

Toxoplasma Polymorphic Effectors Determine Macrophage Polarization and Intestinal Inflammation

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SUMMARY

European and North American strains of the parasite *Toxoplasma gondii* belong to three distinct clonal lineages, type I, type II, and type III, which differ in virulence. Understanding the basis of *Toxoplasma* strain differences and how secreted effectors work to achieve chronic infection is a major goal of current research. Here we show that type I and III infected macrophages, a cell type required for host immunity to *Toxoplasma*, are alternatively activated, while type II infected macrophages are classically activated. The *Toxoplasma* rho-trypanin kinase ROP16, which activates STAT6, is responsible for alternative activation. The *Toxoplasma* dense granule protein GRA15, which activates NF- κ B, promotes classical activation by type II parasites. These effectors antagonistically regulate many of the same genes, and mice infected with type II parasites expressing type I ROP16 are protected against *Toxoplasma*-induced ileitis. Thus, polymorphisms in determinants that modulate macrophage activation influence the ability of *Toxoplasma* to establish a chronic infection.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite capable of infecting a wide range of mammalian hosts including humans. Most *Toxoplasma* strains isolated in Europe and North America belong to just three distinct clonal lineages, the type I, II, and III strains. These strains differ in virulence in mice and likely cause different sequelae in humans (Boothroyd and Grigg, 2002). *Toxoplasma* secretes effector molecules from two secretory organelles, rhoptries and dense granules, into the host cytosol which modulate host signaling pathways and dictate virulence (Reese et al., 2011; Rosowski et al., 2011; Saeij et al., 2006, 2007; Taylor et al., 2006). A major aim in the field is to understand the genetic basis underlying *Toxoplasma* strain differences in virulence and how secreted effector molecules work to achieve chronic infection for the parasite.

A possible strategy to establish a chronic infection is through the modulation of the host's Th1 response, which culminates in the production of IFN- γ , the main mediator of resistance against *Toxoplasma*. However, the Th1 response must also be tightly regulated, otherwise a lethal inflammatory response develops. For example, mice lacking the regulatory cytokines IL-10 or IL-27 die of severe immune pathology when infected with type II strains (Gazzinelli et al., 1996; Stumhofer et al., 2006). Th1 type cytokines (e.g., IFN- γ) synergize with pattern recognition receptors on macrophages to signal for the classical activation (or M1) of macrophages, which exert antimicrobial functions against intracellular pathogens and require the activity of NF- κ B, IRF, and C/EBP δ transcription factors (Medzhitov and Horng, 2009). Macrophages are necessary for host protection to *Toxoplasma* (Dunay et al., 2008), and the IL-12 response by infected macrophages is greatly influenced by the strain type (Robben et al., 2004). The molecular mechanism underlying parasite strain differences in proinflammatory cytokine induction by macrophages has not been resolved.

In contrast, alternatively activated macrophages (or M2) develop in a Th2 cytokine environment (IL-4, IL-13) and are inhibited by Th1 type cytokines (Martinez et al., 2009). M2 macrophages secrete anti-inflammatory molecules that can downregulate Th1 immune processes and are important in the immune response against worm infections. M2 activation is promoted by STAT6 and PPAR γ transcription factors (Odegaard et al., 2007), and mice deficient in IL-4, a potent mediator of M2 polarization, are more susceptible to acute *Toxoplasma* infection (Roberts et al., 1996). IL-4 treatment, as well as TLR stimulation (El Kasmi et al., 2008), alters L-arginine metabolism in macrophages through the induction of the arginase enzyme. L-arginine and the arginase catabolite ornithine can be scavenged by parasites to generate ATP (Cook et al., 2007) and assist their replication (Iniesta et al., 2001). Thus, the ability of *Toxoplasma*, or other protozoan parasites, to induce specific macrophage activation states could have immediate consequences on virulence, local parasite burden, and inflammatory-related pathologies.

Given that *Toxoplasma* elicits a strong Th1 response, it is not known whether the parasite actively counteracts collateral damage following inflammation or if there are strain differences in such a response. In this report, we show that mouse macrophages infected with type I and III *Toxoplasma* strains are

polarized toward an M2 activation state, while type II infected macrophages resemble aspects of M1 activation. This difference is due to polymorphisms in the *Toxoplasma* rhoptry kinase ROP16 and dense granule protein GRA15, which activate STAT6 and NF- κ B signaling pathways, respectively. Finally, type II strains that express ROP16 from the type I strain fail to cause intestinal inflammation in susceptible C57BL/6 (B6) mice. This report provides direct evidence that *Toxoplasma*-infected macrophages can be alternatively activated, and describes parasite-specific factors that work independently of pattern recognition receptors to achieve M1 or M2 activation.

