clin. Immunol. Immunopathol.* 67, 178–183. doi: 10.1006/clin.1993.1062

Coffey, M. J., Sleebs, B. E., Uboldi, A. D., Garnham, A., Franco, M., Marino, N. D., et al. (2015). An aspartyl protease defines a novel pathway for export of *Toxoplasma* proteins into the host cell. *eLife* 4:e10809. doi: 10.7554/eLife.10809

Curt-Varesano, A., Braun, L., Ranquet, C., Hakimi, M. A., and Bougdour, A. (2016). The aspartyl protease TgASP5 mediates the export of the *Toxoplasma* GRA16 and GRA24 effectors into host cells. *Cell. Microbiol.* 18, 151–167. doi: 10.1111/cmi.12498

Dobbin, C. A., Smith, N. C., and Johnson, A. M. (2002). Heat shock protein 70 is a potential virulence factor in murine *Toxoplasma* infection via immunomodulation of host NF-kappa B and nitric oxide. *J. Immunol.* 169, 958–965. doi: 10.4049/jimmunol.169.2.958

El Aamri, F., Remuzgo-Martinez, S., Acosta, F., Real, F., Ramos-Vivas, J., Icardo, J. M., et al. (2015). Interactions of *Streptococcus iniae* with phagocytic cell line. *Microbes Infect.* 17, 258–265. doi: 10.1016/j.micinf.2014.06.006

El Kasmi, K. C., Qualls, J. E., Pesce, J. T., Smith, A. M., Thompson, R. W., Henao-Tamayo, M., et al. (2008). Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat. Immunol.* 9, 1399–1406. doi: 10.1038/ni.1671

Etheridge, R. D., Alaganan, A., Tang, K., Lou, H. J., Turk, B. E., and Sibley, L. D. (2014). The *Toxoplasma* pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice. *Cell Host Microbe* 15, 537–550. doi: 10.1016/j.chom.2014.04.002

Fentress, S. J., Behnke, M. S., Dunay, I. R., Mashayekhi, M., Rommereim, L. M., Fox, B. A., et al. (2010). Phosphorylation of immunity-related GTPases by a *Toxoplasma gondii*-secreted kinase promotes macrophage survival and virulence. *Cell Host Microbe* 8, 484–495. doi: 10.1016/j.chom.2010.11.005

Fox, B. A., Ristuccia, J. G., Gigley, J. P., and Bzik, D. J. (2009). Efficient gene replacements in *Toxoplasma gondii* strains deficient for nonhomologous end joining. *Eukaryot. Cell* 8, 520–529. doi: 10.1128/ec.00357-08

Franco, M., Panas, M. W., Marino, N. D., Lee, M. C., Buchholz, K. R., Kelly, F. D., et al. (2016). A novel secreted protein, MYR1, is central to toxoplasma's manipulation of host cells. *mBio* 7:e02231-15. doi: 10.1128/mBio.02231-15

Gay, G., Braun, L., Brenier-Pinchart, M.-P., Vollaire, J., Josserand, V., Bertini, R.-L., et al. (2016). *Toxoplasma gondii* TgIST co-opts host chromatin repressors dampening STAT1-dependent gene regulation and IFN-γ–mediated host defenses. *J. Exp. Med.* 213, 1779–1798. doi: 10.1084/jem.20160340

Geissmann, F., Gordon, S., Hume, D. A., Mowat, A. M., and Randolph, G. J. (2010). Unravelling mononuclear phagocyte heterogeneity. *Nat. Rev. Immunol.* 10, 453–460. doi: 10.1038/nri2784

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.* 126, 131–138. doi: 10.1016/0003-2697(82)90118-X

Hakimi, M. A., Olias, P., and Sibley, L. D. (2017). *Toxoplasma* effectors targeting host signaling and transcription. *Clin. Microbiol. Rev.* 30, 615–645. doi: 10.1128/cmr.00005-17

Heming, T. A., Tuazon, D. M., Dave, S. K., Chopra, A. K., Peterson, J. W., and Bidani, A. (2001). Post-transcriptional effects of extracellular pH on tumour necrosis factor-alpha production in RAW 246.7 and J774 A.1 cells. *Clin. Sci.* 100, 259–266. doi: 10.1042/cs1000259

Hirst, J. W., Jones, G. G., and Cohn, M. (1971). Characterization of a BALB/c myeloma library. *J. Immunol.* 107, 926–927.

Hunter, C. A., and Sibley, L. D. (2012). Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. *Nat. Rev. Microbiol.* 10, 766–778. doi: 10.1038/nrmicro2858

Khaminets, A., Hunn, J. P., Konen-Waisman, S., Zhao, Y. O., Preukschat, D., Coers, J., et al. (2010). Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. *Cell. Microbiol.* 12, 939–961. doi: 10.1111/j.1462-5822.2010.01443.x

Khan, I. A., Schwartzman, J. D., Matsuura, T., and Kasper, L. H. (1997). A dichotomous role for nitric oxide during acure *Toxoplasma gondii* infection in mice. *Proc. Natl. Acad. Sci. U.S.A.* 94, 13955–13960. doi: 10.1073/pnas.94.25.13955

Lindmark, H., Rosengren, B., Hurt-Camejo, E., and Bruder, C. E. (2004). Gene expression profiling shows that macrophages derived from mouse embryonic stem cells is an improved in vitro model for studies of vascular disease. *Exp. Cell Res.* 300, 335–344. doi: 10.1016/j.yexcr.2004.06.025

Lowenstein, C. J., and Padalko, E. (2004). iNOS (NOS2) at a glance. *J. Cell Sci.* 117(Pt 14), 2865–2867. doi: 10.1242/jcs.01166

Luder, C. G., Algner, M., Lang, C., Bleicher, N., and Gross, U. (2003). Reduced expression of the inducible nitric oxide synthase after infection with *Toxoplasma gondii* facilitates parasite replication in activated murine macrophages. *Int. J. Parasitol.* 33, 833–844. doi: 10.1016/S0020-7519(03)00092-4

MacMicking, J., Xie, Q. W., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15, 323–350. doi: 10.1146/annurev.immunol.15.1.323

Mercier, C., Dubremetz, J. F., Rauscher, B., Lecordier, L., Sibley, L. D., and Cesbron-Delauw, M. F. (2002). Biogenesis of nanotubular network in *Toxoplasma* parasitophorous vacuole induced by parasite proteins. *Mol. Biol. Cell* 13, 2397–2409. doi: 10.1091/mbc.E02-01-0021

Olias, P., Etheridge, R. D., Zhang, Y., Holtzman, M. J., and Sibley, L. D. (2016). *Toxoplasma* effector recruits the Mi-2/NuRD complex to repress STAT1 transcription and block IFN-gamma-dependent gene expression. *Cell Host Microbe* 20, 72–82. doi: 10.1016/j.chom.2016.06.006

Padrao Jda, C., Cabral, G. R., da Silva Mde, F., Seabra, S. H., and DaMatta, R. A. (2014). *Toxoplasma gondii* infection of activated J774-A1 macrophages causes inducible nitric oxide synthase degradation by the proteasome pathway. *Parasitol. Int.* 63, 659–663. doi: 10.1016/j.parint.2014.05.003

Ralph, P., and Nakoinz, I. (1977). Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: enhancement by PPD and LPS. *J. Immunol.* 119, 950–954.

Reese, M. L., Zeiner, G. M., Saeij, J. P., Boothroyd, J. C., and Boyle, J. P. (2011). Polymorphic family of injected pseudokinases is paramount in *Toxoplasma* virulence. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9625–9630. doi: 10.1073/pnas.1015980108

Roberts, F., Roberts, C. W., Ferguson, D. J., and McLeod, R. (2000). Inhibition of nitric oxide production exacerbates chronic ocular toxoplasmosis. *Parasite Immunol.* 22, 1–5. doi: 10.1046/j.1365-3024.2000.00259.x

Saeij, J. P., Boyle, J. P., Coller, S., Taylor, S., Sibley, L. D., Brooke-Powell, E. T., et al. (2006). Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science* 314, 1780–1783. doi: 10.1126/science.1133690

Scharton-Kersten, T. M., Yap, G., Magram, J., and 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, 1261–1273. doi: 10.1084/jem.185.7.1261

Seabra, S. H., de Souza, W., and DaMatta, R. A. (2002). *Toxoplasma gondii* partially inhibits nitric oxide production of activated murine macrophages. *Exp. Parasitol.* 100, 62–70. doi: 10.1006/expr.2001.4675

Seabra, S. H., de Souza, W., and DaMatta, R. A. (2004). *Toxoplasma gondii* exposes phosphatidylserine inducing a TGF-beta1 autocrine effect orchestrating macrophage evasion. *Biochem. Biophys. Res. Commun.* 324, 744–752. doi: 10.1016/j.bbrc.2004.09.114

Sibley, L. D. (2011). Invasion and intracellular survival by protozoan parasites. *Immunol. Rev.* 240, 72–91. doi: 10.1111/j.1600-065X.2010.00990.x

Sibley, L. D., Adams, L. B., Fukutomi, Y., and Krahenbuhl, J. L. (1991). Tumor necrosis factor-alpha triggers antitoxoplasmal activity of IFN-gamma primed macrophages. *J. Immunol.* 147, 2340–2345.

Steinfeldt, T., Konen-Waisman, S., Tong, L., Pawlowski, N., Lamkemeyer, T., Sibley, L. D., et al. (2010). Phosphorylation of mouse immunity-related GTPase (IRG) resistance proteins is an evasion strategy for virulent *Toxoplasma gondii*. *PLoS Biol.* 8:e1000576. doi: 10.1371/journal.pbio.1000576

Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F., and Nathan, C. F. (1991). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. U.S.A.* 88, 7773–7777. doi: 10.1073/pnas.88.17.7773

Taylor, S., Barragan, A., Su, C., Fux, B., Fentress, S. J., Tang, K., et al. (2006). A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. *Science* 314, 1776–1780. doi: 10.1126/science.1133643

Tenter, A. M., Heckeroth, A. R., and Weiss, L. M. (2000). *Toxoplasma gondii*: from animals to humans. *Int. J. Parasitol.* 30, 1217–1258. doi: 10.1016/S0020-7519(00)00124-7

Xie, Q. W., Cho, H., Kashiwabara, Y., Baum, M., Weidner, J. R., Elliston, K., et al. (1994). Carboxyl terminus of inducible nitric oxide synthase. Contribution to NADPH binding and enzymatic activity. *J. Biol. Chem.* 269, 28500–28505.

Zhao, Y., Ferguson, D. J., Wilson, D. C., Howard, J. C., Sibley, L. D., and Yap, G. S. (2009). Virulent *Toxoplasma gondii* evade immunity-related GTPase-mediated parasite vacuole disruption within primed macrophages. *J. Immunol.* 182, 3775–3781. doi: 10.4049/jimmunol.0804190

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

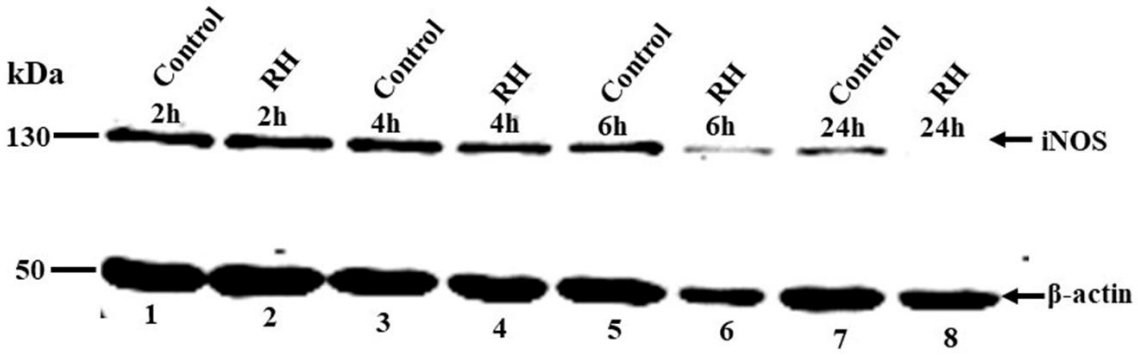
Copyright © 2018 Cabral, Wang, Sibley and DaMatta. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Inhibition of Nitric Oxide Production in Activated Macrophages Caused by *Toxoplasma gondii* Infection Occurs by Distinct Mechanisms in Different Cell Lines

Gabriel R. A. Cabral, Zi Teng Wang, L. David Sibley [*], and Renato A. DaMatta[*]

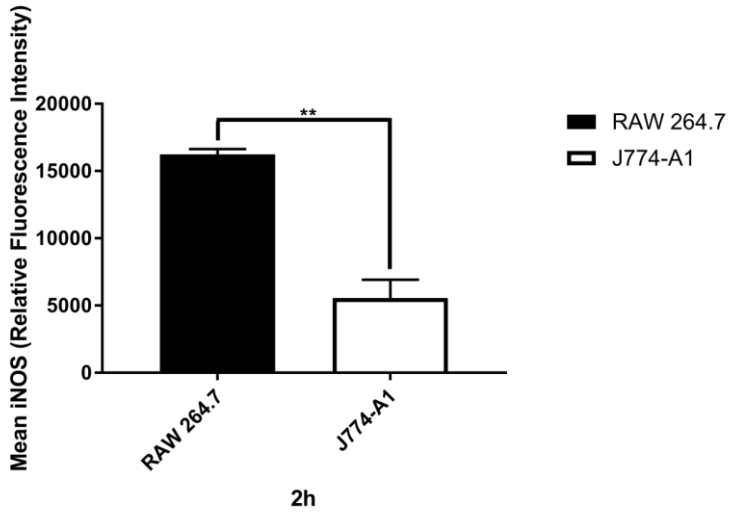
*** Correspondence:** Dr. L. David Sibley: sibley@wustl.edu; Dr. Renato Augusto DaMatta: renato@uenf.br

Supplementary Figures

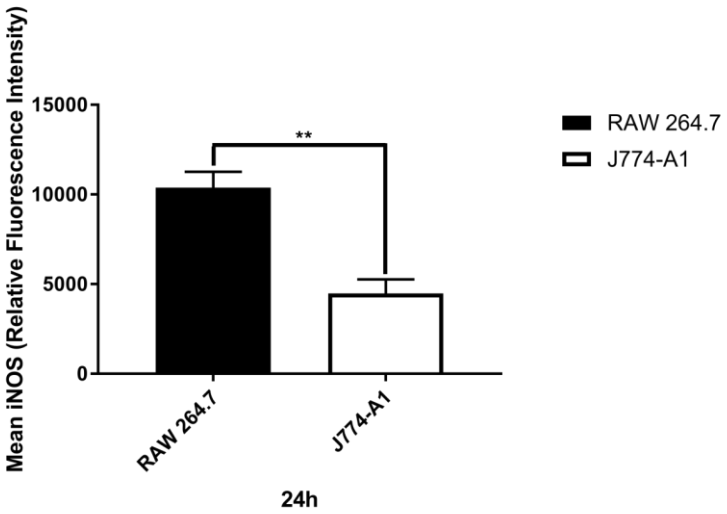


Supplemental figure 1. Western blot detection of iNOS expression in non-infected (Control) and *T. gondii* infected (RH) activated J774-A1 macrophages. β-actin was used as loading control.

A)

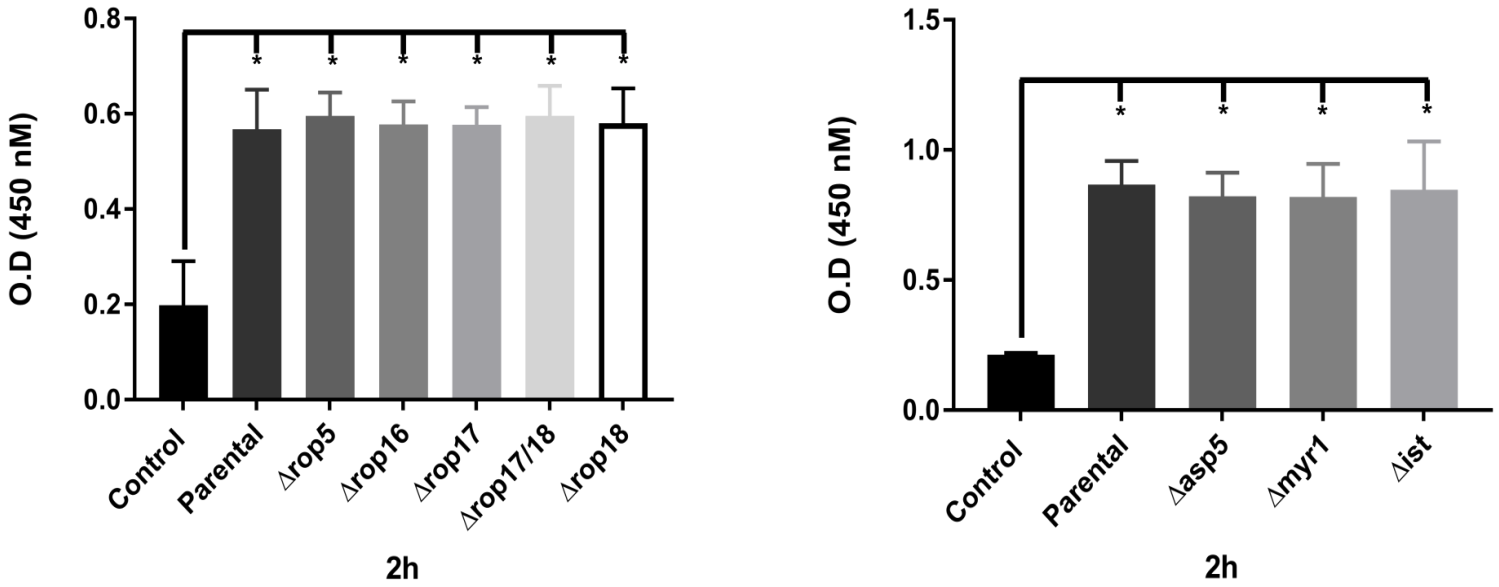


B)

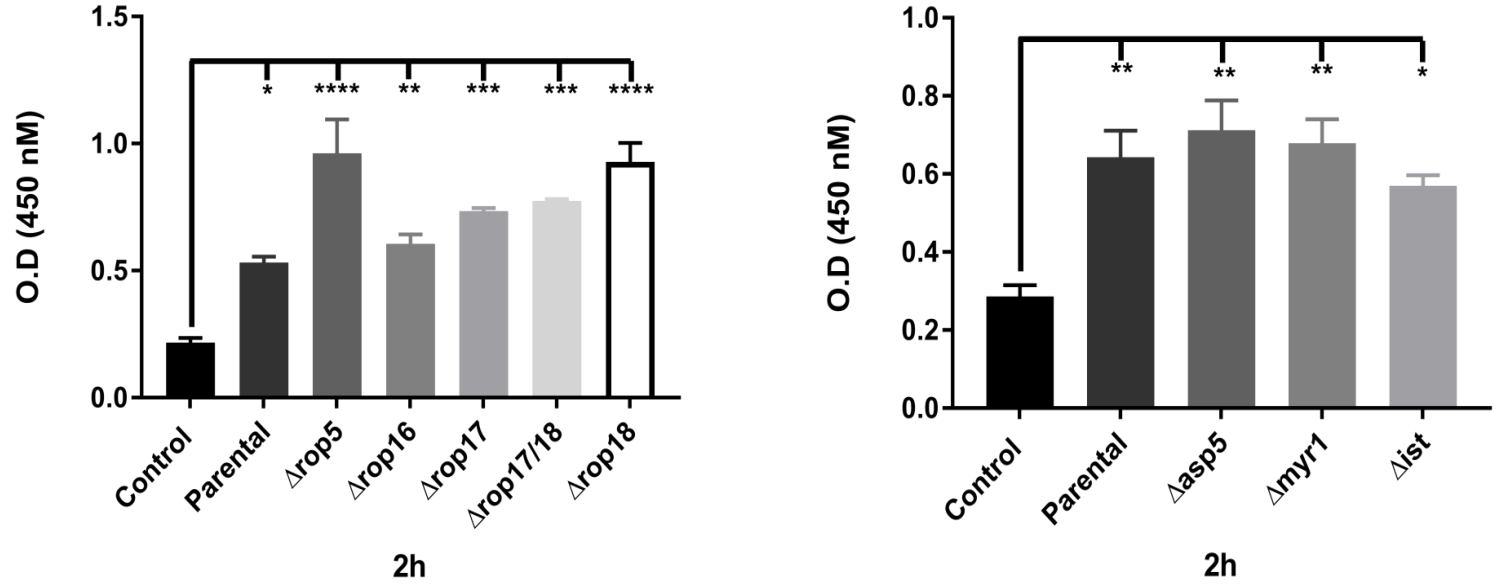


Supplemental figure 2. Relative expression of iNOS in activated RAW 264.7 and J774-A1 macrophages. (A) iNOS expression in control RAW 264.7 and J774-A1 macrophages at 2 h and (B) 24 h post-infection. Mean ± SEM (n = 4 experiments, each with 8 replicates). $^{**}P \leq 0.01$, unpaired Student t test.