RESULTS

***Toxoplasma* Type I and III Strains Induce M2 Activation, while the Type II Strain Induces M1-like Cells**

Toxoplasma strain differences in the modulation of host cells at the site of infection might lead to variation in their ability to disseminate, replicate, or survive. In agreement with an earlier report studying the type I and II strains (Mordue and Sibley, 2003), we observed that all three strains equally infected macrophages during the first 3 days of intraperitoneal (i.p.) infection, and this cell type accounted for approximately 60% of the total infected peritoneal exudate cells (PECs) (see Figure S1 available online). Neutrophils were the next most abundant cell type infected by *Toxoplasma* (15% of the total infected PECs). However, it was the macrophage that provided a niche permissive for parasite replication, because when compared to neutrophils they exhibited a higher GFP intensity (parasites express GFP) (Figure S1) and had more parasites per vacuole when FACS sorted and analyzed under a microscope (≥ 4 compared to 1 parasite per parasitophorous vacuole, macrophages versus neutrophils, data not shown). No differences were observed in cellular recruitment, or infectivity of these or other cell types, over the first 3 days of infection (Figure S1, data not shown).

These observations suggest that parasite differences in virulence might be due in part to how these strains modulate or interact with macrophages. To explore this further, two mouse macrophage cell lines, RAW264.7 and J774, were infected with either the type II or III strain, and gene expression profiles were analyzed by oligonucleotide arrays 18 hr after infection. Dendritic cells are an important link between the innate and adaptive T cell immune responses and can be infected by *Toxoplasma* in vivo (Bierly et al., 2008; John et al., 2009) (data not shown). Thus, the transcriptional response of the dendritic cell line DC2.4 (Shen et al., 1997) to *Toxoplasma* infection was also analyzed. A total of 1173 annotated genes were differentially regulated (2-fold expression level difference) between type II and III infections: 670 in RAW264.7 cells, 238 in J447 cells, and 930 in DC2.4 cells.

Bioinformatic analysis of these gene sets suggested that signaling pathways which intersect with NF- κ B (pattern recognition receptor signaling, TNF, IL-8, and CD40 signaling), IRF (cytosolic receptor and interferon signaling), JAK/STAT transcription factors (interferon and IL-10 signaling), and nuclear receptors (LXR/RXR and glucocorticoid receptors) mediate gene expression differences between type II and III infections (Figure S2). Furthermore, type II-induced genes were significantly enriched in TFBSs for NF- κ B, IRF, Ets, ATF/CREB, and CEBP/AP1 transcription factors, as well as several nuclear

receptors (Table S1). Generally these transcription factors are also activated in LPS-stimulated or M1 macrophages (Medzhitov and Horng, 2009). Conversely, type III-induced genes had significant enrichment for TFBSs of transcription factors involved in hematopoietic cell proliferation, survival, and differentiation (GATA1, E2F, and HOXA9), as well as for NFIL3, a transcription factor which is regulated by STAT6 (Schroder et al., 2002). These results suggest that macrophages and dendritic cells undergo distinct programming when infected by different *Toxoplasma* strain types. Interestingly, many of the genes that differed in expression between cells infected with the type II and III strains are also highly expressed in either M1 or M2 macrophages, respectively (Martinez et al., 2006) (Table 1). Thus, type II and III infected cells appear to have been activated differently, specifically along the M1/M2 axis.

To confirm these observations, a variety of in vitro assays were performed to determine the alternative activation state of macrophages: arginase-I activity, expression of the mannose receptor type C (Mrc1/CD206), and the macrophage galactose/N-acetylgalactosamine-specific lectin (mMgl or Mgl1/2). Macrophages infected with type I or III parasites, but not with type II parasites, had high arginase activity 24 hr after infection (Figure 1A). In general, type III infected macrophages had higher arginase activity than type I infected macrophages. Furthermore, approximately 50% of macrophages infected with either the type I or III strain, but not the type II strain, expressed high levels of CD206 and mMgl (Figure 1B). Experiments with the DC2.4 dendritic cell line produced essentially the same results (Figure 1C). Compared to RAW264.7 cells, the DC2.4 cell line was more sensitive to arginase induction, and under these conditions the type II strain induced a small but significant increase of arginase activity compared to noninfected cells.

In contrast, the type II strain induced the expression of genes that are associated with the M1 phenotype, including many proinflammatory cytokines (Table 1). As has been previously noted (Robben et al., 2004), compared to other strain types, the type II strain highly induces the expression of transcripts for the proinflammatory cytokines IL-1 β , IL-6, and IL-12p40/35 in infected bone marrow-derived macrophages (BMDMs). Our transcriptional profiling largely confirmed these results, and also suggested that *I/23* was consistently induced by the type II strain in all three cell lines tested (Table 1). In agreement with these observations, the type II strain, but not the type I or III strains, elicited a strong IL-23 (p40/p19) and IL-12 (p40/p35) response by infected BMDMs as determined by ELISA (Figure 1D). Not all aspects of M1 activation were observed in type II infected cells. For example, M1 cells are known to produce high amounts of reactive oxygen and nitrogen intermediates. Although we found iNOS message (*Nos2*) to be enriched in type II infected cells (Table 1), compared to LPS/IFN- γ -stimulated BMDMs, infection with the type I, II, or III strains generated little to no amounts of nitric oxide (NO) (data not shown). The inability of *Toxoplasma* to directly induce iNOS protein has similarly been observed (Mordue and Sibley, 2003).

***Toxoplasma* Rhoptry Kinase ROP16 Promotes M2 Activation**

Previously, using human foreskin fibroblasts (HFFs), we demonstrated that the type I and III strains, but not the type II strain,

Table 1. Macrophages and Dendritic Cell Lines Infected with Type II or Type III Parasites Have Gene Expression Profiles Consistent with either a Classical or an Alternative Activation Program

Gene Symbol	Type II versus Type III Fold Change				ALT/CLASS
	Raw	DC	J774	Average	
H2-Eb1 (MHCII)	8.0	18.7	6.2	11.0	CLASS/ALT
Ccr7	7.5	20.5	4.6	10.8	CLASS
Ccl5	14.5	12.2	2.7	9.8	CLASS
Cxcl10	4.0	8.1	2.2	4.8	CLASS
Cxcl11	4.6	3.3	3.4	3.8	CLASS
Ltb (Lymphotoxin B)	4.0	4.6	2.2	3.6	CLASS
Oasl2	2.3	4.6	2.9	3.3	CLASS
Irf7	4.3	3.2	1.9	3.2	CLASS
Traf1	2.6	3.9	2.6	3.1	CLASS
Ptgs2 (COX2)	3.2	2.5	2.3	2.6	CLASS
Tnf	3.1	2.3	2.6	2.6	CLASS
Il15ra	2.1	2.7	2.9	2.6	CLASS
Relb	1.3	4.5	1.8	2.5	CLASS
Cd40	1.6	2.2	3.5	2.4	CLASS
Nos2 (iNOS)	2.7	1.3	3.0	2.3	CLASS
Ccl3	2.9	2.1	1.6	2.2	CLASS
Nfkbie (IkB-ε)	1.3	2.8	2.3	2.1	CLASS
Il23a	3.0	ND	1.2	2.1	CLASS
Stat1	2.3	2.5	1.4	2.1	CLASS
Cxcl16	1.4	3.1	1.7	2.1	CLASS
Tlr2	1.6	2.5	1.8	2.0	CLASS
Arg1	-24.0	-529.9	-138.0	-230.6	ALT
Chi3l4 (Ym2)	-1.3	-182.2	ND	-91.8	ALT
Chi3l3 (Ym1)	ND	-74.9	ND	-74.9	ALT
Mgl2	-61.1	-121.8	-10.1	-64.3	ALT
Ccl24	1.1	-58.1	ND	-28.5	ALT
Mrc1 (CD206)	-13.4	-45.2	-4.2	-20.9	ALT
Ear11	-23.8	-11.9	ND	-17.8	ALT
Pdcd1lg2 (B7-DC)	-3.7	-22.9	ND	-13.3	ALT
Mgl1	-11.7	-14.4	-3.7	-9.9	ALT
Ptgs1 (COX1)	-7.7	-4.6	-2.6	-5.0	ALT
Igf1	-8.2	-3.5	-1.5	-4.4	ALT
Irf4	-1.5	-5.5	ND	-3.5	ALT
Clec7a (Dectin 1)	-1.4	-4.5	-2.7	-2.8	ALT
Retnla (Fizz1)	ND	-2.6	ND	-2.6	ALT
Ccl17	-1.8	-3.3	ND	-2.5	ALT

Gene symbols are depicted for a subset of genes (36 out of 184) that were similarly regulated in a strain-specific manner (at least 2-fold average difference) between the mouse macrophage cell lines RAW264.7 and J774, and the DC2.4 dendritic cell line. The fold difference in expression comparing type II and III infected cells is indicated, where positive numbers indicate the fold increase in type II over III infection and negative values indicate the fold increase of type III over type II infections. Genes were grouped according to their expression level in either alternatively or classically activated macrophages (Biswas and Mantovani, 2010; Gordon, 2003; Martinez et al., 2006, 2009). ND indicates that the gene was not detected above background in that particular cell type. The experiment was performed in duplicate. See also Figure S2 and Table S1 for molecular pathway and TFBSs analysis of these gene sets, respectively.

constitutively activate STAT6, a difference due to polymorphisms in the secreted *Toxoplasma* rho-trypan kinase ROP16 (Saeij et al., 2007). Furthermore, ROP16 can directly phosphorylate the critical Y641 residue required for STAT6 activation (Ong et al., 2010), thus making ROP16 a likely candidate driving strain differences in M2 activation. To investigate this further, we analyzed whether a type I strain that has the *ROP16* gene deleted (type I $\Delta rop16$) (Ong et al., 2010) can induce M2 cells. Unlike wild-type type I (and III) strains, the type I $\Delta rop16$ strain failed to induce the expression of any of the M2-associated markers (arginase, CD206, and mMgl) (Figure 2), which correlated with its inability to phosphorylate STAT6 at any time point investigated (0.5, 1, 2, 4, 8, and 18 hr, data not shown; 24 hr shown in Figures 2C and 2D).