A) J774-A1



B) RAW 264.7



Supplemental figure 3. Analysis of entry by parental and knockout *T. gondii* in activated J774-A1 and RAW 264.7 macrophages at 2 h post-infection. (A) Infection of activated J774-A1 macrophages by parental (RHΔku80) and mutant strains of *T. gondii*. Mean ± SEM (n = 3 experiments, each with 12 replicates). (B) Infection of activated RAW 264.7 macrophages by parental (RHΔku80) and mutant strains of *T. gondii*. Mean ± SEM (n= 3 experiments, each with 12 replicates). **P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001, one-way ANOVA with Tukey post-test.

Trabalho 3

Gabriel R. de Abreu Cabral, Joaquim T. Xavier Junior, and Renato A. DaMatta[1,*]

[1]Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Brazil

Enhancement of arginase 1 expression and activity in infected macrophages is important for *Toxoplasma gondii* replication

Gabriel R. de Abreu Cabral[1,a], Joaquim Teixeira Xavier[1,a], Gustavo Lázzaro Rezende[2], and Renato A. DaMatta[1]*

1. Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Brazil, 28013-602

2. Laboratório de Química e Função de Proteínas e Peptídeos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Brazil, 28013-602

a. These authors contributed equally to this work.

* Corresponding author: renato@uenf.br, 55 22 2739 7310

Abstract

Toxoplasma gondii is an obligate intracellular parasite with widespread distribution and capacity to infect virtually any nucleated cell. It has been proposed that *T. gondii* induces arginase 1 (ARG1) expression in host cells as an immune evasion strategy, but the role of ARG1 for *T. gondii* development in non-activate or alternatively activated mouse macrophage cell lines was never explored. Here, we evaluated the expression pattern and activity of ARG1 in non-activate and alternatively activated RAW 264.7 and peritoneal (Swiss and C57BL/6 mice) macrophages after infection with *T. gondii*. In addition, the role of ARG1 in the development of *T. gondii* in these cells was determined. No differences in the pattern of ARG1 expression was observed between non-activate and alternatively activated macrophages. However, *T. gondii* infection enhanced ARG1 expression in all non-activate or alternatively activated mouse macrophage cell lines used. ARG1 activity was higher in alternative activated macrophages and in peritoneal cells compared to RAW 264.7. After infection, ARG1 activity enhanced only in non-activate RAW 264.7 and Swiss peritoneal macrophages; but did not alter in alternatively activated macrophages. L-arginine supplementation of culture medium favored *T. gondii* replication only in RAW 264.7 macrophages. In an opposite way, treatment of RAW 264.7 macrophages with the ARG1 inhibitor Nor-NOHA reduced the infection rate of *T. gondii* in a dose dependent way especially in alternative activated cells. Taken together our findings indicate that the enhancement of ARG1 expression and activity may be a general mechanism induced by *T. gondii* to replicate and survive inside the host cell *in vitro*. This may be related to the host L-arginine dependence of *T. gondii* since it lacks the enzymes required to synthetize this amino acid.

Keywords: *Toxoplasma gondii*, macrophages, arginase 1, L-arginine, evasion mechanism

Introduction

Toxoplasmosis is an important zoonosis with global distribution caused by *Toxoplasma gondii*, an obligate intracellular parasite (Tenter et al., 2000). *T. gondii* is able to infect virtually any nucleated cell from warm blooded vertebrate

animals (Tenter et al., 2000). Macrophages are important cells from the immune system, are extremely heterogeneous (Geissmann et al., 2010) and highly responsive to pathogen infections. According to the stimuli received, macrophages can assume specific activation profile as “classically activated” macrophages, when stimulated with proinflammatory cytokines such as IFN-γ (Stuehr & Marletta, 1987) or pathogen molecules as lipopolysaccharides (Stuehr & Marletta, 1985). After activation, these cells become highly microbicidal, controlling *T. gondii* replication through different mechanisms as reactive oxygen species (Murray & Cohn, 1979), nitric oxide (Adams et al., 1990; Sibley et al., 1991), and immunity related GTPases (Zhao et al., 2009; Khaminets et al., 2010). Macrophages can assume an “alternatively activated” (M2) profile when stimulated with anti-inflammatory cytokines such as IL-4, IL-10, TGF-β (Munder et al., 1999; Barksdale et al., 2004) and 8-Br-cAMP (Sheldon at al., 2013). M2 macrophages have low microbicidal capacity, and are characterized by an anti-inflammatory and tissue repair response (Mills et al., 2000; Mills, 2012). This dual nature characteristic of host defense versus tissue repair must be maintained in a fine balance, able to control pathogens, but avoiding damage to the host. However, parasites modulate activation profiles of macrophages as an evasion mechanism (Khan et al., 1995; Butcher & Denkers, 2002; Jensen et al., 2011; Woods et al., 2013; Cabral & DaMatta, 2017).

The modulation of host activation profile by pathogens has been studied, including *T. gondii* infections (Khan et al., 1995; Butcher and Denkers, 2002; Butcher et al., 2011; Jensen et al., 2011). It has been proposed that arginase 1 (ARG1), an important marker of M2 macrophages, can be used by *T. gondii* to subvert the host immune system. ARG1 convert L-arginine in L-ornitine and urea. L-ornitine can be converted in polyamines that are used by cells, including intracellular parasites, for replication (Pfaff et al., 2005; Cook et al., 2007). Furthermore, L-arginine is the substrate for ARG1 and inducible nitric oxide synthase, the enzyme responsible for nitric oxide production, thus, both enzymes compete for the same substrate (El-Gayar et al., 2003; Murray, 2011; Jensen et al., 2011). During host cell invasion, *T. gondii* from type I and III strains secrets ROP16, an important virulence factor that phosphorylates

STAT6. Phosphorylated STAT6 migrate to host nucleus, inducing host ARG1 activity, benefitting *T. gondii* (Jensen et al., 2011; Murray, 2011). Incontrast, C57BL/6 mice, with macrophages lacking ARG1, infected with *T. gondii* type II strain have less weight loss and did not show signs of disease, indicating that host ARG1 is detrimental for *T. gondii*. In addition, ARG1 knockout macrophages produced more nitric oxide in response to inflammatory stimuli during *T. gondii* infection (El-Kasmi et al., 2008). Li and colleagues also showed that blocking ARG1 activity in peritoneal macrophages from Balb/c mice decreases the replication of *T. gondii* type I strain (Li et al., 2012). Furthermore, mice macrophages are naturally less resistant to *T. gondii* infection than rat macrophages (Müllner et al., 2002) because express more ARG1 and produce more polyamines (Cook et al., 2007), confirming the major role of these two factors in *T. gondii* replication. However, the benefits of ARG1 activity to *T. gondii* still remain inconclusive and controversial. Woods et al. (2013) have shown that ARG1 is an important enzyme to host protection in *T. gondii* infections. This finding is related to MAP kinase phosphatase-2 (MKP-2), an important class of conserved enzymes responsible for down modulation of ARG1 expression and, at the same time, to up regulate inducible nitric oxide synthase expression (Woods et al., 2013). Thus, playing a major role in *T. gondii* infection control. Inhibition of ARG1 with nor-NOHA in C57BL/6 MKP-$2^{-/-}$ increased parasite burdens indicating that ARG1 has a protective role for the host (Woods et al., 2013). Furthermore, the study of the role of host ARG1 for *T. gondii* in different mouse macrophage cell lines has not been explored.

The aim of this work is to contribute with the role of ARG1 on *T. gondii* infected macrophages. This study reveals that ARG1 was constitutively expressed in the three mouse macrophages cell lines tested, and after infection ARG1 expression increased in all macrophages cell lines, independently if non-activate or alternatively activated. Interestingly, ARG1 expression did not enhance in M2 activated macrophages, as well as the ARG1 activity in these cells after infection. Furthermore, experiments of culture medium supplementation with L-arginine favored *T. gondii* replication, and inhibition of ARG1 with nor-NOHA reduced the number of parasite in infected cells. Taken

together, these results reveal that host ARG1 has a major role in *T. gondii* replication *in vitro*.

Materials and methods

Biosecurity and Institutional Safety Procedures

This project was approved by the ethics committee in animal use of the Universidade Estadual do Norte Fluminense Darcy Ribeiro, protocol number 98 and 301.

Macrophages and activation

Macrophages from C57BL/6 and Swiss mice were obtained by peritoneal lavage with 10 ml of PBS containing 3 % fetal bovine serum (PBS-FBS) (FBS - Gibco, 12657-029). RAW 264.7 macrophages were cultivated in Dulbecco's modified Eagle's medium (DMEM - Sigma, D1152) supplemented with 10% FBS in an incubator (Laboven - L212) at 37°C in 5% CO_2 atmosphere. Reaching confluence, RAW 264.7 macrophages were removed from flasks (Kasvi - K11-1050) using a cell scraper (Kasvi - K26-0025). RAW 264.7 macrophages were kindly provided by Dr. Elena Lassounskaia (LBR-UENF). Macrophages were centrifuged at 500*g* for 10 min at 25°C, counted and seeded at the density of 5 x 10^5 cells per well in a 24 well-plate. After 2h of adherence, cells were washed and cultured in DMEM with 10% FBS (non-activated - M0) or activated in a M2 profile with IL-4 (2.5 µg/mL) and Br-AMPc (0.075 mM) in DMEM with 10% FBS and cultured for 24h in an incubator at 37°C in 5% CO_2 atmosphere.

Toxoplasma gondii and macrophage infection

T. gondii of the RH strain was maintained by serial passage in 2-3 days in the peritoneal cavity of Swiss mice. The peritoneal lavage was performed with 5 ml of PBS, followed by centrifugation at 1000*g*, 4°C for 10 min to obtain tachyzoites. After 24 h of activation, macrophages were washed twice with PBS, DMEM was added, and cells were infected with a 5:1 *T. gondii* macrophage ratio at 37°C. After 2 h of infection, cells were washed with PBS and DMEM supplemented with 10% FBS and activators were added and macrophages further cultivated for 24 and 48 h.

Immunofluorescence Assay

Macrophages were seeded over coverslips in 24-well plates (TPP, Switzerland), activated and infected as described. Cells were fixed for 30 min with PBS containing 4% formaldehyde, permeabilized for 15 min in PBS containing 0.1% Triton X-100, incubated for 30 min with PBS containing 100 mM of NH_4Cl (Sigma-Aldrich, United States), and washed 3 times with PBS containing 1.5% Bovine Serum Albumin (PBS-BSA, Sigma-Aldrich, United States). Cells were incubated for 1 h with anti-ARG1-mouse monoclonal antibody (DB 610708, United States) diluted 1:200 in PBS, washed twice in PBS-BSA and incubated with goat anti-mouse IgG monoclonal antibody conjugated to TRITC (Sigma-Aldrich, United States), diluted 1:400 in PBS. Cells were mounted with Prolong Gold antifade reagent with DAPI (Life Technologies, United States) and visualized with a Zeiss Axioplan epifluorescence microscope with a 100x Plan Apochromat lens (numerical aperture of 1.40; Carl Zeiss, Inc., Germany), equipped with an AxioCam MRc5 camera (CarlZeiss, Inc., Germany). Tachyzoites were visualized by differential interference contrast microscopy (DIC). Images were acquired using Axiovision v4.1 (Carl Zeiss, Inc., Germany).

ARG 1 activity

The ARG1 activity was evaluated based on the urea produced by macrophages reveled by α-isonitrosopropiophenone (Sigma-Aldrich, I3502) (Corraliza et al. 1994). To evaluate the enzymatic activity of ARG1, RAW 264.7 and peritoneal macrophages from Swiss and C57BL/6 mice were seeded at the density of 1 x 10^6 cells per well in 24-well plates, activated and infected as described. After 24 and 48 h of infection, cells were washed once in PBS, lysed with a lysing buffer containing 50 mM Tris-HCl, 0.1 %Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich, P8340), pH 7.4, and incubated in a shaker (Cientec - 151) at 200 rpm for 40 min at room temperature. The samples were transferred to Eppendorf tubes, centrifuged at 13,000 g, part of the supernatant collected for protein dosage, and ARG1 was activated by adding 10mM $MnCl_2$ and heating the samples at 56 °C for 20 min. After ARG1 activation, 0.5 M L-arginine (Sigma) (pH 9.7) was added, samples incubated at 37°C, and at 10, 20 and 30 min samples were collected. The reaction was stopped with the addition of an acid solution containing H_2SO_4, H_3PO_4, and H_2O in 1:3:7 proportion, urea

production was revealed by adding 9% α-isonitrosopropiophenone and heating the samples at 100°C for 40 min. Samples and a urea standard curve were read in a plate reader (Molecular Devices) at 540 nm.

Bright-field microscopy

Activated macrophages infected for 2 h, in triplicates, were washed twice in PBS and incubated with Ng-hydroxy-Nor-L-Arginine (Nor-NOHA) at 10, 50 and 100 µM for 24 h, and parasite development evaluated by direct cell counting. For experiments with L-arginine supplementation, cells were incubated with DMEM supplemented with 10 % FBS and activators and 1280 mg/L of L-arginine for 24h (Sigma-Aldrich, United States). Control cells received no arginine supplementation. After the experiments, infected macrophages were fixed for 30 min with PBS containing 4% formaldehyde, washed with PBS and stained with Giemsa (Sigma-Aldrich - P3288) diluted in distilled water in 1:10 proportion for 10 min. Samples were dehydrated in acetone-xylol, mounted in Entellan (Merck - 1079610100) and visualized with the Zeiss Axioplan microscope with a 100x Plan Apochromat lens.

Evaluation of *T. gondii* growth

After Giemsa staining, 100 cells were counted per coverslip, a total of 300 cells per slide, considering the number of infected or non-infected macrophages and the number of parasites per macrophage. The percentage of infected macrophages and the mean of parasite per macrophage were calculated. The mean number of parasite per cell and the percentage of infected cells were multiplied to obtain the infection rate.

Statistical analysis

Possible differences in the means were assessed by one-way or two-way ANOVA performed with Prism 7 (GraphPad Software Inc., United States). $P \leq 0.05$ was the cutoff considered minimum for significance.

Results

ARG1 expression is not altered in M2 macrophages

The pattern of ARG1 expression in the used mouse macrophage cell lines in M0 or after M2 activation was analyzed by immunofluorescence. M0 and M2 macrophages of all three cell lines used expressed similar level or ARG1

(**Figure 1**). ARG1 was most expressed in the centrosome and irradiated to the macrophages cortex (**Figure 1**).

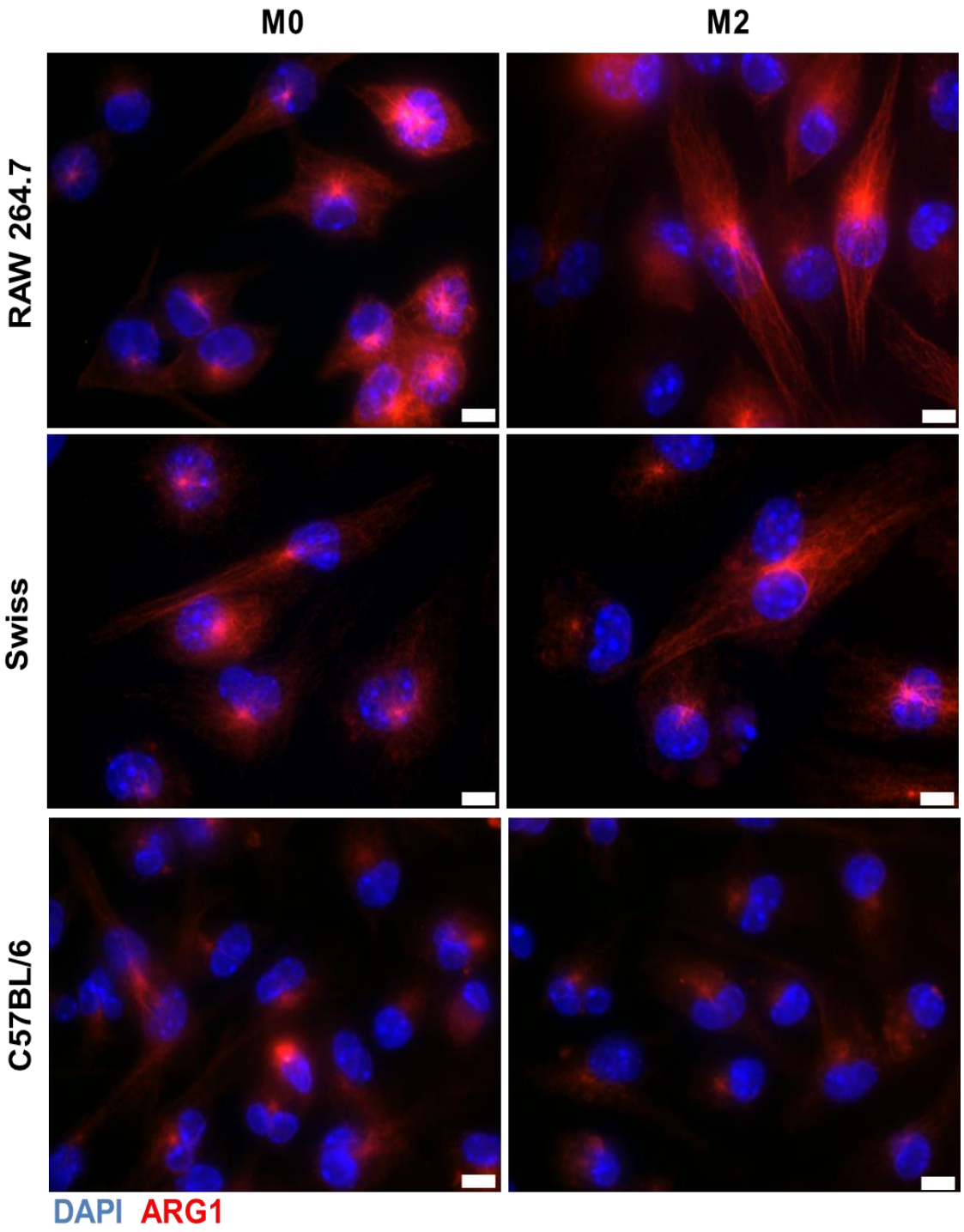


Figure 1. Immunofluorescence detection of ARG1 expression (red) in non-activated (M0) or macrophages (DAPI - blue) activated with IL-4 and 8-Br-AMPc (M2) at 24 h post-activation in RAW 264.7 and mouse peritoneal (Swiss, C57BL/6) macrophage cells lines. Scale bar = 10 μm.