Gene expression profiling of BMDMs infected with the type I or type I $\Delta rop16$ strains indicated that ROP16 controlled the expression (≥ 2 -fold) of 538 unique annotated genes. Indeed, the most differentially regulated genes were those associated with the M2 phenotype, with *Arg1* at the top of the list of ROP16-induced genes (132-fold) (Table S2A). Other M2 markers including *Cd206*, *Mgl2*, and *Cdh1* (E-cadherin) were also dependent on ROP16. Surface receptors that were induced by ROP16 were confirmed by FACS, which included the B7 family members B7-DC (*Pdcd1lg2*) and B7-H1 (*Cd274*), as well as the C-type lectin Dectin-1 (*Clec7a*) (Figure 2E). The M2 signature could clearly be seen in a TFBS analysis of ROP16-regulated genes, which implicated STAT6 and other M2 transcriptional regulators (Table S2B). In summary, the M2 activation program induced by *Toxoplasma* is controlled by ROP16.

To confirm that ROP16-mediated differences in macrophage activation occur *in vivo*, we infected BALB/c mice *i.p.* with type I, type II, or type I $\Delta rop16$ parasites and stained for M2 markers 21 hr postinfection. In type I-challenged animals, infected CD11b⁺ PECs (which includes monocytes/macrophages and granulocytes) stained positive for phospho-STAT6 and expressed the mannose receptor CD206 (Figures 2F and 2G), and this was dependent on type I *ROP16*. In contrast, type II infected cells did not express CD206 and were less likely to stain positive for phospho-STAT6 (Figures 2F and 2G). These data show that strain-specific induction of M2 cells is apparent *in vivo* and that the rho-trypan kinase ROP16 plays a fundamental role in this process.

***Toxoplasma* Dense Granule Protein GRA15 Promotes M1 Activation**

Recently, we identified a dense granule protein GRA15, encoded by the type II strain (*GRA15_{II}*), that is secreted into the host cytosol and causes the activation and nuclear translocation of host p50/REL-A NF- κ B heterodimers (Rosowski et al., 2011). Furthermore, *GRA15_{II}* accounted for most of the IL-12p40 response by infected macrophages *in vitro*, which prompted us to explore in greater detail the role of *GRA15_{II}* in M1 activation. Therefore, gene expression profiles of type II and type II $\Delta gra15$ -infected BMDMs were analyzed. Genes that were dependent (≥ 2 -fold) on the expression of *Toxoplasma GRA15_{II}* were those typically induced in M1 cells (Table S3A) and included many proinflammatory cytokines (*Il23a*, *Il6*, *Il12a*, *Il12b*, *Il1a*, and *Tnf*), ligands for T cell costimulatory receptors (*Cd40*, *Cd80*, *Cd70*, *Tnfsf9*, and *Tnfsf4*), NF- κ B modulators (*Nfkb1e*, *Nfkb1b*, *Nfkb1z*),