T. gondii infection alter the ARG1 expression levels in macrophages

Different works report that *T. gondii* alters the ARG1 expression levels in macrophages as an evasion mechanism to survive inside the cell (El-Kasmi et al., 2008; Butcher et al., 2011; Murray et al., 2011). ARG1 expression was evaluated to determine whether after 24h of *T. gondii* infection the expression of the enzyme was altered. *T. gondii* infection enhanced ARG1 expression in all macrophage cell lines evaluated, independently if the macrophages was M0 or M2 activated (**Figure 2 A and B**).

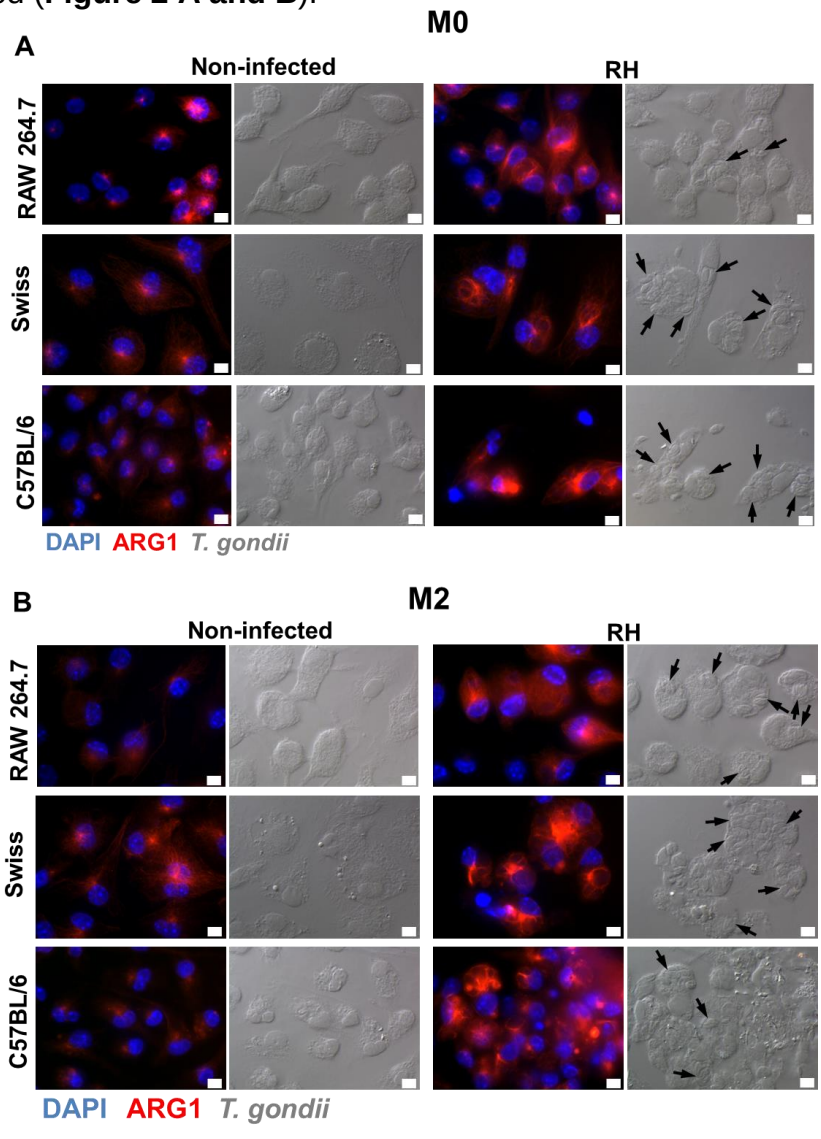


Figure 2. Immunofluorescence detection of ARG1 expression in mouse macrophage cell lines at 24 h post-infection with *T. gondii*. Immunofluorescence and DIC images. **(A)** ARG1 expression (red) in non-activated macrophages (M0), non-infected or *T. gondii* (RH) infected cells (DAPI - blue). **(B)** ARG1 expression (red) in macrophages activated with IL-4 and 8-Br-AMPc (M2), non-infected or *T. gondii* (RH) infected cells (DAPI - blue). Scale bar = 10 µm.

ARG1 activity enhancement is dependent on the activation profile of the infected macrophage

To evaluate whether the activation profile of macrophages infected with *T. gondii* influenced ARG1 activity, M0 and M2 macrophages were infected and ARG1 activity evaluated. Interestingly, differences in ARG1 activity were detected depending on the cell line and if in a M2 activation profile as expected (**Figure 3**). In all non-infected macrophage cell lines M2 macrophages presented a higher ARG1 activity (2 to 8-fold) when compared to M0 macrophages (Figure 3). Both peritoneal macrophage cell lines presented a 30-fold higher ARG1 activity than RAW 264.7 macrophages (Figure 3). *T. gondii* infection increased ARG1 activity by 4 fold in M0 RAW 264.7 macrophages (**Figure 3 A)** and by 1.35 fold in M0 Swiss peritoneal macrophages (**Figure 3 B**). The infection of M2 macrophages by *T. gondii* did not induce an enhancement of ARG1 expression in all macrophages used in this work (**Figure 3**). Interestingly, infection of M0 C57BL/6 peritoneal macrophages by *T. gondii* did not altered the ARG1 activity pattern (**Figure 3 C**).

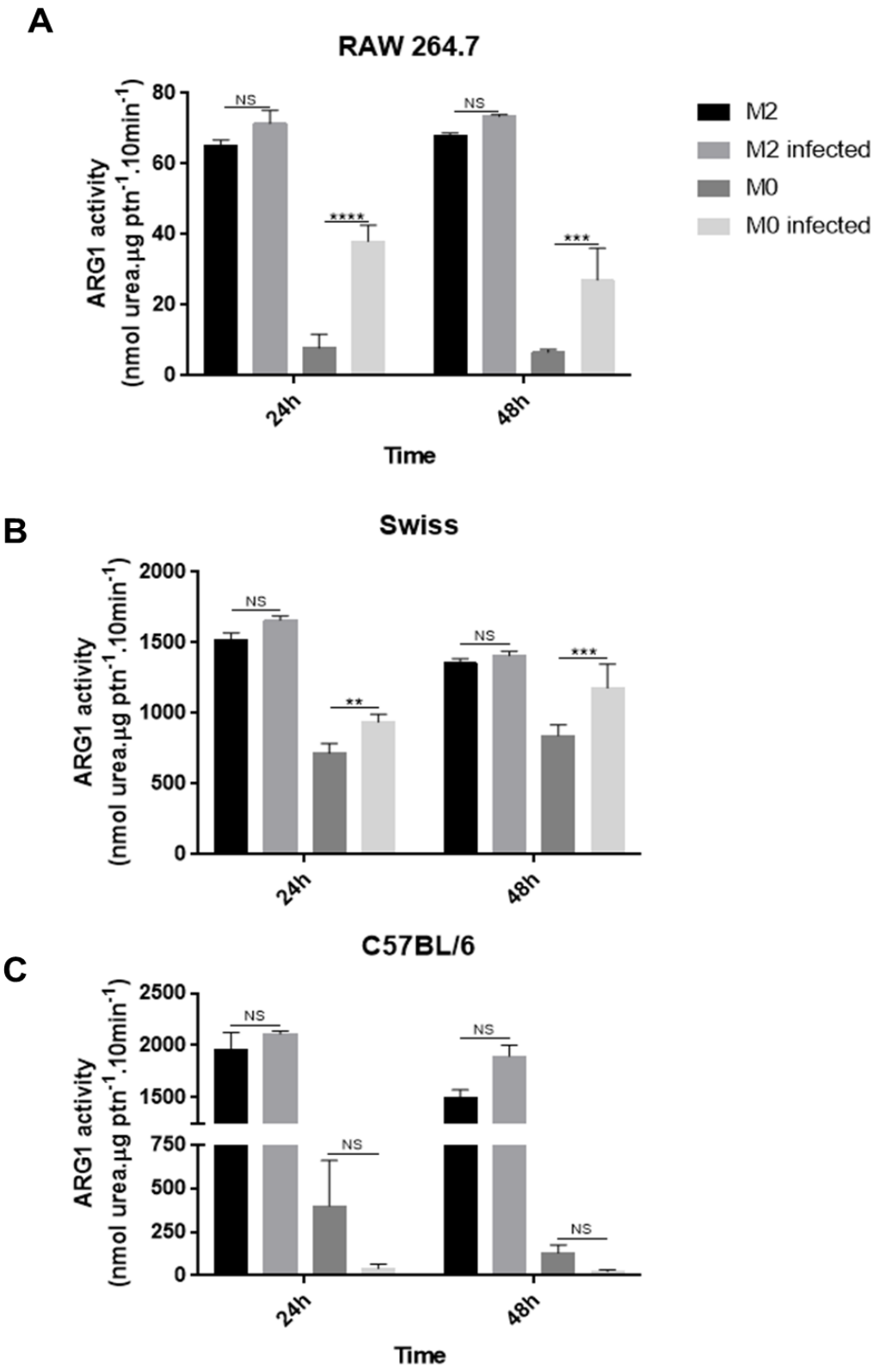


Figure 3. Arginase 1 (ARG1) activity of macrophages activated with IL-4 and 8-Br-AMPc (M2), or non-activated (M0) infected or not with *T. gondii*. ARG1 activity in RAW 264.7 **(A)**, Swiss **(B)** and C57BL/6 **(C)** peritoneal mice macrophages. Mean ± standard deviation (n = 3 independent experiments, each with 3 replicates). Significantly different by two-way ANOVA with Tukey post-test, *P=0.0109; **P=0.0062; ***P=0.0002; ****P<0.0001; NS = Not significant.

Supplementation of culture medium with L-arginine benefits *T. gondii* replication in RAW 264.7 macrophages only

To evaluate whether the availability of L-arginine, the ARG1 substrate crucial for polyamine production, influence the replication of *T. gondii*, M2 macrophages were incubated with extra L-arginine for 24h and parasite development evaluated. In M2 RAW 264.7 macrophages, the increased availability of L-arginine in the culture medium benefited the parasite replication, enhancing the number of infected cells (**Figure 4 A**) but without change in the number of tachyzoites per cell (**Figure 4 B**), nor enhancing the infection rate (**Figure 4 C**). Interestingly, in Swiss and C57BL/6 peritoneal macrophages the number of infected cells (**Figure 4 D and G**), the mean number of tachyzoites per cell (**Figure 4 E and H**) and the infection rate did not alter after L-arginine supplementation (**Figure 4 F and I**).

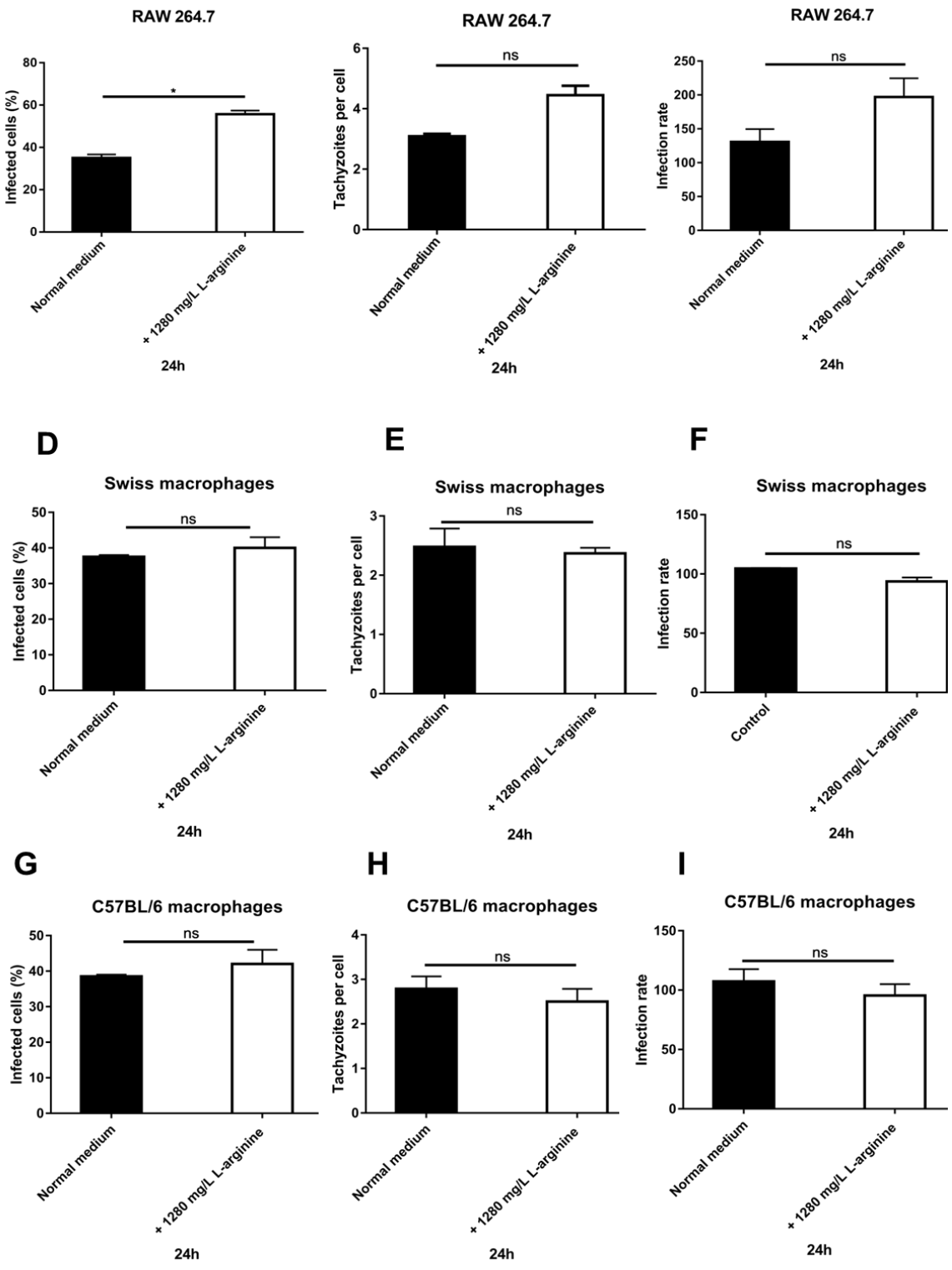


Figure 4. Evaluation of *T. gondii* growth in RAW 264.7 and mouse peritoneal (Swiss, C57BL/6) macrophages cells lines activated with IL-4 and 8-Br-AMPc cultured with normal medium or medium supplemented with L-arginine (+ 1280 mg/L L-arginine). Percentage of infected macrophages (**A, D and G**), mean number of tachyzoites per infected macrophages (**B, E and H**) and infection rate (**C, F and I**). Mean ± SD (n = 2 independent experiments, each with 3 replicates). *P ≤ 0.05, two-way ANOVA with Tukey post-test, ns (not significant).

Inhibition of ARG1 activity with nor-NOHA decrease *T. gondii* replication in macrophages

To evaluate whether ARG1 inhibition is prejudice to *T. gondii*, M0 and M2 RAW 264.7 macrophages were treated or not with nor-NOHA after infection and growth assayed. Treatment with nor-NOHA reduced parasites growth independently if M0 or M2 (**Figure 5**).

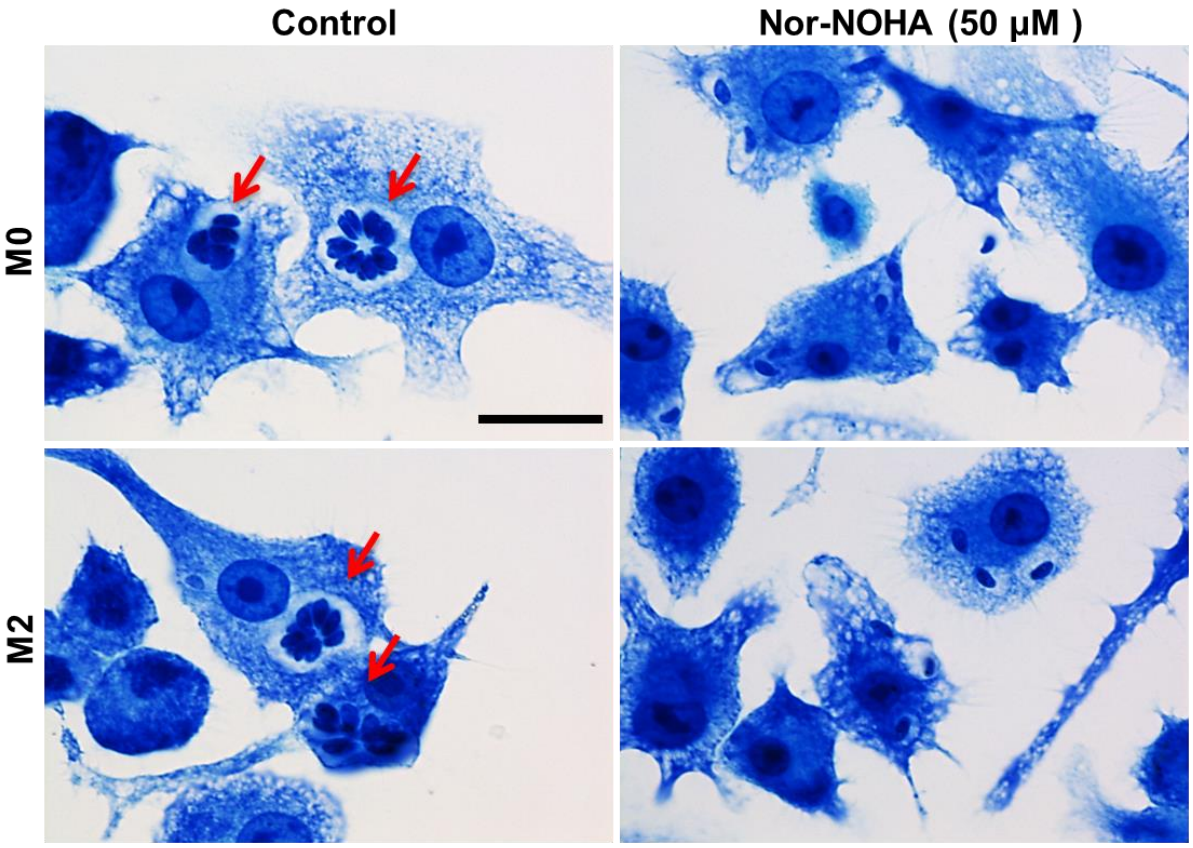


Figure 5. Bright-field microscopy of Giemsa stained non-activated (M0) or activated with IL-4 and 8-Br-AMPc (M2) RAW 264.7 macrophages infected with *T. gondii* for 24h, non-treated (control) or treated with 50 µM of Nor-NOHA. Rosette formed by the multiplication of parasites (arrow). Scale Bar = 10 µm.

Quantification showed that treatment of macrophages in M0 and M2 activation with 50 µM of nor-NOHA strongly reduced the percentage of infected cells (**Figure 6 A and B**), as well as the mean number of tachyzoites per cell only in M2 macrophages (**Figure 6 D**) and, thus, reduced the infection rate (**Figure 6 E and F**), indicating that ARG1 activity have an important role in *T. gondii* infection. Furthermore, RAW 264.7 macrophages with the M2 activation profile seems to be more sensible to the ARG1 inhibition, as all parameters evaluated in these experiments were strongly reduced in these cells (**Figure 6 B, D and F**).

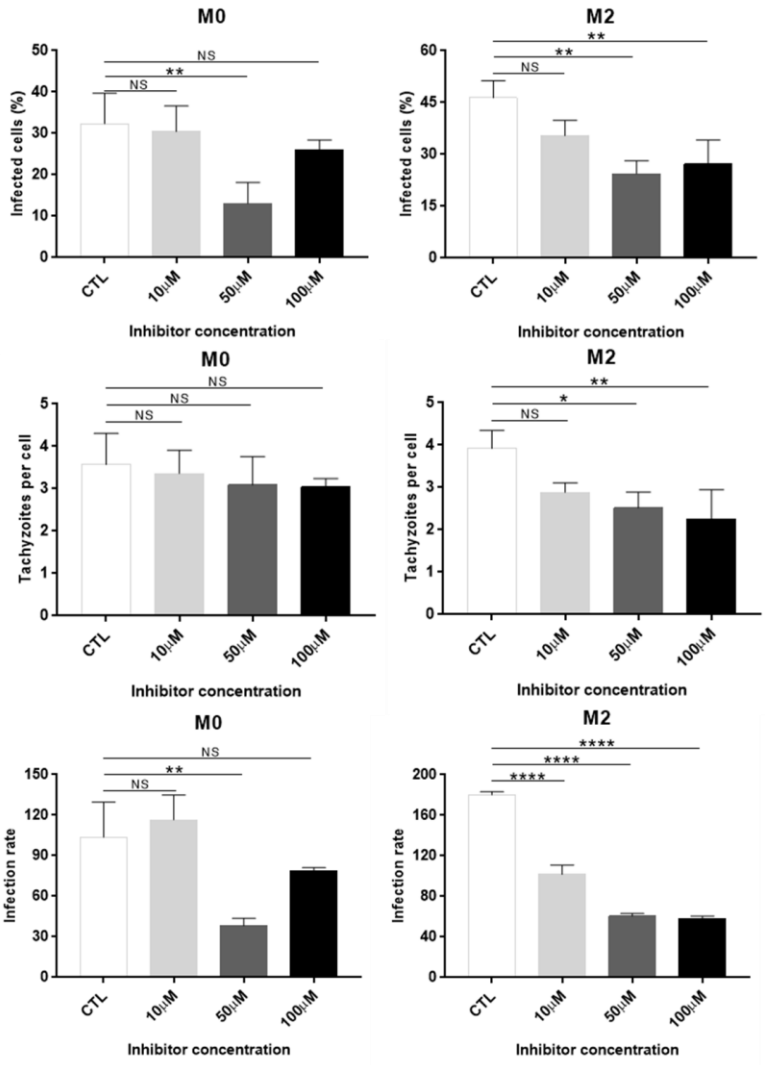


Figure 6. Evaluation of *T. gondii* growth in non-activated (M0) or activated with IL-4 and 8-Br-AMPc (M2) RAW 264.7 macrophages treated or not with Nor-NOHA. Percentage of infected macrophages (**A** and **B**), number of tachyzoites per infected macrophages (**C** and **D**) and infection rate (**E** and **F**). Representative experiment (n = 3). Significantly different by One Way ANOVA with Tukey post-test, *P=0.0147; **P=0.0028; ****P<0.0001; NS (not significant).

Discussion

Toxoplasma gondii is an obligate intracellular parasite that lacks the enzymes required for *de novo* arginine biosynthesis, requiring host cell L-arginine and its derivatives for replication (Fox et al., 2004; Butcher et al., 2011). Thus, *T. gondii* probably depends on ARG1 for host survival. To better understand ARG1 importance for the biology of this parasite, we evaluated in M0 and M2 mice macrophages ARG1 expression and activity after *T. gondii* infection, and parasite development in M2 infected macrophages supplemented with L-arginine or treated with an ARG1 inhibitor. No difference in ARG1 expression was detected between M0 and M2 activated macrophages, in all macrophages tested. However, infection of all macrophages cell lines in both M0 and M2 activated macrophages enhanced ARG1 expression. In addition, ARG1 activity only increased in M0 RAW 264.7 and Swiss peritoneal macrophages. L- arginine supplementation increased parasite growth only in M2 RAW 264.7 macrophages and inhibition of ARG1 decreased parasite growth in this macrophage cell line. Our findings suggest that the enhancement of ARG1 expression and activity in *T. gondii* infected macrophage may be a general evasion mechanism benefiting parasite development.

ARG1 was localized in the centrosome and irradiated to the macrophage cell periphery. This expression pattern did not vary between M0 and M2 macrophages nor between the macrophage cell lines used. The expression pattern of ARG1 was similar to microtubules distribution in macrophages, suggesting colocalization of these proteins. As far as we know, there are a few reports showing the localization of ARG1 within different cell types including macrophages. ARG1 is described as “cytosolic protein” in peritoneal rat macrophages after lipopolysaccharides stimulation (Sonoki et al., 1997), in ARG1 transfected rat astrocytes (Lee et al., 2003), in rat skeletal muscle cells (Buchwalow et al., 2005), in mouse and human embryonic stem cell (Cohen et al., 2010), in rat microglia/macrophages after rat brain injury (Kim et al., 2013) and in M0 and M2 canine blood-derived macrophages (Heinrich et al., 2017). However, these articles do not explore the ARG1 localization and only in Cohen et al. (2010), and in the BD Biosciences and Sinobiologica anti ARG1 prospects, high resolution images show ARG1 localization analogous to the

result we obtained (e.g. similar distribution to microtubule). The localization of ARG1 and microtubules should be further assayed in a confocal laser scanning microscope to determine if ARG1 is in fact colocalized to this cytoskeleton structure as suggested. In addition, nocodazole may be used to interfere with microtubule polymerization to determine if ARG1 localization, expression and activity may change. We could not see differences in ARG1 expression between M0 and M2 macrophages. This seems odd, but is similar to M0 and M2 canine blood-derived macrophages where no differences in mean expression percentages of cells were reported (Heinrich et al., 2017). Because, M2 macrophages have a higher ARG1 activity in comparison to M0, we suggest that the activity of this enzyme is not controlled by the amount of ARG1 expressed, but by a regulation mechanism not yet described. Further experiments are necessary to determine these possibilities.

Previous report showed that the enhancement of ARG1 expression in the host may be an evasion mechanism of *T. gondii*, as ARG1 catalyze the synthesis of polyamines (Nichols & Prosser, 1980; Tjandrawinata et al., 1994), important molecules used for cell replication (Heby, 1981), including *T. gondii* development (Seabra et al., 2004; Pfaff et al., 2005; Cook et al., 2007). Studies with rat and mouse peritoneal macrophages revealed differences in ARG1 expression and activity between these two macrophages cell lines (Li et al., 2012). Mouse peritoneal macrophages have more ARG1 expression and activity when compared to rat macrophages, leading to higher susceptibility to the parasite (Li et al., 2012). Thus, we decided to investigate the ARG1 expression and activity in both M0 and M2 activated macrophages from distinct mouse macrophage cell lines. In all mouse macrophages used independently if M0 or M2, ARG1 expression increased after *T. gondii* infection. Although no differences in ARG1 expression was found between M0 and M2 macrophages, infection by the parasite caused a higher expression of this enzyme suggesting that *T. gondii* was capable of up-regulating ARG1 expression. This may be due to the presence of ROP16, an effector protein that in type I *T. gondii* strains, as RH used here, increases ARG1 expression (Jensen et al., 2011; Butcher et al., 2011). The increase in ARG1 expression may be beneficial to the parasite as

shown before (Chang et al., 1998; Tenu et al., 1999; Pfaff et al., 2005; Cook et al., 2007).

ARG1 activity was higher in all M2 macrophages tested in this work. This is in accordance to pervious published results indicating that ARG1 is a M2 macrophage marker (Pauleau, et al., 2004). Peritoneal macrophages had a much higher ARG1 activity when compared to RAW 264.7 macrophages. Because ARG1 is a M2 marker, this is another example of the higher inflammatory capacity of RAW 264.7 macrophages in comparison to peritoneal macrophages or J774.A1 macrophages as shown recently (Cabral et al., 2018). This may help to explain why L-arginine and ARG1 inhibition only changed parasite growth in RAW 264.7 macrophages instead of peritoneal macrophages (see below).

ARG1 activity enhanced in M0 macrophages after infection, but not in M2 macrophages, indicating that the activation profile of the macrophage influenced the modulation of ARG1 activity by the parasite. This difference may be explained by the already higher ARG1 activity of the M2 macrophages in comparison to M0. It seems that in M2 macrophages ARG1 is already at its maximum activity. Thus, parasite infection may no longer increase its activity. Whereas in M0 macrophages, the infection could further increase ARG1 activity as demonstrated here.

Major role of ARG1 in parasite survival and replication has been reported for *Mycobacterium tuberculosis* (El Kasmi et al., 2008), *Trypanosoma brucei* (Gobert et al., 2000), *Schistosoma mansoni* (Abdallahi et al., 2001), and *T. gondii* (Pfaff et al., 2005; Cook et al., 2007, El Kasmi et al., 2008, Jensen et al., 2011; Li et al., 2012; Woods et al., 2013). ARG1 use L-arginine as substrate to produce L-ornitine, important substrate used by ornithine decarboxylase to produce polyamines, molecules used by *T. gondii* for replication (Cook et al., 2007), although, in M0 macrophages ornithine decarboxylase is not modulated by *T. gondii* infection (Seabra et al., 2004). As L-arginine has a major role in *T. gondii* development, we supplemented the culture medium with this amino acid and assayed parasite growth. Interestingly, only in M2 RAW 264.7 macrophages an enhancement in the percentage of infected cell was detected, suggesting that L-arginine benefits parasite development probably by polyamine

production. However, no differences in *T. gondii* development were observed in the Swiss and C57BL/6 peritoneal macrophages. This result suggest differences in the capacity of some specific mouse macrophage cell lines to metabolize L-arginine, since *T. gondii* is auxotrophic for this amino acid, depending exclusively from the host to obtain this essential molecule. This could be also due to an intrinsic characteristic of RAW 264.7 macrophages to be highly inflammatory (Cabral et al., 2018), as further confirmed here by lower ARG1 activity when compared to peritoneal macrophages. Because ARG1 activity is lower in RAW 264.7 macrophages, the capacity of L-arginine supplementation to influence parasite growth may be more discernible in this cell type. Thus, the extra supplementation was only influential in this cell type probably by higher polyamine production. This possibility should be further explored by measuring the concentration of polyamines in this cell after supplementation.

Overall, the enhancement of ARG1 activity may be an important mechanism for parasite survival. To confirm our hypothesis, we blocked the ARG1 activity with Nor-NOHA, an ARG1 specific inhibitor, and evaluated the replication of *T. gondii*. Treatment of M0 and M2 RAW 264.7 macrophages with Nor-NOHA reduced the infection rate, indicating that ARG1 have an important role in *T. gondii* survival and replication. These results match with previous reports showing that deletion of ARG1 from the host is prejudicial to *T. gondii* replication and survival, probably because of the reduction of polyamines biosynthesis and enhancement of nitric oxide production (El-Kasmi et al., 2008; Murray, 2011; Li et al., 2012). Furthermore, it has been shown that blocking ARG1 activity in BALB/c mice peritoneal macrophages (Li et al., 2012) and in a dendritic cell line (Jensen et al., 2011) reduces the number of parasite per cell, similar to ours results.

Overall, our findings reveal that all mouse macrophage cell lines used here express similar levels of ARG1, exhibiting no difference between M0 and M2 activated macrophages. Interestingly, *T. gondii* infection induces an enhancement of ARG1 expression in the cell lines analyzed, indicating that this strategy could be a general phenomenon to survive inside the host. The enhancement of ARG1 expression in host cells by *T. gondii* can be interpreted

as an immune evasion strategy of the parasite, as ARG1 catalyse L-ornitine synthesis, an important molecule used by ornithine decarboxylase to synthetize polyamines. It has been reported that polyamines are used by *T. gondii* for replication (Cook et al., 2007, Seabra et al., 2004). Our results also shows that ARG1 activity impairment by a specific inhibitor prejudice *T. gondii* replication, indicating that ARG1 have a major role in parasite survival. These results may contribute to the paradigm of the main role of host ARG1 in *T. gondii* infection.

Author contributions

GC and JX performed the experiments. GC, JX, GR and RD wrote the manuscript. GR and RD designed the experiments and revised the manuscript critically. All authors read and approved the final manuscript.

Funding

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. GC was supported by a fellowship from CAPES. JX was supported by a fellowship from the Brazilian Federal Agency, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). RD is supported by CNPq.

Acknowledgments

We are grateful to Dr. Elena Lassounskaia (LBR-UENF) that provided the RAW 264.7 macrophages, and Dr. Jorge Hudson Petreski for helpful advice. We would like to thank Andrèa Carvalho César for proof reading the manuscript.

References

Abdallahi OM, Bensalem H, Augier R, Diagana M, De Reggi M, Gharib B. Arginase expression in peritoneal macrophages and increase in circulating polyamine levels in mice infected with Schistosoma Mansoni. Cell. Mol. Life Sci. 2001; 58, 1350-1357.

Adams LB, Hibbs JB Jr, Taintor RR, Krahenbuhl JL. Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii* . Role for synthesis of inorganic nitrogen oxides from L-arginine." The Journal of Immunology.1990; 144(7): 2725-2729.

Barksdale AR, Bernard AC, Maley ME, Gellin GL, Kearney PA, Boulanger BR, Tsuei BJ, Ochoa JB. Regulation of arginase expression by T-helper II cytokines and isoproterenol. Surgery. 2004; 135:527–35.

Buchwalow IB, Minin EA, Samoilova VE, Boecker W, Wellner M, Schmitz W, Neumann J, Punkt K. Compartmentalization of NO signaling cascade in skeletal muscles. Biochem Biophys Res Commun. 2005; 330:615-21.

Butcher BA, Fox BA, Rommereim LM, Kim SG, Maurer KJ, Yarovinsky F, Herbert DR, Bzik DJ, Denkers EY. *Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. PLoS Pathog. 2011; Sep;7:e1002236.

Butcher BA, Denkers EY. Mechanism of entry determines ability of *Toxoplasma gondii* to inhibit macrophage proinflammatory cytokine production. Infect Immun. 2002; 70, 5216-5224.

Cabral GRA, DaMatta RA. Antimicrobial Activities of Macrophages and *Toxoplasma gondii* Evasion Mechanisms. Chapter 3. In *Toxoplasma gondii: Dangers, Life Cycle and Research*. Editor Sean L. Hall, 1 Ed. Hauppauge, NY, Nova Science Publishers, INC, 2017; P 39-84.

Cabral GRA, Wang ZiT, Sibley LD, DaMatta RA. Inhibition of nitric oxide production in activated macrophages caused by *Toxoplasma gondii* infection occurs by distinct mechanisms in different mouse macrophage cell lines. Frontiers in microbiology. 2018; 9.

Cohen NY, Ram SE, Shufaro Y, Rachmilewitz J, Reubinoff B. Human embryonic stem cells suppress T cell responses via arginase I-dependent mechanism. J Immunol. 2010 Feb 1;184(3):1300-8.

Cook T, Roos D, Morada M, Zhu G, Keithly JS, Feagin JE, Wu G, Yarlett N. Divergent polyamine metabolism in the Apicomplexa. 2007; Microbiology (Reading, England) 153(Pt 4):1123-30.

Corraliza IM, Campo ML, Soler G, Modolell M. Determination of arginase activity in macrophages: a micromethod. J Immunol Methods. 1994 Sep 14;174(1-2):231-5.

Chang CI, Liao JC, Kuo L. Arginase modulates nitric oxide production in activated macrophages. Am J Physiol. 1998 Jan; 274 (1 Pt 2):H342-8.

El-Gayar S, Thüring-Nahler H, Pfeilschifter J, Röllinghoff M, Bogdan C. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. J. Immunol. 2003; (171) 4561-4568.

El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, Henao-Tamayo M, Basaraba RJ, König T, Schleicher U, Koo MS, Kaplan G, Fitzgerald KA, Tuomanen EI, Orme IM, Kanneganti TD, Bogdan C, Wynn TA, Murray PJ. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol. 2008; Dec;9 (12):1399-406.

Fox BA, Gigley JP, Bzik DJ. *Toxoplasma gondii* lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation. Parasitol. 2004; Mar 9;34(3):323-31.

Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science. 2010; 327:656-661, 2010.