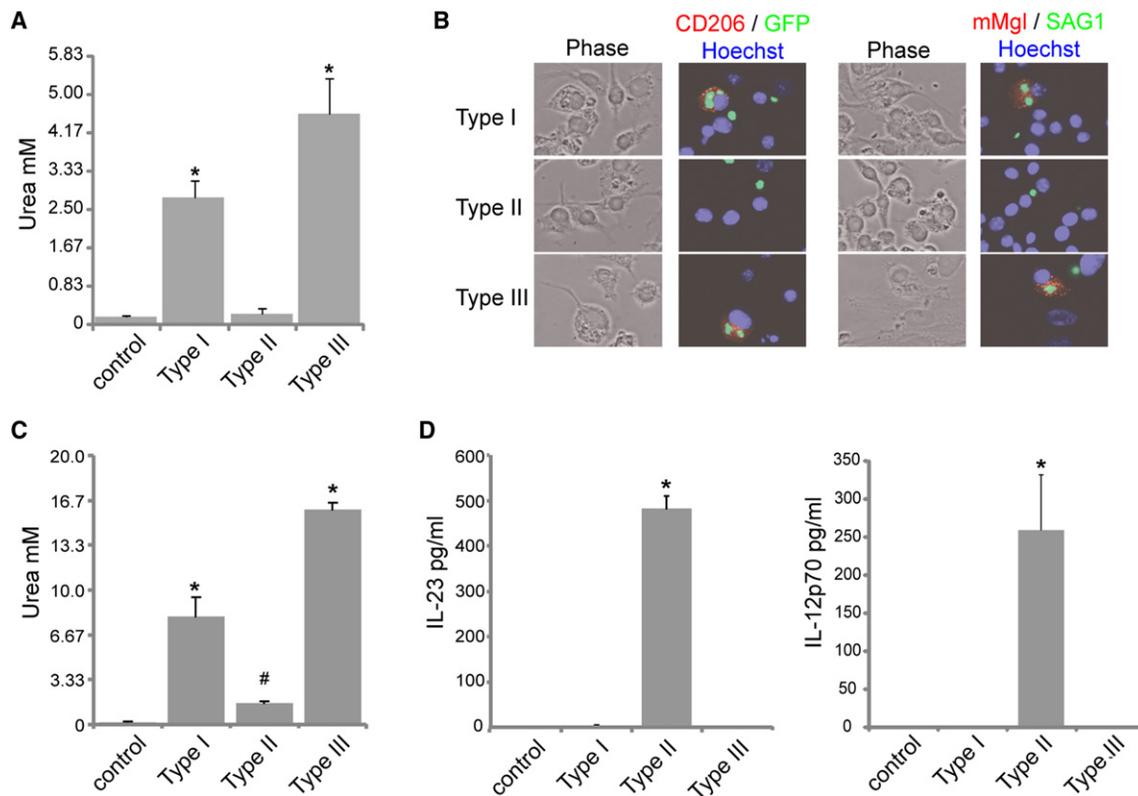


Figure 1. *Toxoplasma* Strain-Specific Induction of Markers Associated with either M1 or M2 Macrophages

(A) RAW264.7 macrophages were infected with the type I (RH), II (Pru), or III (CEP) *Toxoplasma* strains (moi = 5), and 24 hr after infection arginase activity was measured in the lysates of infected and uninfected macrophages by determining the conversion of L-arginine to urea in 1 hr (Student's t test, * $p < 0.006$, type I or III versus type II). Similar results were obtained with other strains of the three clonal lineages (GT1, ME49, and VEG, data not shown). Error bars represent a standard deviation (SD). The relative percentage of infected macrophages following i.p. infection with the three strain types can be seen in Figure S1.

(B) RAW264.7 macrophages were infected with either the type I, II, or III *Toxoplasma* strains that expressed GFP (moi = 0.5), and 1 day after infection macrophages were fixed, permeabilized, and stained with antibodies against either the macrophage mannose receptor (CD206, also called Mrc1, stained red) or the galactose/N-acetylgalactosamine-specific lectin (mMgl/Mgl2, stained red). Nuclei were stained with Hoechst (blue). These results are representative of at least three experiments.

(C) As in (A), but DC2.4 dendritic cells were assayed (Student's t test, * $p < 0.02$, type I, or III versus type II; # $p < 0.002$, type II versus uninfected control). Error bars, +SD.

(D) IL-23 (p40/p19) and IL-12 (p40/p35 or "IL-12p70") cytokine production by type I, II, or III infected BMDMs were determined by ELISA 24 hr after infection (Student's t test, * $p < 0.03$ type II versus type I or III). These results are representative of at least three experiments. Error bars, +SD.

and some of the M1-associated chemokines (*Cxcl1* and *Cxcl11*). Transcripts for *Ii10* and the IL-27/35 subunit *Ebi3*, which are necessary to prevent inflammatory damage following *Toxoplasma* infection (Gazzinelli et al., 1996; Stumhofer et al., 2006), were also highly dependent on the expression of *GRA15_{II}* (Table S3A). Interestingly, *Ii23a* was the most differentially regulated gene transcript between these strains (58-fold), and secretion of IL-23 (p40/19), IL-12 (p40/35), and IL-10 by infected BMDMs was entirely dependent on the expression of type II *GRA15* (Figure 3C). TFBS analysis of the 710 genes that were regulated by *GRA15_{II}* also revealed a significant enrichment of TFBSs for transcription factors known to be activated in M1 macrophages (Medzhitov and Horng, 2009), which include NF- κ B, IRF, C/EBP, PU1, and ATF (Table S2B). *GRA15_{II}* also repressed many genes that are associated with M2 activation (*Mrc1*, *Mgl1*, *Ccl24*, *Ear11*, *Ptgs1*) (Table S3A). In particular, *PPAR γ* , which encodes a transcription factor that promotes

M2 activation (Odegaard et al., 2007), was the gene most repressed by *GRA15_{II}*. In summary, many aspects of M1 activation by the type II strain were largely controlled by *GRA15*.