Gobert AP, Daulouede S, Lepoivre M, Boucher JL, Bouteille B, Buguet A, Cespuglio R, Veyret B, Vincendeau P. L-arginine availability modulates local nitric oxide production and parasite killing in experimental trypanosomiasis. Infect. Immun. 2000; 68, 4653-4657.

Heby O. Role of polyamines in the control of cell proliferation and differentiation. Differentiation; research in biological diversity. 1981; (1):1-20.

Heinrich F, Lehmbecker A, Raddatz BB, Kegler K, Tipold A, Stein VM, Kalkuhl A, Deschl U, Baumgärtner W, Ulrich R, Spitzbarth I. Morphologic, phenotypic, and transcriptomic characterization of classically and alternatively activated canine blood-derived macrophages *in vitro*. PLoS One. 2017 Aug 17;12(8):e0183572.

Jensen KD, Wang Y, Wojno ED, Shastri AJ, Hu K, Cornel L, Boedec E, Ong YC, Chien YH, Hunter CA, Boothroyd JC, Saeij JP. Toxoplasma polymorphic effectors determine macrophage polarization and intestinal inflammation. Cell Host Microbe. 2011, 16, 472-483.

Kim H, Ahn M, Choi S, Kim M, Sim KB, Kim J, Moon C, Shin T. Potential role of fibronectin in microglia/macrophage activation following cryoinjury in the rat brain: an immunohistochemical study. Brain Res. 2013 Mar 28;1502:11-9.

Khan I, Matsuura T, Kasper LH. IL-10 mediated immunosuppression following primary infection with *Toxoplasma gondii* in mice. Parasite Immunol. 1995, 17,185-195.

Khaminets A, Hunn JP, Könen-Waisman, S, Zhao YO, Preukschat D, Coers J, Boyle JP, Ong YC, Boothroyd JC, Reichmann G, Howard JC. Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. Cell Microbiol; 2010, 12, 939-961**.**

Lee J, Ryu H, Ferrante RJ, Morris SM Jr, Ratan RR. Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. Proc Natl Acad Sci U S A. 2003; Apr 15;100 (8):4843-8.

Li Z, Zhao ZJ, Zhu XQ, Ren QS, Nie FF, Gao JM, Gao XJ, Yang TB, Zhou WL, Shen JL, Wang Y, Lu FL, Chen XG, Hide G, Ayala FJ, Lun ZR. Differences in iNOS and Arginase Expression and Activity in the Macrophages of Rats Are Responsible for the Resistance against *T. gondii* Infection. PLoS One. 2012; 7(4): e35834.

Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol.2000; 164(12): 6166-6173.

Mills CD. M1 and M2 Macrophages: Oracles of Health and Disease. Crit Rev Immunol. 2012; 32(6):463-88.

Murray HW, Cohn ZA. Macrophage oxygen-dependent antimicrobial activity. I. Susceptibility of *Toxoplasma gondii* to oxygen intermediates. J Exp Med. 1979; 150(4): 938-949.

Murray PJ. Macrophages as a battleground for toxoplasma pathogenesis. Cell Host Microbe. 2011; 9, 445-447.

Munder M, Eichmann K, Moran JM, Centeno F, Soler G, Modolell M. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. J Immunol. 1999;163:3771–7.

Müllner N, Lázár A, Hrabák A. Enhanced utilization and altered metabolism of arginine in inflammatory macrophages caused by raised nitric oxide synthesis. Int J Biochem Cell Biol. 2002 Sep;34(9):1080-90.

Nichols WK, Prosser FH. Induction of ornithine decarboxylase in macrophages by bacterial lipopolysaccharides (LPS) and mycobacterial cell wall material. Life sciences; 1980, 27(11):913-20.

Pauleau AL, Rutschman R, Lang R, Pernis A, Watowich SS, Murray PJ. Enhancer-mediated control of macrophage-specific arginase I expression. J. Immunol; 2004; 172, 7565–7573.

Pfaff AW, Villard O, Klein JP, Mousli M, Candolfi E. Regulation of Toxoplasma gondii multiplication in BeWo trophoblast cells: cross-regulation of nitric oxide production and polyamine biosynthesis. International journal for parasitology 2005; 35(14):1569-76.

Seabra SH, DaMatta RA, de Mello FG, de Souza W. Endogenous polyamine levels in macrophages is sufficient to support growth of *Toxoplasma gondii*. J Parasitol. 2004 90(3):455-60.

Sibley LD, Adams LB, Fukutomi Y, Krahenbuhl JL. Tumor necrosis factor-alpha triggers antitoxoplasmal activity of IFN-gamma primed macrophages. The Journal of Immunology; 1991; 147(7): 2340-2345.

Sonoki T, Nagasaki A, Gotoh T, Takiguchi M, Takeya M, Matsuzaki H, Mori M. Coinduction of nitric-oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues in vivo by lipopolysaccharide. J Biol Chem. 1997 272(6):3689-93.

Sheldon KE, Shandilya H, Kepka-Lenhart D, Poljakovic M, Ghosh A, Morris SM Jr. Shaping the murine macrophage phenotype: IL-4 and cyclic AMP synergistically activate the arginase I promoter. J Immunol. 2013, 191(5):2290-8.

Stuehr DJ, Marletta, M.A. Mammalian nitrite biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. Proc. Natl. Acad. Sci. U.S.A;1985; 82, 7738–7742.

Stuehr DJ, Marletta, MA. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines or interferon-g. J. Immunol. 1987; 139, 518–525.

Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma gondii*: from animal to humans. Int J Parasitol. 2000., 30: 1217-1258.

Tenu JP, Lepoivre M, Moali C, Brollo M, Mansuy D, Boucher JL. Effects of the new arginase inhibitor N (omega)-hydroxy-nor-L-arginine on NO synthase activity in murine macrophages. Nitric Oxide. 1999 Dec;3 (6):427-38.

Tjandrawinata RR, Hawel L, 3rd, Byus CV. Regulation of putrescine export in lipopolysaccharide or IFN-gamma-activated murine monocytic-leukemic RAW 264 cells. Journal of immunology (Baltimore, Md : 1950) 1994, 152(6):3039-52.

Woods S, Schroeder J, Mcgachy HA, Plevin R, Roberts CW, Alexander J. MAP kinase phosphatase-2 plays a key role in the control of infection with *Toxoplasma gondii* by modulating iNOS and arginase-1 activities in mice. PLoS Pathog 2013; 9, e1003535.

Zhao YO, Khaminets A, Hunn JP, Howard JC. Disruption of the *Toxoplasma gondii* parasitophorous vacuole by IFN gamma-inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death. PLoS Pathog. 2009, e1000288.

5- Discussão

Toxoplasma gondii é um dos parasitos mais bem adaptados do mundo, fato demonstrado por sua alta prevalência mundial e capacidade de infectar praticamente todas as células de animais vertebrados de sangue quente (Tenter et al, 2000). Macrófagos são importantes células do sistema imune (Geissmann et al, 2010), possuem heterogeneidade funcional e fenotípica (Gomez Perdiguero et al, 2015; Sorokin et al, 1992) e alta capacidade de controlar a replicação de patógenos, como *T. gondii* (Khan et al, 1997). Estas células controlam a replicação de *T. gondii* através de diversos mecanismos microbicidas, como fagocitose (Jones et al, 1972), espécies reativas de oxigênio (Murray & Cohn, 1979) e NO (Adams et al, 1990), e GTPases relacionadas com a imunidade, como as IRGs e GBPs (Khaminets et al, 2010). Porém, durante o processo de coevolução com o hospedeiro, *T. gondii* desenvolveu diversos mecanismos de evasão do sistema imune (Khaminets et al, 2010). Grande parte do sucesso na evasão do sistema imune é mediado por fatores de virulência secretados durante o processo de invasão da célula hospedeira, como proteínas das róptrias e dos grânulos densos. Parte destes fatores de virulência migra para o núcleo da célula hospedeira controlando a expressão gênica (Bougdour et al, 2013; Gay & Braun, 2016; Olias et al, 2016). Sabe-se que a iNOS desaparece em macrófagos peritoneais de camundongo ativados no modo M1 após infecção por *T. gondii* (Seabra et al, 2002; Seabra et al, 2004), através da via do proteossomo na linhagem J774-A1 (da Cruz Padrão et al, 2014). No entanto, não se sabe qual é o possível efetor secretado pelo parasito responsável por modular a iNOS e se há diferenças nesta modulação em diferentes linhagens de macrófagos. Ademais, estudos recentes mostram que após infecção com *T. gondii*, ARG1 é induzida na célula hospedeira, o que beneficia o parasito (Jensen et al, 2011; Li et al, 2012). Este fato ocorre, pois a iNOS compete com a ARG1 pelo substrato L-arginina (Tenu et al, 1999), e ao mesmo tempo, ARG1 catalisa a síntese de poliaminas, moléculas importantes para a replicação celular, incluindo de *T. gondii* (Cook et al, 2007). Nessa tese, revisamos alguns mecanismos microbicidas que controlam e, portanto, são burlados por *T. gondii*, verificamos fatores de virulência na evasão do sistema

microbicida baseado na produção de NO, e avaliamos a expressão e atividade de ARG1 e crescimento do parasito em macrófagos M2.

Toxoplasma gondii causa a toxoplasmose, pois consegue escapar de praticamente todos os mecanismos microbicidas descritos até então. É curioso notar que enquanto se descreviam mecanismos microbicidas, logo em seguida era demostrado que *T. gondii* conseguia escapar dos mesmos (Cabral & DaMatta, 2017). Durante os anos 70 do século XX, ainda estava em discussão como *T. gondii* entrava na célula (Jones & Hirsch, 1972). Jones e Hirsch (1972) descrevem essa entra em células hospedeiras por um processo fagocítico e demostram que se o parasito está vivo, não ocorre fusão com o sistema endolisossomal, mas se morto, a fusão corre e o parasito é destruído por digestão lisossomal. Aproveitando dos estudos iniciais de ativação macrofágica (Nathan, 1983), foi demostrado que a produção de radicais de oxigênio eram responsáveis pelo controle da multiplicação de *T. gondii* em macrófagos peritoneais de camundongos que foram previamente injetados com bactérias mortas (Murray and Cohn, 1979). No entanto, o parasito apresenta grande capacidade de resistir a esses radicais de oxigênio, pois detém uma gama de proteínas antioxidantes (Sibley et al., 1986; Ding et al., 2004). Portanto, também burlam esse sistema microbicida. O que não se sabia na época era que esses macrófagos ativados não produziam só esses radicais, mas também NO! No início dos anos 90 do século passado, houve uma explosão de conhecimento sobre o NO (Koshland, 1992) e vários artigos foram publicados mostrando que esse radical controlava o crescimento de parasitos intracelulares como *T. gondii* (Adams et al., 1990). No entanto, coube ao nosso grupo demostrar que *T. gondii* consegue inibir a produção de NO (Seabra et al., 2002). Mais recentemente, via recombinação sexuada de distintas cepas no intestino de gatos, descobriu-se que algumas ROPs eram fatores de virulência, mas não se sabiam o que essas proteínas faziam na célula hospedeira. Um novo sistema microbicida foi descoberto na tentativa de se desvendar como as ROPs funcionavam como fatores de virulência. Esse sistema microbicida envolve GTPases sendo exclusivo em algumas espécies de roedores, o que sugere que esses hospedeiros devem ser os mais adaptados ao *T. gondii* (Gazzinelli et al, 2014). É curioso como descobertas

são feitas, e a história das descobertas dos mecanismos evasivos de *T. gondii* é um belo exemplo.

A produção de NO é modulada negativamente em macrófagos ativados e infectados com *T. gondii* (Seabra et al, 2002), de maneira dependente de TGF-β (Seabra et al, 2004). Em 2014 foi descrito que a iNOS, enzima responsável pela síntese de NO, é degradada via proteossomo após infecção de macrófagos ativados por *T. gondii* (da Cruz Padrão et al, 2014). Estudos recentes revelaram que a inibição de NO ocorre através de diferentes mecanismos, dependendo do tipo celular infectado (Cabral et al, 2018). Em macrófagos J774-A1, a infecção por *T. gondii* induz redução no número de células iNOS positiva e o desaparecimento da iNOS (Cabral et al, 2018), possivelmente pela via do proteossomo, reduzindo os níveis de NO produzidos (da Cruz Padrão et al, 2014). Em macrófagos RAW 264.7 infectados por *T. gondii* não há desaparecimento da iNOS ou redução no número de células iNOS positiva, e ainda sim, há redução dos níveis de NO produzidos (Cabral et al, 2018). Ademais, diferentes fatores de virulência secretados pelas róptrias, como ROP5, ROP16, ROP17, ROP18 ou grânulos densos, como ASP5, MYR1 e TgIST não participam como efetores na modulação do NO (Cabral et al, 2018). Estes resultados indicam que: 1- existem outros mecanismos de inibição do NO além da degradação da iNOS via proteossomo (Cabral et al, 2018; da Cruz Padrão et al, 2014); 2- o mecanismo de inibição de NO pode variar de acordo com a linhagem de macrófago (Cabral et al, 2018); 3- existe outro(s) efetor (es) ainda não descrito na literatura que é responsável pela modulação do NO em macrófagos infectados com *T. gondii* (Cabral et al, 2018). Um desses efetores pode ser a fosfatidilserina (PS) que é um importante fosfolipídeo de membrana celular. Tem sido descrito que a PS pode estar envolvida na inibição da produção de NO em macrófagos peritoneais de camundongos Suíços ativados e infectados com *T. gondii* (Seabra et al, 2004). Células apoptóticas expõem PS, que quando em contato com macrófagos induz a produção do TGF-β, um potente desativador que age de maneira autócrina e parácrina (Ashcroft, 1999). Estudos mostram que *T. gondii* expõe a PS em sua membrana, mimetizando uma célula apoptótica, o que leva a desativação do macrófago e consequente redução na produção de NO

nestas células, beneficiando o parasito (Seabra et al., 2004a). O fato de não termos achado um efetor proteico na inibição da produção de NO, é um indicativo que a PS pode ser mesmo uma molécula importante nesse processo.

ARG1 é uma enzima citosólica manganês dependente que catalisa a síntese de L-ornitina e ureia a partir da hidrólise de L-arginina (Ash, 2004), sendo induzida por citocinas anti-inflamatórias como IL-4, IL-6, IL-10, IL-13 e TGF-β (Barksdale et al, 2004; Munder et al, 1999). Ademais, L-arginina é o substrato de duas vias metabólicas distintas mediada pela iNOS (Klatt et al, 1996) e ARG1 (Kossel & Dakin, 1904). iNOS utiliza L-arginina como substrato para síntese NO e citrulina (Klatt et al, 1996); já a ARG1 utiliza L-arginina para síntese de ureia e L-ornitina, na qual esta última pode sofrer ação da ornitina descarboxilase para síntese de poliaminas (Kossel & Dakin, 1904). Tem-se descrito o papel das poliaminas como importantes moléculas na proliferação celular de *T. gondii* (Cook et al, 2007; Pfaff et al, 2005). Deste modo, a indução da ARG1 na célula infectada por *T. gondii* pode ser interpretada como um mecanismo de evasão do sistema imune hospedeiro (El Kasmi et al, 2008), já que a iNOS e ARG1 competem pela L-arginina (Tenu et al, 1999) e ao mesmo tempo o parasito pode utilizar as poliaminas para replicação celular (Cook et al, 2007; Pfaff et al, 2005). Porém, ainda existe grande discussão acerca do papel da indução da ARG1 na célula infectada por *T. gondii*. Aqui, avaliamos o padrão de expressão da ARG1 em três linhagens de macrófagos após ativação M2, seu perfil de expressão nessas células M0 e M2 após infecção por *T. gondii* e seu papel na replicação *in vitro* do parasito após suplementação de arginina e inibição farmacológica de macrófagos M2. Experimentos de imunofluorescência revelaram que ARG1 é expressa constitutivamente em diferentes linhagens de macrófagos, como RAW 264.7 e macrófagos peritoneais de camundongos suíços e C57BL/6, não sofrendo alteração de expressão mesmo após ativação por citocinas anti-inflamatórias como IL-4. Interessantemente, houve aumento na expressão de ARG1 em todas as linhagens de macrófagos testadas após infecção por *T. gondii*, independente se não ativadas ou ativadas no perfil M2. Como *T. gondii* é auxotrófico para L-arginina, o aumento na expressão de ARG1 na célula infectada pode favorecer a replicação do parasito, isto é, este parasito é

incapaz de sintetizar este aminoácido, obtendo-o exclusivamente via hospedeiro (Fox et al, 2004). Isto corrobora com o fato de *T. gondii* não possuir enzimas chave da síntese de L-arginina, dependendo exclusivamente das enzimas do hospedeiro (Fox et al, 2004). Estes resultados indicam que o aumento da expressão de ARG1 na célula infectada por *T. gondii* pode ser um mecanismo geral de evasão do sistema imune, beneficiando o parasito. Experimentos de atividade enzimática da ARG1 revelaram que a infecção por *T. gondii* aumenta a atividade desta enzima na célula infectada, corroborando com os resultados anteriores. Ademais, experimentos de suplementação do meio de cultura com L-arginina revelaram aumento na porcentagem de células infectadas em macrófagos RAW 264.7, sugerindo que a maior disponibilidade de substrato beneficia a replicação de *T. gondii*. Experimentos de inibição da atividade de ARG1 com Nor-NOHA revelaram redução no número de parasitos e no número de células infectadas após tratamento dos macrófagos com o inibidor específico da ARG1. Ademais, El Kasmi et al. (2008) demonstraram que camundongos C57BL/6 *knockout* para ARG1 apresentam maior sobrevida em infecções com *T. gondii*, indicando o papel chave de ARG1 para replicação e sobrevivência de *T. gondii* . De modo similar, Li et al. (2012) demonstraram que o bloqueio da atividade de ARG1 em macrófagos peritoneais de camundongos Balb/c diminui a replicação de cepas tipo I de *T. gondii* . Suplementação do meio de cultura de macrófagos RAW 264.7 com L-arginina aumentou o número de células infectadas, sugerindo que a maior disponibilidade de substrato para ARG1 beneficia o desenvolvimento de *T. gondii*, provavelmente pelo aumento na produção de poliaminas (Cook et al, 2007). Este resultado pode estar relacionado com o fato de que macrófagos de camundongos são naturalmente menos resistentes a infecção com *T. gondii*, devido a maior expressão de ARG1 e consequentemente, maior produção de poliaminas, aumento a susceptibilidade dessas células ao parasito (Cook et al, 2007; Henrique Seabra et al, 2004). Interessantemente, o mesmo fenômeno do aumento no número de parasitos após suplementação do meio de cultura com L-arginina não se reproduziu em macrófagos peritoneais de camundongos Suíços e C57BL/6, indicando que o fenômeno pode variar de acordo com o tipo celular.

Toxoplasma gondii, agente causador da toxoplasmose, é um parasito intracelular obrigatório de ampla distribuição mundial (Tenter et al, 2000). Macrófagos são células chave do sistema imune e realizam o controle da replicação de *T. gondii* quando ativados com IFN-γ, e um segundo sinal por TNF-α ou LPS (Adams et al, 1990; Sibley et al, 1991), através de diversos mecanismos microbicidas (Cabral & DaMatta, 2017), como produção de espécies reativas de oxigênio (Murray & Cohn, 1979), produção de NO via iNOS (Khan et al, 1997) e IRGs (Butcher et al, 2011; Khaminets et al, 2010; Zhao et al, 2009). No entanto, durante o processo coevolutivo com o seu hospedeiro, *T. gondii* desenvolveu diversos mecanismos de evasão do hospedeiro, para subverter o sistema imune, como a inibição da fusão lisossomal (Jones et al, 1972), inibição de espécies reativas de oxigênio (Abdallahi et al, 2001; Murray & Cohn, 1980; Wilson et al, 1980), inibição de NO (Seabra et al, 2002) e degradação da iNOS (Cabral et al, 2018; da Cruz Padrão et al, 2014) e inibição da atividade das IRGs (Behnke et al, 2011; Fentress et al, 2010; Steinfeldt et al, 2010). Grande parte do sucesso evolutivo adquirido por *T. gondii* na evasão de mecanismos microbicidas do hospedeiro está diretamente relacionado com a os fatores de virulência secretados pelo parasito. Durante o processo de invasão da célula hospedeira, *T. gondii* secreta o conteúdo de organelas secretoras especializadas como róptrias e grânulos densos, que subvertem o sistema imune hospedeiro (Bougdour et al, 2013; Braun et al, 2013; Carruthers & Sibley, 1997; Etheridge et al, 2014; Olias et al, 2016). Fatores de virulência provenientes das róptrias, como ROP5, ROP 17 e ROP18 formam complexos proteicos que inibem a atividade de IRGs (Behnke et al, 2011; Etheridge et al, 2014; Saeij et al, 2006). Ademais, fatores de virulência secretados pelos grânulos densos, como TgIST transloca para o núcleo do hospedeiro, controlando a transcrição dependente de IFN-γ, bloqueando a ativação celular (Olias et al, 2016). Embora o papel destes e outros fatores de virulência estejam descritos na evasão de diversos mecanismos microbicidas, ainda não se sabe qual fator de virulência é responsável pela degradação da iNOS e consequente inibição da produção de NO em macrófagos ativados (Cabral et al, 2018). Resultados recentes revelam que fatores de virulência provenientes das

róptrias, como ROP5, ROP 16, ROP17, ROP18, e fatores de virulência provenientes dos grânulos densos, como ASP5, MYR1, e TgIST não estão envolvidos na evasão microbicida do NO, sugerindo um novo fator de virulência a ser elucidado (Cabral et al, 2018). Outro importante fator de virulência secretado pelas róptrias é a ROP16, proteína efetora que ativa STAT3 e STAT6 resultando na inibição de citocinas pró-inflamatórias e indução de ARG1 (Butcher et al, 2011). ARG1 sintetiza L-ornitina, molécula utilizada para produção de poliaminas e que são utilizadas pelo *T. gondii* para replicação (Cook et al, 2007; Pfaff et al, 2005). Porém, existe contradição quanto ao benefício da ARG1 do hospedeiro para *T. gondii* (Woods et al, 2013). Tem sido demonstrado que o papel da ARG1 pode variar em experimentos *in vivo* x *in vitro* e até mesmo de acordo com o tipo celular do hospedeiro (Li et al, 2012; Murray, 2011; Woods et al, 2013). Estes dados ressaltam que ainda existem lacunas no conhecimento acerca do real papel da ARG1 em infecção com *T. gondii*, e que os dados obtidos podem variar de acordo com o tipo celular do hospedeiro, da cepa de *T. gondii* utilizada, ou do tipo de experimento realizado (*in vivo* x *in vitro*), evidenciando a complexidade da interação parasito - célula hospedeira no estudo do paradigma iNOS versus ARG1.

6- Conclusão

I. *Toxoplasma gondii* apresenta diferentes mecanismos evasivos contra macrófagos;
II. A inibição de NO parece ser um mecanismo geral de evasão de *T. gondii* que ocorre em diferentes linhagens de macrófagos;
III. O mecanismo pelo qual ocorre a inibição de NO pode variar de acordo com a linhagem de macrófago;
IV. Ainda são se tem descrito na literatura qual efetor de *T. gondii* é responsável pelo fenótipo de inibição do NO;
V. ARG1 é expressa constitutivamente em diferentes linhagens de macrófagos;
VI. A expressão de ARG1 aumenta em macrófagos infectados com *T. gondii*, independente do perfil de ativação destas células;

VII. A atividade enzimática de ARG1 aumenta em macrófagos M0 infectados com *T. gondii*, mas não em macrófagos M2;

VIII. Suplementação do meio de cultura de macrófagos RAW 264.7 ativados em perfil M2 beneficiou o desenvolvimento de *T. gondii in vitro*, como representado pelo aumento no número de parasitos e no número de células infectadas quando comparados aos macrófagos não suplementados com L-arginina;

IX. Inibição farmacológica de ARG1 com Nor-NOHA prejudica a replicação de *T. gondii*, como representado pela redução no número de parasitos e no número de células infectadas.

7-Referências

Abdallahi, O. M., et al. (2001). "Arginase expression in peritoneal macrophages and increase in circulating polyamine levels in mice infected with *Schistosoma mansoni*." Cell Mol Life Sci 58(9): 1350-1357.

Achbarou, A., et al. (1991). "Differential targeting of dense granule proteins in the parasitophorous vacuole of *Toxoplasma gondii*." Parasitology 103 Pt 3: 321-329.

Adams, L., et al. (1990). "Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii* . Role for synthesis of inorganic nitrogen oxides from L-arginine." The Journal of Immunology 144(7): 2725-2729.

Adjogble, K. D., et al. (2004). "GRA9, a new *Toxoplasma gondii* dense granule protein associated with the intravacuolar network of tubular membranes." Int J Parasitol 34(11): 1255-1264.

Ahn, H. J., et al. (2005). "Host cell binding of GRA10, a novel, constitutively secreted dense granular protein from *Toxoplasma gondii*." Biochem Biophys Res Commun 331(2): 614-620.

Alaganan, A., et al. (2014). "*Toxoplasma* GRA7 effector increases turnover of immunity-related GTPases and contributes to acute virulence in the mouse." Proc Natl Acad Sci U S A 111(3): 1126-1131.

Alexander, D. L., et al. (2005). "Identification of the moving junction complex of *Toxoplasma gondii*: a collaboration between distinct secretory organelles." PLoS Pathog 1(2): e17.

Andreesen, R., et al. (1988). "Human macrophage maturation and heterogeneity: restricted expression of late differentiation antigens in situ." Cell Tissue Res 253(2): 271-279.

Ash, D. E. (2004). "Structure and function of arginases." J Nutr 134(10 Suppl): 2760S-2764S; discussion 2765S-2767S.

Ashcroft, G. S. (1999). "Bidirectional regulation of macrophage function by TGF-β." Microbes and Infection 1(15): 1275-1282.

Barksdale, A. R., et al. (2004). "Regulation of arginase expression by T-helper II cytokines and isoproterenol." Surgery 135(5): 527-535.

Barragan, A. and L. D. Sibley (2003). "Migration of *Toxoplasma gondii* across biological barriers." Trends Microbiol 11(9): 426-430.
Barrangou, R., et al. (2007). "CRISPR provides acquired resistance against viruses in prokaryotes." Science 315(5819): 1709-1712.
Baum, J., et al. (2006). "A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites." J Biol Chem 281(8): 5197-5208.
Behnke, M. S., et al. (2012). "The polymorphic pseudokinase ROP5 controls virulence in *Toxoplasma gondii* by regulating the active kinase ROP18." PLoS Pathog 8(11): e1002992.
Behnke, M. S., et al. (2011). "Virulence differences in *Toxoplasma* mediated by amplification of a family of polymorphic pseudokinases." Proc Natl Acad Sci U S A 108(23): 9631-9636.
Bekpen, C., et al. (2005). "The interferon-inducible p47 (IRG) GTPases in vertebrates: loss of the cell autonomous resistance mechanism in the human lineage." Genome Biol 6(11): R92.
Bermudes, D., et al. (1994). "Cloning of a cDNA encoding the dense granule protein GRA3 from *Toxoplasma gondii* ." Mol Biochem Parasitol 68(2): 247-257.
Bierly, A. L., et al. (2008). "Dendritic cells expressing plasmacytoid marker PDCA-1 are Trojan horses during *Toxoplasma gondii* infection." J Immunol 181(12): 8485-8491.
Biswas, S. K., et al. (2012). "Macrophage polarization and plasticity in health and disease." Immunol Res 53(1-3): 11-24.
Black, M. W. and J. C. Boothroyd (2000). "Lytic cycle of *Toxoplasma gondii*." Microbiol Mol Biol Rev 64(3): 607-623.
Blanchette, J., et al. (2003). "Signalling events involved in interferon-gamma-inducible macrophage nitric oxide generation." Immunology 108(4): 513-522.
Boddey, J. A., et al. (2013). "Role of plasmepsin V in export of diverse protein families from the *Plasmodium falciparum* exportome." Traffic 14(5): 532-550.
Boddey, J. A., et al. (2010). "An aspartyl protease directs malaria effector proteins to the host cell." Nature 463(7281): 627-631.
Bogdan, C. (2000). "The function of type I interferons in antimicrobial immunity." Curr Opin Immunol 12(4): 419-424.
Bogdan, C. (2015). "Nitric oxide synthase in innate and adaptive immunity: an update." Trends in immunology 36(3): 161-178.
Bojar, I. and J. Szymanska (2010). "Environmental exposure of pregnant women to infection with *Toxoplasma gondii* -state of the art." Ann Agric Environ Med 17(2): 209-214.
Bougdour, A., et al. (2013). "Host cell subversion by *Toxoplasma* GRA16, an exported dense granule protein that targets the host cell nucleus and alters gene expression." Cell Host Microbe 13(4): 489-500.
Bougdour, A., et al. (2014). "*Toxoplasma* exports dense granule proteins beyond the vacuole to the host cell nucleus and rewires the host genome expression." Cell Microbiol 16(3): 334-343.
Bradley, P. J. and L. D. Sibley (2007). "Rhoptries: an arsenal of secreted virulence factors." Curr Opin Microbiol 10(6): 582-587.

Braun, L., et al. (2013). "A *Toxoplasma* dense granule protein, GRA24, modulates the early immune response to infection by promoting a direct and sustained host p38 MAPK activation." J Exp Med 210(10): 2071-2086.
Bredt, D. S., et al. (1991). "Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase." Nature 351(6329): 714-718.
Brown, K. M., et al. (2017). "Plasma Membrane Association by N-Acylation Governs PKG Function in *Toxoplasma gondii* ." MBio 8(3).
Butcher, B. A., et al. (2011). "*Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control." PLoS Pathog 7(9): e1002236.
Cabral G.R.A, DaMatta R.A (2017). “Antimicrobial Activities of Macrophages and *Toxoplasma gondii* ” Evasion Mechanisms. Chapter 3. In *Toxoplasma gondii*: Dangers, Life Cycle and Research. Editor Sean L. Hall, 1 Ed. Hauppauge, NY, Nova Science Publishers, INC, P 39-84.
Cabral, G. R. A. et al. (2018). "Inhibition of nitric oxide production in activated macrophages caused by *Toxoplasma gondii* infection occurs by distinct mechanisms in different mouse macrophage cell lines." Frontiers in microbiology 9.
Cama, E., et al. (2003). "Human arginase II: crystal structure and physiological role in male and female sexual arousal." Biochemistry 42(28): 8445-8451.
Carey, K. L., et al. (2000). "Identification and molecular characterization of GRA8, a novel, proline-rich, dense granule protein of *Toxoplasma gondii* ." Mol Biochem Parasitol 105(1): 25-37.
Carruthers, V. B. (2002). "Host cell invasion by the opportunistic pathogen *Toxoplasma gondii* ." Acta Trop 81(2): 111-122.
Carruthers, V. B., et al. (1999). "Secretion of micronemal proteins is associated with toxoplasma invasion of host cells." Cell Microbiol 1(3): 225-235.
Carruthers, V. B. and L. D. Sibley (1997). "Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts." Eur J Cell Biol 73(2): 114-123.
Casado, M., et al. (1997). "Expression of the calcium-independent cytokine-inducible (iNOS) isoform of nitric oxide synthase in rat placenta." Biochem J 324 (Pt 1): 201-207.
Chang, C. I., et al. (1998). "Arginase modulates nitric oxide production in activated macrophages." Am J Physiol 274(1 Pt 2): H342-348.
Chartrain, N. A., et al. (1994). "Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene." J Biol Chem 269(9): 6765-6772.
Chavez-Galan, L., et al. (2015). "Much More than M1 and M2 Macrophages, There are also CD169(+) and TCR(+) Macrophages." Front Immunol 6: 263.
Chorro, L., et al. (2009). "Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network." J Exp Med 206(13): 3089-3100.
Churchill, G. A. and R. W. Doerge (1994). "Empirical threshold values for quantitative trait mapping." Genetics 138(3): 963-971.
Coffey, M. J. and B. E. Sleebs (2015). "An aspartyl protease defines a novel pathway for export of Toxoplasma proteins into the host cell." 4.

Cong, L., et al. (2013). "Multiplex genome engineering using CRISPR/Cas systems." Science 339(6121): 819-823.
Cook, T., et al. (2007). "Divergent polyamine metabolism in the Apicomplexa." Microbiology 153(Pt 4): 1123-1130.
Cooper, E. L. (2010). "Evolution of immune systems from self/not self to danger to artificial immune systems (AIS)." Phys Life Rev 7(1): 55-78.
Cooper, M. D. and M. N. Alder (2006). "The evolution of adaptive immune systems." Cell 124(4): 815-822.
Cooper, S. J. (2008). "From Claude Bernard to Walter Cannon. Emergence of the concept of homeostasis." Appetite 51(3): 419-427.
Courret, N., et al. (2006). "CD11c- and CD11b-expressing mouse leukocytes transport single *Toxoplasma gondii* tachyzoites to the brain." Blood 107(1): 309-316.
Crocker, P. R. and S. Gordon (1986). "Properties and distribution of a lectin-like hemagglutinin differentially expressed by murine stromal tissue macrophages." J Exp Med 164(6): 1862-1875.
Cunningham, T. (1982). "Pancarditis in acute toxoplasmosis." Am J Clin Pathol 78(3): 403-405.
Curthoys, N. P. and M. Watford (1995). "Regulation of glutaminase activity and glutamine metabolism." Annu Rev Nutr 15: 133-159.
Curt-Varesano, A., et al. (2016). "The aspartyl protease TgASP5 mediates the export of the Toxoplasma GRA16 and GRA24 effectors into host cells." Cell Microbiol 18(2): 151-167.
da Cruz Padrão, J., et al. (2014). "*Toxoplasma gondii* infection of activated J774-A1 macrophages causes inducible nitric oxide synthase degradation by the proteasome pathway." Parasitology international 63(5): 659-663.
Da Gama, L. M., et al. (2004). "Reduction in adhesiveness to extracellular matrix components, modulation of adhesion molecules and in vivo migration of murine macrophages infected with *Toxoplasma gondii* ." Microbes and Infection 6(14): 1287-1296.
Daffos, F., et al. (1988). "Prenatal management of 746 pregnancies at risk for congenital toxoplasmosis." N Engl J Med 318(5): 271-275.
Darnell, J. E., Jr., et al. (1994). "Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins." Science 264(5164): 1415-1421.
de Koning-Ward, T. F., et al. (2009). "A newly discovered protein export machine in malaria parasites." Nature 459(7249): 945-949.
del Rio-Hortega, P. (1932). Cytology & [and] Cellular Pathology of the Nervous System, Hoeber.
Demar, M., et al. (2012). "Acute toxoplasmoses in immunocompetent patients hospitalized in an intensive care unit in French Guiana." Clinical Microbiology and Infection 18(7): E221-E231.
Desmonts, G. (1982). "Acquired toxoplasmosis in pregnant women. evaluation of the frequency of transmission of Toxoplasma and of congenital toxoplasmosis." Lyon Med 248: 115-123.

Di Cristina, M. and V. B. Carruthers (2018). "New and emerging uses of CRISPR/Cas9 to genetically manipulate apicomplexan parasites." Parasitology 145(9): 1119-1126.
DiCarlo, J. E., et al. (2013). "Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems." Nucleic Acids Res 41(7): 4336-4343.
Dizikes, G. J., et al. (1986). "Isolation of human liver arginase cDNA and demonstration of nonhomology between the two human arginase genes." Biochem Biophys Res Commun 141(1): 53-59.
Dobrowolski, J. M. and L. D. Sibley (1996). "Toxoplasma invasion of mammalian cells is powered by the actin cytoskeleton of the parasite." Cell 84(6): 933-939.
Donald, R. G. and D. S. Roos (1993). "Stable molecular transformation of *Toxoplasma gondii* : a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria." Proc Natl Acad Sci U S A 90(24): 11703-11707.
Dowling, D. P., et al. (2008). "Evolution of the arginase fold and functional diversity." Cell Mol Life Sci 65(13): 2039-2055.
Downs, J. A. and S. P. Jackson (2004). "A means to a DNA end: the many roles of Ku." Nat Rev Mol Cell Biol 5(5): 367-378.
Drapier, J. C. and J. B. Hibbs, Jr. (1986). "Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron-sulfur prosthetic group and is reversible." J Clin Invest 78(3): 790-797.
Dubey, J. P., et al. (2007). "Molecular and biological characterization of *Toxoplasma gondii* isolates from free-range chickens from Guyana, South America, identified several unique and common parasite genotypes." Parasitology 134(Pt 11): 1559-1565.
Dubey, J. P. and C. Beattie (1988). Toxoplasmosis of animals and man, CRC Press, Inc.
Dubey, J. P. and J. K. Frenkel (1972). "Cyst-induced toxoplasmosis in cats." J Protozool 19(1): 155-177.
Dubey, J. P., et al. (2012). "Toxoplasmosis in humans and animals in Brazil: high prevalence, high burden of disease, and epidemiology." Parasitology 139(11): 1375-1424.
Dubey, J. P., et al. (1998). "Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts." Clin Microbiol Rev 11(2): 267-299.
Dubey, J. P., et al. (1970). "The *Toxoplasma gondii* oocyst from cat feces." J Exp Med 132(4): 636-662.
Eckersten, D. and R. Henningsson (2012). "Nitric oxide (NO)--production and regulation of insulin secretion in islets of freely fed and fasted mice." Regul Pept 174(1-3): 32-37.
El Kasmi, K. C., et al. (2008). "Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens." Nat Immunol 9(12): 1399-1406.
Etheridge, R. D., et al. (2014). "The *Toxoplasma* pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice." Cell Host Microbe 15(5): 537-550.