Inducing the M2 Phenotype with Type II-Engineered Parasites

To test whether a simple combination of *GRA15* and *ROP16* will determine the M1/M2 phenotype of infected macrophages, a series of type II parasites was generated that expressed or lacked the STAT3/6- and/or NF- κ B-activating versions of these genes. Gene expression profiles, proinflammatory cytokine secretion, and arginase activity of BMDMs infected with type II, type II +*ROP16_{II}*, type II Δ *gra15*, and type II Δ *gra15* +*ROP16_{II}* parasites were analyzed. Type II +*ROP16_{II}*-infected macrophages possessed significantly higher arginase activity than type II infected RAW264.7 cells (data not shown) and BMDMs (Figure 3B), but lower than the response elicited by the type III

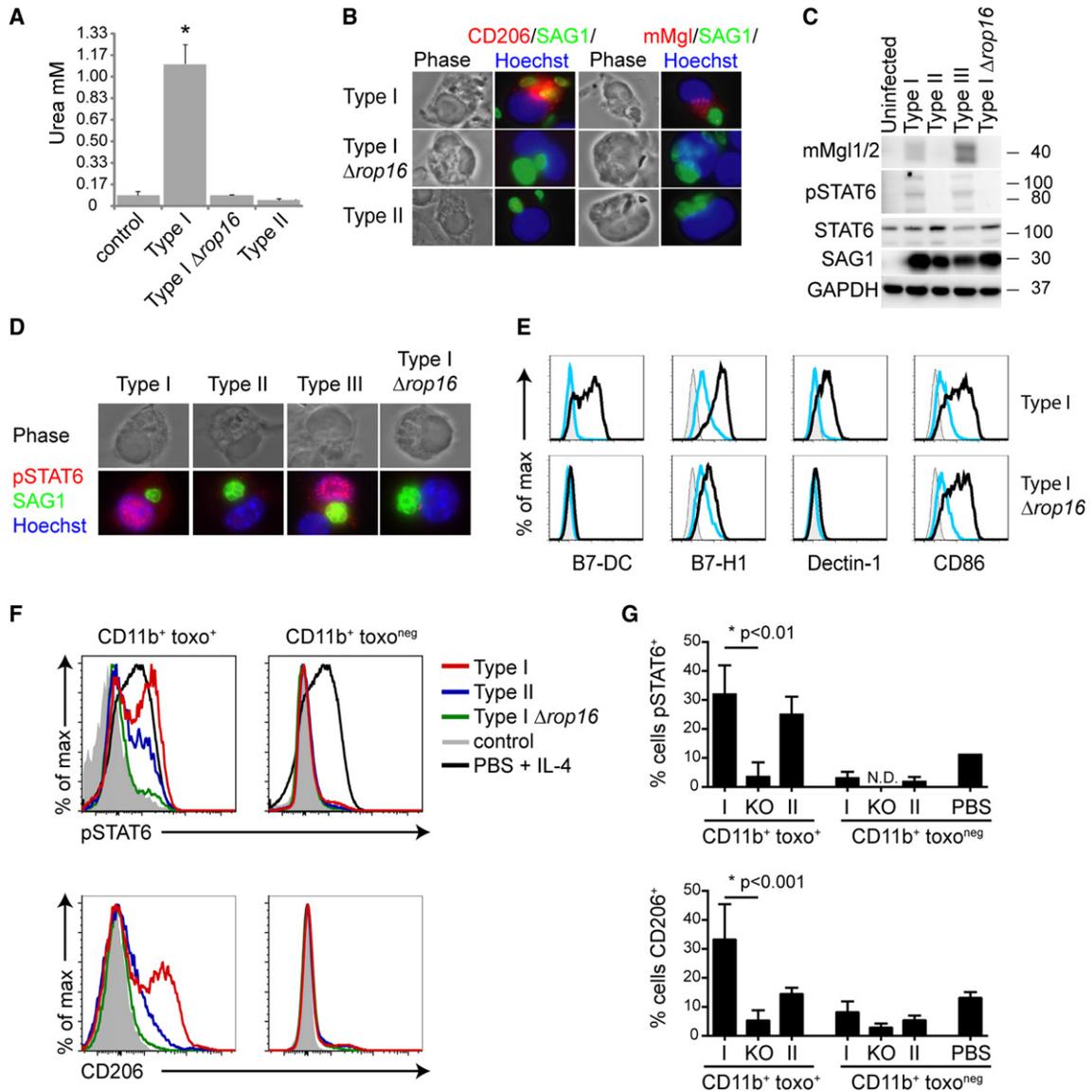


Figure 2. Toxoplasma-Secreted Rhopty Kinase ROP16 Mediates Alternative Macrophage Activation

(A) RAW264.7 macrophages were mock infected (control) or infected (moi = 5) with type I, type I $\Delta rop16$, or type II parasites, and 24 hr later arginase activity was determined (Student's t test, * $p < 0.05$, type I versus type I $\Delta rop16$ or type II). Error bars, +SD.