Evans, T. J., et al. (1996). "Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria." Proc Natl Acad Sci U S A 93(18): 9553-9558.
Fang, F. C. (2004). "Antimicrobial reactive oxygen and nitrogen species: concepts and controversies." Nat Rev Microbiol 2(10): 820-832.
Fayer, R. (1972). "Penetration of cultured cells by Eimeria meleagrimitis and E. tenella sporozoites." J Parasitol 58(5): 921-927.
Fentress, S. J., et al. (2010). "Phosphorylation of immunity-related GTPases by a *Toxoplasma gondii* -secreted kinase promotes macrophage survival and virulence." Cell Host Microbe 8(6): 484-495.
Ferguson, D. J. (2004). "Use of molecular and ultrastructural markers to evaluate stage conversion of *Toxoplasma gondii* in both the intermediate and definitive host." Int J Parasitol 34(3): 347-360.
Fleetwood, A. J., et al. (2007). "Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation." J Immunol 178(8): 5245-5252.
Flynn, N. E., et al. (2002). "The metabolic basis of arginine nutrition and pharmacotherapy." Biomed Pharmacother 56(9): 427-438.
Fox, B. A., et al. (2011). "Type II *Toxoplasma gondii* KU80 knockout strains enable functional analysis of genes required for cyst development and latent infection." Eukaryot Cell 10(9): 1193-1206.
Fox, B. A., et al. (2004). "*Toxoplasma gondii* lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation." Int J Parasitol 34(3): 323-331.
Fox, B. A., et al. (2009). "Efficient gene replacements in *Toxoplasma gondii* strains deficient for nonhomologous end joining." Eukaryot Cell 8(4): 520-529.
Franco, M., et al. (2016). "A Novel Secreted Protein, MYR1, Is Central to Toxoplasma's Manipulation of Host Cells." MBio 7(1): e02231-02215.
Frenkel, J. (1973). "*Toxoplasma* in and around us." Bioscience 23(6): 343-352.
Frenkel, J. K., et al. (1970). "*Toxoplasma gondii* in cats: fecal stages identified as coccidian oocysts." Science 167(3919): 893-896.
Gao, J., et al. (1997). "An interferon-gamma-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-gamma and lipopolysaccharide." J Biol Chem 272(2): 1226-1230.
Gautier, E. L., et al. (2012). "Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages." Nat Immunol 13(11): 1118-1128.
Gay, G. and L. Braun (2016). "*Toxoplasma gondii* TgIST co-opts host chromatin repressors dampening STAT1-dependent gene regulation and IFN-gamma-mediated host defenses." 213(9): 1779-1798.
Geissmann, F., et al. (2010). "Development of monocytes, macrophages, and dendritic cells." Science 327(5966): 656-661.
Ghafourifar, P. and C. Richter (1997). "Nitric oxide synthase activity in mitochondria." FEBS Lett 418(3): 291-296.

Ghorbal, M., et al. (2014). "Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system." Nat Biotechnol 32(8): 819-821.
Ginhoux, F., et al. (2010). "Fate mapping analysis reveals that adult microglia derive from primitive macrophages." Science 330(6005): 841-845.
Gobert, A. P., et al. (2000). "L-Arginine availability modulates local nitric oxide production and parasite killing in experimental trypanosomiasis." Infect Immun 68(8): 4653-4657.
Gold, D. A., et al. (2015). "The Toxoplasma Dense Granule Proteins GRA17 and GRA23 Mediate the Movement of Small Molecules between the Host and the Parasitophorous Vacuole." Cell Host Microbe 17(5): 642-652.
Goldman, M., et al. (1958). "Reproduction of *Toxoplasma gondii* by internal budding." J Parasitol 44(2): 161-171.
Gomez Perdiguero, E., et al. (2015). "Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors." Nature 518(7540): 547-551.
Greenlee, J. E., et al. (1975). "Adult toxoplasmosis presenting as polymyositis and cerebellar ataxia." Ann Intern Med 82(3): 367-371.
Grzybek, M., et al. (2018). "The CRISPR/Cas9 system sheds new lights on the biology of protozoan parasites." Appl Microbiol Biotechnol 102(11): 4629-4640.
Hakansson, S., et al. (2001). "Toxoplasma evacuoles: a two-step process of secretion and fusion forms the parasitophorous vacuole." Embo j 20(12): 3132-3144.
Hakimi, M. A., et al. (2017). "Toxoplasma Effectors Targeting Host Signaling and Transcription." Clin Microbiol Rev 30(3): 615-645.
Hammoudi, P. M., et al. (2015). "Fundamental Roles of the Golgi-Associated Toxoplasma Aspartyl Protease, ASP5, at the Host-Parasite Interface." PLoS Pathog 11(10): e1005211.
Hashimoto, D., et al. (2013). "Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes." Immunity 38(4): 792-804.
Heby, O. (1981). "Role of polyamines in the control of cell proliferation and differentiation." Differentiation 19(1): 1-20.
Henrique Seabra, S., et al. (2004). "Endogenous polyamine levels in macrophages is sufficient to support growth of *Toxoplasma gondii* ." Journal of Parasitology 90(3): 455-460
Herzfeld, A. and S. M. Raper (1976). "The heterogeneity of arginases in rat tissues." Biochem J 153(2): 469-478.
Hill, D. and J. Dubey (2014). "Toxoplasmosis: Reston, Va." US Geological Survey Circular 1389(2014): 84.
Hiller, N. L., et al. (2004). "A host-targeting signal in virulence proteins reveals a secretome in malarial infection." Science 306(5703): 1934-1937.
Holland, GN.; Wilhelmus, KR., editors. Ocular Infection and Immunity. St. Louis: Mosby-Year Book; 1996. p. 1183-1223.
Hoppe, H. C., et al. (2000). "Targeting to rhoptry organelles of *Toxoplasma gondii* involves evolutionarily conserved mechanisms." Nat Cell Biol 2(7): 449-456.

Howe, D. K. and L. D. Sibley (1995). "*Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease." J Infect Dis 172(6): 1561-1566.
Howe, D. K., et al. (1996). "Acute virulence in mice is associated with markers on chromosome VIII in *Toxoplasma gondii* ." Infect Immun 64(12): 5193-5198
Hu, K., et al. (2002). "A novel polymer of tubulin forms the conoid of *Toxoplasma gondii* ." J Cell Biol 156(6): 1039-1050.
Hu, X. and L. B. Ivashkiv (2009). "Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases." Immunity 31(4): 539-550.
Hughes, J. M., and D. G. Colley. 2000. Preventing congenital toxoplasmosis. Morb. Mortal. Wkly. Rep. 49(RR02):57-75.
Hume, D. A. (2008). "Differentiation and heterogeneity in the mononuclear phagocyte system." Mucosal Immunol 1(6): 432-441.
Hume, D. A. and S. Gordon (1983). "Mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. Identification of resident macrophages in renal medullary and cortical interstitium and the juxtaglomerular complex." J Exp Med 157(5): 1704-1709.
Hunn, J. P., et al. (2011). "The immunity-related GTPases in mammals: a fast-evolving cell-autonomous resistance system against intracellular pathogens." Mamm Genome 22(1-2): 43-54.
Hunter, C. A. and L. D. Sibley (2012). "Modulation of innate immunity by *Toxoplasma gondii* virulence effectors." Nat Rev Microbiol 10(11): 766-778.
Huynh, M. H. and V. B. Carruthers (2009). "Tagging of endogenous genes in a *Toxoplasma gondii* strain lacking Ku80." Eukaryot Cell 8(4): 530-539.
Ignarro, L. J., et al. (1987). "Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide." Proc Natl Acad Sci U S A 84(24): 9265-9269.
Ishino, Y., et al. (1987). "Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product." J Bacteriol 169(12): 5429-5433.
Jansen, R., et al. (2002). "Identification of genes that are associated with DNA repeats in prokaryotes." Mol Microbiol 43(6): 1565-1575.
Jenkins, S. J., et al. (2013). "IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1." J Exp Med 210(11): 2477-2491.
Jensen, K. D., et al. (2011). "Toxoplasma polymorphic effectors determine macrophage polarization and intestinal inflammation." Cell Host Microbe 9(6): 472-483.
Jinek, M., et al. (2012). "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." Science 337(6096): 816-821.
Jones, J., et al. (2003). "Congenital toxoplasmosis." Am Fam Physician 67(10): 2131-2138.
Jones, J. L. and J. P. Dubey (2010). "Waterborne toxoplasmosis--recent developments." Exp Parasitol 124(1): 10-25.

Jones, J. L., et al. (2007). "*Toxoplasma gondii* infection in the United States, 1999–2004, decline from the prior decade." The American journal of tropical medicine and hygiene 77(3): 405-410.
Jones, N. G., et al. (2017). "Secreted protein kinases regulate cyst burden during chronic toxoplasmosis." Cell Microbiol 19(2).
Jones, T. C., et al. (1972). "The interaction between *Toxoplasma gondii* and mammalian cells. I. Mechanism of entry and intracellular fate of the parasite." J Exp Med 136(5): 1157-1172.
Kamijo, R., et al. (1993). "Generation of nitric oxide and induction of major histocompatibility complex class II antigen in macrophages from mice lacking the interferon gamma receptor." Proc Natl Acad Sci U S A 90(14): 6626-6630.
Kanyo, Z. F., et al. (1996). "Structure of a unique binuclear manganese cluster in arginase." Nature 383(6600): 554-557.
Kappe, S., et al. (1999). "Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites." J Cell Biol 147(5): 937-944.
Kappe, S. H., et al. (2004). "Apicomplexan gliding motility and host cell invasion: overhauling the motor model." Trends Parasitol 20(1): 13-16.
Kawai, T., et al. (1999). "Unresponsiveness of MyD88-deficient mice to endotoxin." Immunity 11(1): 115-122.
Keeley, A. and D. Soldati (2004). "The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa." Trends Cell Biol 14(10): 528-532.
Kepka-Lenhart, D., et al. (2000). "Arginase I: a limiting factor for nitric oxide and polyamine synthesis by activated macrophages?" Am J Physiol Regul Integr Comp Physiol 279(6): R2237-2242.
Khaminets, A., et al. (2010). "Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole." Cell Microbiol 12(7): 939-961.
Khan, I. A., et al. (1997). "A dichotomous role for nitric oxide during acute *Toxoplasma gondii* infection in mice." Proc Natl Acad Sci U S A 94(25): 13955-13960.
Kim, K., et al. (1993). "Gene replacement in *Toxoplasma gondii* with chloramphenicol acetyltransferase as selectable marker." Science 262(5135): 911-914.
Kim, S. K., et al. (2007). "*Toxoplasma gondii* dysregulates IFN-gamma-inducible gene expression in human fibroblasts: insights from a genome-wide transcriptional profiling." J Immunol 178(8): 5154-5165.
Kishimoto, J., et al. (1992). "Localization of brain nitric oxide synthase (NOS) to human chromosome 12." Genomics 14(3): 802-804.
Klatt, P., et al. (1996). "Determination of nitric oxide synthase cofactors: heme, FAD, FMN, and tetrahydrobiopterin." Methods Enzymol 268: 358-365.
Kossel, A. and H. Dakin (1904). "Über die Arginase." Hoppe-Seyler´ s Zeitschrift für physiologische Chemie 41(4): 321-331.
Koshland DE Jr. The molecule of the year. Science. 1992 258(5090):1861, PMID: 147090)
Kramer, O. H. and T. Heinzel (2010). "Phosphorylation-acetylation switch in the regulation of STAT1 signaling." Mol Cell Endocrinol 315(1-2): 40-48.

Krebs, H. A. and K. Henseleit (1932). "Untersuchungen uber die Harnstoffbildung im Tierkörper." Hoppe-Seyler s Zeitschrift für physiologische Chemie 210(1-2): 33-66.
Kuriyama, K. and S. Ohkuma (1995). "Role of nitric oxide in central synaptic transmission: effects on neurotransmitter release." Jpn J Pharmacol 69(1): 1-8.
Landau, G., et al. (2010). "The role of polyamines in supporting growth of mammalian cells is mediated through their requirement for translation initiation and elongation." J Biol Chem 285(17): 12474-12481.
Lander, N., et al. (2015). "CRISPR/Cas9-Induced Disruption of Paraflagellar Rod Protein 1 and 2 Genes in Trypanosoma cruzi Reveals Their Role in Flagellar Attachment." MBio 6(4): e01012.
Lavin, Y., et al. (2014). "Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment." Cell 159(6): 1312-1326.
Lebrun, M., et al. (2005). "The rhoptry neck protein RON4 re-localizes at the moving junction during *Toxoplasma gondii* invasion." Cell Microbiol 7(12): 1823-1833.
Lecordier, L., et al. (1999). "Transmembrane insertion of the *Toxoplasma gondii* GRA5 protein occurs after soluble secretion into the host cell." Mol Biol Cell 10(4): 1277-1287.
Lekutis, C., et al. (2001). "Surface antigens of *Toxoplasma gondii* : variations on a theme." Int J Parasitol 31(12): 1285-1292.
Li, G., et al. (2009). "The evolutionarily dynamic IFN-inducible GTPase proteins play conserved immune functions in vertebrates and cephalochordates." Mol Biol Evol 26(7): 1619-1630.
Li, Z., et al. (2017). "Decidual Macrophage Functional Polarization during Abnormal Pregnancy due to *Toxoplasma gondii :* Role for LILRB4." Front Immunol 8: 1013.
Li, Z., et al. (2012). "Differences in iNOS and arginase expression and activity in the macrophages of rats are responsible for the resistance against T. gondii infection." PLoS One 7(4): e35834.
Lichanska, A. M., et al. (1999). "Differentiation of the mononuclear phagocyte system during mouse embryogenesis: the role of transcription factor PU.1." Blood 94(1): 127-138.
Lingelbach, K. and K. A. Joiner (1998). "The parasitophorous vacuole membrane surrounding Plasmodium and Toxoplasma: an unusual compartment in infected cells." J Cell Sci 111 (Pt 11): 1467-1475.
Lipton, S. A., et al. (1993). "A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds." Nature 364(6438): 626-632.
Long, S., et al. (2017). "A conserved ankyrin repeat-containing protein regulates conoid stability, motility and cell invasion in *Toxoplasma gondii* ." 8(1): 2236.
Lopatin, A. N., et al. (1994). "Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification." Nature 372(6504): 366-369.
Lorenz, E., et al. (2002). "Toll-like receptor 4 (TLR4)-deficient murine macrophage cell line as an in vitro assay system to show TLR4-independent signaling of Bacteroides fragilis lipopolysaccharide." Infect Immun 70(9): 4892-4896.
Lowenstein, C. J., et al. (1993). "Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide." Proc Natl Acad Sci U S A 90(20): 9730-9734.

Lowenstein, C. J. and E. Padalko (2004). "iNOS (NOS2) at a glance." J Cell Sci 117(Pt 14): 2865-2867.
Luft, B. J. and J. S. Remington (1992). "Toxoplasmic encephalitis in AIDS." Clin Infect Dis 15(2): 211-222.
Lykins, J., et al. (2016). "Understanding Toxoplasmosis in the United States Through "Large Data" Analyses." Clin Infect Dis 63(4): 468-475.
Ma, D. and F. Liu (2015). "Genome Editing and Its Applications in Model Organisms." Genomics Proteomics Bioinformatics 13(6): 336-344.
MacMicking, J., et al. (1997). "Nitric oxide and macrophage function." Annu Rev Immunol 15: 323-350.
Maeno, Y., et al. (1990). "A study on the vital reaction in wounded skin: simultaneous determination of histamine and polyamines in injured rat skin by high-performance liquid chromatography." Forensic Sci Int 46(3): 255-268.
Makhijani, K. and K. Bruckner (2012). "Of blood cells and the nervous system: hematopoiesis in the Drosophila larva." Fly (Austin) 6(4): 254-260.
Mali, P. and K. M. Esvelt (2013). "Cas9 as a versatile tool for engineering biology." 10(10): 957-963.
Mantovani, A., et al. (2006). "Role of tumor-associated macrophages in tumor progression and invasion." Cancer Metastasis Rev 25(3): 315-322.
Mao, Z., et al. (2008). "Comparison of nonhomologous end joining and homologous recombination in human cells." DNA Repair (Amst) 7(10): 1765-1771.
Marraffini, L. A. (2015). "CRISPR-Cas immunity in prokaryotes." Nature 526(7571): 55-61.
Marsden, P. A., et al. (1993). "Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene." J Biol Chem 268(23): 17478-17488.
Martin, A. M., et al. (2007). "The *Toxoplasma gondii* parasitophorous vacuole membrane: transactions across the border." J Eukaryot Microbiol 54(1): 25-28.
Martinez, F. O. and S. Gordon (2014). "The M1 and M2 paradigm of macrophage activation: time for reassessment." F1000Prime Rep 6: 13.
Martinez, F. O., et al. (2008). "Macrophage activation and polarization." Front Biosci 13: 453-461.
McCabe, R. E., et al. (1987). "Clinical spectrum in 107 cases of toxoplasmic lymphadenopathy." Rev Infect Dis 9(4): 754-774.
Mebius, R. E. and G. Kraal (2005). "Structure and function of the spleen." Nat Rev Immunol 5(8): 606-616.
Mercier, C., et al. (2005). "Dense granules: are they key organelles to help understand the parasitophorous vacuole of all apicomplexa parasites?" Int J Parasitol 35(8): 829-849.
Metchnikoff, E. (1883). "Untersuchungen über die mesodermalen Phagocyten einiger Wirbeltiere." Biol Zentralbl 3: 560-565.
Metchnikoff, E. (1892). Leçons sur la pathologie comparée de l'inflammation: faites à l'Institut Pasteur en avril et mai 1891, G. Masson.
Mills, C. D., et al. (2000). "M-1/M-2 macrophages and the Th1/Th2 paradigm." J Immunol 164(12): 6166-6173.
Mills, C. D. and K. Ley (2014). "M1 and M2 macrophages: the chicken and the egg of immunity." J Innate Immun 6(6): 716-726.