(B) As in (A), except the macrophages were fixed and stained with antibodies to the parasite surface antigen SAG-1 (green) and the M2 markers CD206 or mMgl. Hoechst dye was used to stain nuclei (blue).

(C) Western blot of infected RAW264.7 cell lysates using antibodies against mMgl1/2, tyrosine-phosphorylated STAT6 (pSTAT6), or total STAT6. Antibodies against GAPDH and SAG-1 were used for host cell and parasite loading controls, respectively.

(D) One day after infection with the indicated strain types, RAW264.7 macrophages were fixed, permeabilized, and stained with antibodies against pSTAT6 and SAG-1. Nuclei were stained with Hoechst (blue).

(E) BMDMs were infected with GFP expressing type I or type I $\Delta rop16$ parasites and stained with PE-labeled antibodies to the surface receptors B7-H1 (PD-L1), B7-DC (PD-L2), Dectin-1 (Clec7a), or CD86 (which is not regulated by ROP16). Histogram plots depict the relative surface expression of these markers on infected GFP⁺ (black lines) and noninfected GFP^{neg} (blue lines) BMDMs in the same well. Isotype staining with Rat IgG2a antibodies is also shown (shaded histogram).

(F) BALB/c mice were infected i.p. with type I, type I $\Delta rop16$, or type II parasites. Twenty-one hours postinfection, PECs were harvested and stained for CD11b and the SAG-1 parasite surface antigen. Infected (tox⁺) and uninfected (tox^{neg}) CD11b⁺ PECs were analyzed for their expression of pSTAT6 or CD206 by histogram (gray, "minus one" staining control, where cells are stained with all staining reagents except the anti-STAT6 or CD206 primary antibodies; red, type I; blue, type II; green, type I $\Delta rop16$; black, PECs from an uninfected mouse stimulated with recombinant murine IL-4 for 10 min at 37°C). Data are representative of two independent experiments (n = 3).

(G) Bar graphs depict the percentage of positively staining CD11b⁺ PECs as analyzed in (F) (KO, type I $\Delta rop16$). N.D., not detected. Error bars, +SD. ANOVA one-way analysis of variance was used to determine statistical significance. The percentage of infected CD11b⁺ cells was similar in type I, type II, and type I $\Delta rop16$ -infected mice (data not shown).

See Table S2A for a list of genes regulated by ROP16, as well as Table S2B for a TFBS analysis of this gene set.

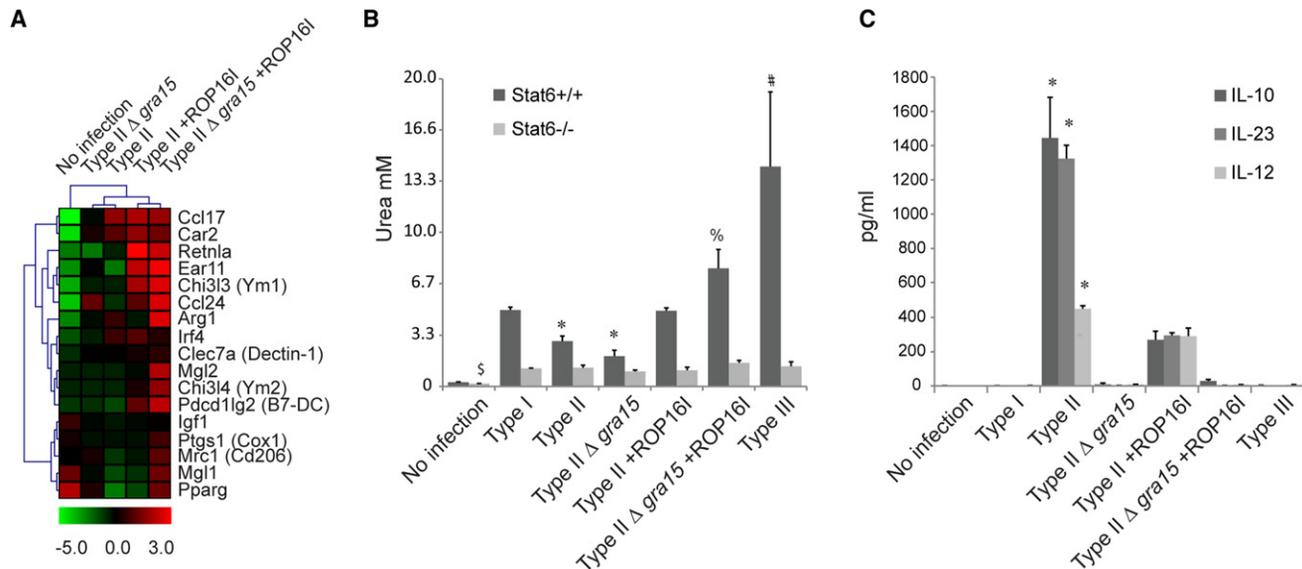


Figure 3. GRA15 and ROP16 Determine M1/M2 Activation

(A) Gene cluster analysis of uninfected and infected BMDMs with the indicated type II *Toxoplasma* strains for markers of alternative activation. For a list of genes that are regulated by GRA15 and a TFBS analysis of this gene set, see Tables S3A and S2B, respectively. For a list of genes that are coregulated by ROP16 and GRA15, see Table S3B.