Mineo, J. R. and L. H. Kasper (1994). "Attachment of *Toxoplasma gondii* to host cells involves major surface protein, SAG-1 (P30)." Exp Parasitol 79(1): 11-20.
Molestina, R. E. and A. P. Sinai (2005). "Detection of a novel parasite kinase activity at the *Toxoplasma gondii* parasitophorous vacuole membrane capable of phosphorylating host IkappaBalpha." Cell Microbiol 7(3): 351-362.
Moncada, S. (1999). "Nitric oxide: discovery and impact on clinical medicine." J R Soc Med 92(4): 164-169.
Montoya, J. G. and O. Liesenfeld (2004). "Toxoplasmosis." Lancet 363(9425): 1965-1976.
Montoya, J. G. and J. S. Remington (1996). "Toxoplasmic chorioretinitis in the setting of acute acquired toxoplasmosis." Clin Infect Dis 23(2): 277-282.
Moore, J. K. and J. E. Haber (1996). "Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae." Mol Cell Biol 16(5): 2164-2173.
Morisaki, J. H., et al. (1995). "Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell." J Cell Sci 108 (Pt 6): 2457-2464.
Morris, S. M., Jr. (2007). "Arginine metabolism: boundaries of our knowledge." J Nutr 137(6 Suppl 2): 1602s-1609s.
Morris, S. M., Jr., et al. (1997). "Human type II arginase: sequence analysis and tissue-specific expression." Gene 193(2): 157-161.
Mosmann, T. R., et al. (1986). "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." J Immunol 136(7): 2348-2357.
Munder, M., et al. (1999). "Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells." J Immunol 163(7): 3771-3777.
Murohara, T., et al. (1998). "Nitric oxide synthase modulates angiogenesis in response to tissue ischemia." J Clin Invest 101(11): 2567-2578.
Murray, H. and Z. Cohn (1980). "Macrophage oxygen-dependent antimicrobial activity. III. Enhanced oxidative metabolism as an expression of macrophage activation." Journal of Experimental medicine 152(6): 1596-1609.
Murray, H. W. and Z. A. Cohn (1979). "Macrophage oxygen-dependent antimicrobial activity. I. Susceptibility of *Toxoplasma gondii* to oxygen intermediates." J Exp Med 150(4): 938-949.
Murray, P. J. (2011). "Macrophages as a battleground for Toxoplasma pathogenesis." Cell Host Microbe 9(6): 445-447.
Nathan, C. and M. U. Shiloh (2000). "Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens." Proc Natl Acad Sci U S A 97(16): 8841-8848.
Nathan, C. F., et al. (1983). "Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity." J Exp Med 158(3): 670-689.
NICHOLS, B. A. and M. L. CHIAPPINO (1987). "Cytoskeleton of *Toxoplasma gondii* 1." J Protozool 34(2): 217-226.
Nichols, W. K. and F. H. Prosser (1980). "Induction of ornithine decarboxylase in macrophages by bacterial lipopolysaccharides (LPS) and mycobacterial cell wall material." Life Sci 27(11): 913-920.

Nicole, C. and L. Manceaux (1908). "Sur une infection corps de Leishman (ou organisms voisins) du gondii: on an infection by Leishman bodies in the gondii." Compt rend acad de sc 147: 763-766.
Nicolle, C. (1909). "C. Nicolle and L. Manceaux, Sur un protozoaire nouveau du gondi." CR Acad. Sci 148: 369.
Nussenblatt, R. B. and R. Belfort, Jr. (1994). "Ocular toxoplasmosis. An old disease revisited." Jama 271(4): 304-307.
Olias, P., et al. (2016). "Toxoplasma Effector Recruits the Mi-2/NuRD Complex to Repress STAT1 Transcription and Block IFN-gamma-Dependent Gene Expression." Cell Host Microbe 20(1): 72-82.
Orkin, S. H. and L. I. Zon (2008). "Hematopoiesis: an evolving paradigm for stem cell biology." Cell 132(4): 631-644.
Ossorio, P. N., et al. (1994). "A soluble secretory protein of the intracellular parasite *Toxoplasma gondii* associates with the parasitophorous vacuole membrane through hydrophobic interactions." J Biol Chem 269(21): 15350-15357.
Ouzounis, C. A. and N. C. Kyrpides (1994). "On the evolution of arginases and related enzymes." J Mol Evol 39(1): 101-104.
Palmer, R. M., et al. (1987). "Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor." Nature 327(6122): 524-526.
Palmieri, L., et al. (2001). "Citrin and aralar1 are Ca(2+)-stimulated aspartate/glutamate transporters in mitochondria." Embo j 20(18): 5060-5069.
Pappas, G., et al. (2009). "Toxoplasmosis snapshots: global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis." Int J Parasitol 39(12): 1385-1394.
Pawlowski, J., et al. (2012). "CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms." PLoS Biol 10(11): e1001419.
Perrella, M. A., et al. (1999). "High mobility group-I(Y) protein facilitates nuclear factor-kappaB binding and transactivation of the inducible nitric-oxide synthase promoter/enhancer." J Biol Chem 274(13): 9045-9052.
Pfaff, A. W., et al. (2005). "Regulation of *Toxoplasma gondii* multiplication in BeWo trophoblast cells: cross-regulation of nitric oxide production and polyamine biosynthesis." Int J Parasitol 35(14): 1569-1576.
Pfefferkorn, E. R. and L. H. Kasper (1983). "*Toxoplasma gondii* : genetic crosses reveal phenotypic suppression of hydroxyurea resistance by fluorodeoxyuridine resistance." Exp Parasitol 55(2): 207-218.
Pfefferkorn, L. C. and E. Pfefferkorn (1980). "*Toxoplasma gondii* : genetic recombination between drug resistant mutants." Exp Parasitol 50(3): 305-316.
Poltorak, A., et al. (1998). "Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region." Blood Cells Mol Dis 24(3): 340-355.
Puellmann, K., et al. (2006). "A variable immunoreceptor in a subpopulation of human neutrophils." Proc Natl Acad Sci U S A 103(39): 14441-14446.
Recalcati, S., et al. (2010). "Differential regulation of iron homeostasis during human macrophage polarized activation." Eur J Immunol 40(3): 824-835.

Reczkowski, R. S. and D. E. Ash (1992). "EPR evidence for binuclear manganese(II) centers in rat liver arginase." Journal of the American Chemical Society 114(27): 10992-10994.
Reese, M. L., et al. (2014). "The Toxoplasma pseudokinase ROP5 is an allosteric inhibitor of the immunity-related GTPases." J Biol Chem 289(40): 27849-27858.
Reese, M. L., et al. (2011). "Polymorphic family of injected pseudokinases is paramount in Toxoplasma virulence." Proc Natl Acad Sci U S A 108(23): 9625-9630.
Remington, J., et al. (2001). "Infectious diseases of the fetus and newborn infant." Journal of Perinatology 21(8): 571-571.
Remington, J. S. (1974). "Toxoplasmosis in the adult." Bull N Y Acad Med 50(2): 211-227.
Roholt, O. A., Jr. and D. M. Greenberg (1956). "Liver arginase. IV. Effect of pH on kinetics of manganese-activated enzyme." Arch Biochem Biophys 62(2): 454-470.
Roos, D. S., et al. (1994). "Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii* ." Methods Cell Biol 45: 27-63.
Rosowski, E. E., et al. (2011). "Strain-specific activation of the NF-kappaB pathway by GRA15, a novel *Toxoplasma gondii* dense granule protein." J Exp Med 208(1): 195-212.
Rosowski, E. E., et al. (2014). "*Toxoplasma gondii* Inhibits gamma interferon (IFN-gamma)- and IFN-beta-induced host cell STAT1 transcriptional activity by increasing the association of STAT1 with DNA." Infect Immun 82(2): 706-719.
Russo, I., et al. (2010). "Plasmepsin V licenses Plasmodium proteins for export into the host erythrocyte." Nature 463(7281): 632-636.
Sabin, A. B. and P. K. Olitsky (1937). "TOXOPLASMA AND OBLIGATE INTRACELLULAR PARASITISM." Science 85(2205): 336-338.
Sadak, A., et al. (1988). "Characterization of a family of rhoptry proteins of *Toxoplasma gondii* ." Mol Biochem Parasitol 29(2-3): 203-211.
Saeij, J. P., et al. (2006). "Polymorphic secreted kinases are key virulence factors in toxoplasmosis." Science 314(5806): 1780-1783.
Saeij, J. P., et al. (2005). "Bioluminescence imaging of *Toxoplasma gondii* infection in living mice reveals dramatic differences between strains." Infect Immun 73(2): 695-702.
Saeij, J. P., et al. (2007). "Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue." Nature 445(7125): 324-327.
Saini, R., et al. (2006). "Nitric oxide synthase localization in the rat neutrophils: immunocytochemical, molecular, and biochemical studies." J Leukoc Biol 79(3): 519-528.
Samokhvalov, I. M. (2014). "Deconvoluting the ontogeny of hematopoietic stem cells." Cell Mol Life Sci 71(6): 957-978.
Scallan, E., et al. (2011). "Foodborne illness acquired in the United States--major pathogens." Emerg Infect Dis 17(1): 7-15.
Scharton-Kersten, T. M., et al. (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.

Schneider, A. G., et al. (2013). "*Toxoplasma gondii* triggers phosphorylation and nuclear translocation of dendritic cell STAT1 while simultaneously blocking IFNgamma-induced STAT1 transcriptional activity." PLoS One 8(3): e60215.
Schulz, C., et al. (2012). "A lineage of myeloid cells independent of Myb and hematopoietic stem cells." Science 336(6077): 86-90.
Seabra, S. H., et al. (2002). "*Toxoplasma gondii* partially inhibits nitric oxide production of activated murine macrophages." Exp Parasitol 100(1): 62-70.
Seabra, S. H., et al. (2004). "*Toxoplasma gondii* exposes phosphatidylserine inducing a TGF-β1 autocrine effect orchestrating macrophage evasion." Biochem Biophys Res Commun 324(2): 744-752.
Sehgal, A., et al. (2005). "Peculiarities of host cholesterol transport to the unique intracellular vacuole containing Toxoplasma." Traffic 6(12): 1125-1141.
Sen, R. and D. Baltimore (1986). "Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism." Cell 47(6): 921-928.
Shen, B., et al. (2014). "Efficient gene disruption in diverse strains of *Toxoplasma gondii* using CRISPR/CAS9." MBio 5(3): e01114-01114.
Shuai, K., et al. (1993). "A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma." Science 261(5129): 1744-1746.
Sibley, L., et al. (1991). "Tumor necrosis factor-alpha triggers antitoxoplasmal activity of IFN-gamma primed macrophages." The Journal of Immunology 147(7): 2340-2345.
Sibley, L. D. (2009). "Development of forward genetics in *Toxoplasma gondii* ." Int J Parasitol 39(8): 915-924.
Sibley, L. D. and J. W. Ajioka (2008). "Population structure of *Toxoplasma gondii* : clonal expansion driven by infrequent recombination and selective sweeps." Annu Rev Microbiol 62: 329-351.
Sibley, L. D. and J. C. Boothroyd (1992). "Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage." Nature 359(6390): 82-85.
Sibley, L. D., et al. (1992). "Generation of a restriction fragment length polymorphism linkage map for *Toxoplasma gondii* ." Genetics 132(4): 1003-1015.
Sibley, L. D., et al. (1985). "Phagosome acidification blocked by intracellular *Toxoplasma gondii* ." Nature 315(6018): 416-419.
Sidik, S. M., et al. (2014). "Efficient genome engineering of *Toxoplasma gondii* using CRISPR/Cas9." PLoS One 9(6): e100450.
Sinai, A. P. and K. A. Joiner (1997). "Safe haven: the cell biology of nonfusogenic pathogen vacuoles." Annu Rev Microbiol 51: 415-462.
Sleebs, B. E., et al. (2014). "Inhibition of Plasmepsin V activity demonstrates its essential role in protein export, PfEMP1 display, and survival of malaria parasites." PLoS Biol 12(7): e1001897.
Soldati, D. and J. C. Boothroyd (1993). "Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii* ." Science 260(5106): 349-352.
Sorokin, S. P., et al. (1992). "Macrophage development: II. Early ontogeny of macrophage populations in brain, liver, and lungs of rat embryos as revealed by a lectin marker." Anat Rec 232(4): 527-550.

Spillman, N. J., et al. (2015). "Protein export into malaria parasite-infected erythrocytes: mechanisms and functional consequences." Annu Rev Biochem 84: 813-841.
Splendore, A. (1908). "Un nuovo protozoa parassita de'conigli. incontrato nelle lesioni anatomiche d'une malattia che ricorda in molti punti il Kala-azar dell'uomo. Nota preliminare pel." Rev Soc Sci Sao Paulo 3: 109-112.
Stamler, J. S. (1994). "Redox signaling: nitrosylation and related target interactions of nitric oxide." Cell 78(6): 931-936.
Stein, M., et al. (1992). "Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation." J Exp Med 176(1): 287-292.
Steinfeldt, T., et al. (2010). "Phosphorylation of mouse immunity-related GTPase (IRG) resistance proteins is an evasion strategy for virulent *Toxoplasma gondii* ." PLoS Biol 8(12): e1000576.
Stuehr, D. J. and M. A. Marletta (1985). "Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide." Proc Natl Acad Sci U S A 82(22): 7738-7742.
Stuehr, D. J. and M. A. Marletta (1987). "Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma." J Immunol 139(2): 518-525.
Su, C., et al. (2002). "Identification of quantitative trait loci controlling acute virulence in *Toxoplasma gondii* ." Proc Natl Acad Sci U S A 99(16): 10753-10758.
Suss-Toby, E., et al. (1996). "Toxoplasma invasion: the parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fission pore." Proc Natl Acad Sci U S A 93(16): 8413-8418.
Suzuki, Y., et al. (1989). "Importance of endogenous IFN-gamma for prevention of toxoplasmic encephalitis in mice." J Immunol 143(6): 2045-2050.
Swantek, J. L., et al. (2000). "IL-1 receptor-associated kinase modulates host responsiveness to endotoxin." J Immunol 164(8): 4301-4306.
Tauber, A. I. (2003). "Metchnikoff and the phagocytosis theory." Nat Rev Mol Cell Biol 4(11): 897-901.
Taylor, P. R., et al. (2005). "Macrophage receptors and immune recognition." Annu Rev Immunol 23: 901-944.
Taylor, S., et al. (2006). "A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii* ." Science 314(5806): 1776-1780.
Taylor-Robinson, A. W., et al. (1993). "The role of TH1 and TH2 cells in a rodent malaria infection." Science 260(5116): 1931-1934.
Teixeira-Silva, A., et al. (2017). "The end-joining factor Ku acts in the end-resection of double strand break-free arrested replication forks." Nat Commun 8(1): 1982.
Tenter, A. M., et al. (2000). "*Toxoplasma gondii* : from animals to humans." Int J Parasitol 30(12-13): 1217-1258.
Tenu, J. P., et al. (1999). "Effects of the new arginase inhibitor N(omega)-hydroxy-nor-L-arginine on NO synthase activity in murine macrophages." Nitric Oxide 3(6): 427-438.
Thomas, D. D., et al. (2008). "The chemical biology of nitric oxide: implications in cellular signaling." Free Radic Biol Med 45(1): 18-31.

Thompson, L. H. and D. Schild (2001). "Homologous recombinational repair of DNA ensures mammalian chromosome stability." Mutat Res 477(1-2): 131-153.
Tjandrawinata, R. R., et al. (1994). "Regulation of putrescine export in lipopolysaccharide or IFN-gamma-activated murine monocytic-leukemic RAW 264 cells." J Immunol 152(6): 3039-3052.
Torpier, G., et al. (1993). "*Toxoplasma gondii* : differential location of antigens secreted from encysted bradyzoites." Exp Parasitol 77(1): 13-22.
Valdez, L. B., et al. (2005). "Functional activity of mitochondrial nitric oxide synthase." Methods Enzymol 396: 444-455.
van Furth, R. and Z. A. Cohn (1968). "The origin and kinetics of mononuclear phagocytes." J Exp Med 128(3): 415-435.
van Furth, R., et al. (1972). "The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells." Bull World Health Organ 46(6): 845-852.
Vivier, E. and A. Petitprez (1969). "[The outer membrane complex and its development at the time of the formation of daughter cells in *Toxoplasma gondii*]." J Cell Biol 43(2): 329-342.
Vockley, J. G., et al. (1996). "Cloning and characterization of the human type II arginase gene." Genomics 38(2): 118-123.
Vodovotz, Y., et al. (1995). "Vesicle membrane association of nitric oxide synthase in primary mouse macrophages." J Immunol 154(6): 2914-2925.
Volkman, A. and J. L. Gowans (1965). "THE PRODUCTION OF MACROPHAGES IN THE RAT." Br J Exp Pathol 46: 50-61.
Wallon, M. and F. Peyron (2018). "Congenital Toxoplasmosis: A Plea for a Neglected Disease." Pathogens 7(1).
Webb, J. L., et al. (2001). "Macrophage nitric oxide synthase associates with cortical actin but is not recruited to phagosomes." Infect Immun 69(10): 6391-6400.
Wei, L. H., et al. (2000). "IL-4 and IL-13 upregulate arginase I expression by cAMP and JAK/STAT6 pathways in vascular smooth muscle cells." American Journal of Physiology-Cell Physiology 279(1): C248-C256.
Weiss, L. M. and K. Kim (2000). "The development and biology of bradyzoites of *Toxoplasma gondii* ." Front Biosci 5: D391-405.
Weiss, L. M. and K. Kim (2011). *Toxoplasma gondii* : the model apicomplexan. Perspectives and methods, Elsevier.
Wilson, C. B., et al. (1980). "Development of adverse sequelae in children born with subclinical congenital Toxoplasma infection." Pediatrics 66(5): 767-774.
Windmueller, H. G. and A. E. Spaeth (1981). "Source and fate of circulating citrulline." Am J Physiol 241(6): E473-480.
Wolf, A., et al. (1939). "HUMAN TOXOPLASMOSIS: OCCURRENCE IN INFANTS AS AN ENCEPHALOMYELITIS VERIFICATION BY TRANSMISSION TO ANIMALS." Science 89(2306): 226-227.
Woods, S., et al. (2013). "MAP kinase phosphatase-2 plays a key role in the control of infection with *Toxoplasma gondii* by modulating iNOS and arginase-1 activities in mice." PLoS Pathog 9(8): e1003535.
Xie, Q. W., et al. (1992). "Cloning and characterization of inducible nitric oxide synthase from mouse macrophages." Science 256(5054): 225-228.

Xie, Q. W., et al. (1993). "Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide." J Exp Med 177(6): 1779-1784.
Yamamoto, M., et al. (2009). "A single polymorphic amino acid on *Toxoplasma gondii* kinase ROP16 determines the direct and strain-specific activation of Stat3." J Exp Med 206(12): 2747-2760.
Zaobornyj, T. and P. Ghafourifar (2012). "Strategic localization of heart mitochondrial NOS: a review of the evidence." Am J Physiol Heart Circ Physiol 303(11): H1283-1293.
Zhang, J., et al. (2011). "Ku80 gene is related to non-homologous end-joining and genome stability in Aspergillus niger." Curr Microbiol 62(4): 1342-1346.
Zhao, Y. O., et al. (2009). "Disruption of the *Toxoplasma gondii* parasitophorous vacuole by IFNgamma-inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death." PLoS Pathog 5(2): e1000288.