(B) Arginase activity of wild-type (dark bars) or *Stat6*^{-/-} BMDMs (light bars) infected for 20 hr with the indicated parasite strains. Error bars, +SD (Student's t test, **p* < 0.03, indicated sample versus all other infected wild-type BMDMs; #*p* < 0.01, type III versus all other infected wild-type BMDMs; %*p* = 0.06, type II $\Delta gra15$ +ROP16_I versus type II +ROP16_I; §*p* < 0.03 uninfected *Stat6*^{-/-} versus all other infected *Stat6*^{-/-} macrophages; Infected wild-type versus *Stat6*^{-/-} BMDMs was significantly different for each parasite strain, *p* < 0.02, data not shown).

(C) IL-12p70, IL-23 (p40/p19), and IL-10 cytokine secretion by parasite-infected BMDMs was determined by ELISA 20 hr after infection (Student's t test, **p* < 0.03, type II versus all other samples). Error bars, +SD. For a further analysis of the ability of GRA15_{II} and ROP16_I to induce M1 and M2 markers in the context of polarizing environments in vitro and in vivo, see Figure S3.

strain (Figure 3B). Furthermore, by removing GRA15 from the type II +ROP16_I strain (i.e., type II $\Delta gra15$ +ROP16_I), arginase activity of infected BMDMs was enhanced (Figure 3B, *p* = 0.06), suggesting that GRA15 can inhibit arginase-1 induction by ROP16. Although the expression of the other arginase isozyme, Arg2, was dependent on *Toxoplasma* GRA15_{II} (5.7-fold) (Table S3A), only a small decrease of urea production was observed between macrophages infected with type II $\Delta gra15$ and type II parasites (Figure 3B), implicating Arg1 as the major producer of urea following *Toxoplasma* infection. Arginase activity was also determined in *Stat6* knockout BMDMs. In keeping with an earlier report that *Toxoplasma* can induce the expression of Arg1 independently of STAT6 (El Kasmi et al., 2008), *Toxoplasma* elicited urea production in *Stat6*^{-/-} BMDMs, albeit at levels much lower than in wild-type BMDMs (Figure 3B). Importantly, strain differences in urea production were absent in *Stat6*^{-/-} BMDMs, suggesting that polymorphisms in ROP16 affect signaling via STAT6 to achieve strain-specific differences in the macrophage arginase response.

To obtain a complete picture of the macrophage response to these strains, the gene expression profiles of BMDMs infected with type II, type II $\Delta gra15$, type II +ROP16_I, and type II $\Delta gra15$ +ROP16_I parasites were compared. Indeed, there was significant overlap between ROP16- and GRA15-regulated genes, as many genes were either synergistically or antagonistically regulated by both GRA15 and ROP16 (Table S3B). For example, M1 cytokines that were highly induced by GRA15_{II}

were downregulated following type II +ROP16_I infection (Figure 3C), likely because ROP16 can inhibit the activation of NF- κ B by type II strains (Rosowski et al., 2011). Conversely, many M2 markers were more highly expressed in BMDMs infected with type II $\Delta gra15$ +ROP16_I, than with type II +ROP16_I parasites (Figures 3A and 3B), indicating that activation of NF- κ B can inhibit the expression of M2 markers, possibly through its effect on PPAR γ . Finally, GRA15 and ROP16 retained the ability to induce M1 or M2 markers in polarizing environments in vitro and in vivo (see discussion in Figure S3). Thus, a combination of GRA15 and ROP16 can control macrophage activation along the M1/M2 axis.

Arginase Promotes *Toxoplasma* Replication

Recently, it was demonstrated that arginase-I expression in macrophages is a susceptibility factor during *Toxoplasma* infection and that Arg1^{-/-} macrophages produce more NO in response to inflammatory stimuli (El Kasmi et al., 2008). iNOS, like arginase, uses L-arginine as its substrate, but converts it to L-citrulline and NO, which is required for long-term control of *Toxoplasma* infection (Scharton-Kersten et al., 1997). We therefore investigated whether the induction of arginase by *Toxoplasma* ROP16 has a similar effect on the production of NO by macrophages. By adding different concentrations of L-arginine to L-arginine-free medium, it was determined that below ~40 mg/L of L-arginine there is a rapid decline in NO production by mouse macrophages stimulated with LPS and IFN- γ (Figure 4A) (commercial DMEM contains 84 mg/L L-arginine, mouse serum

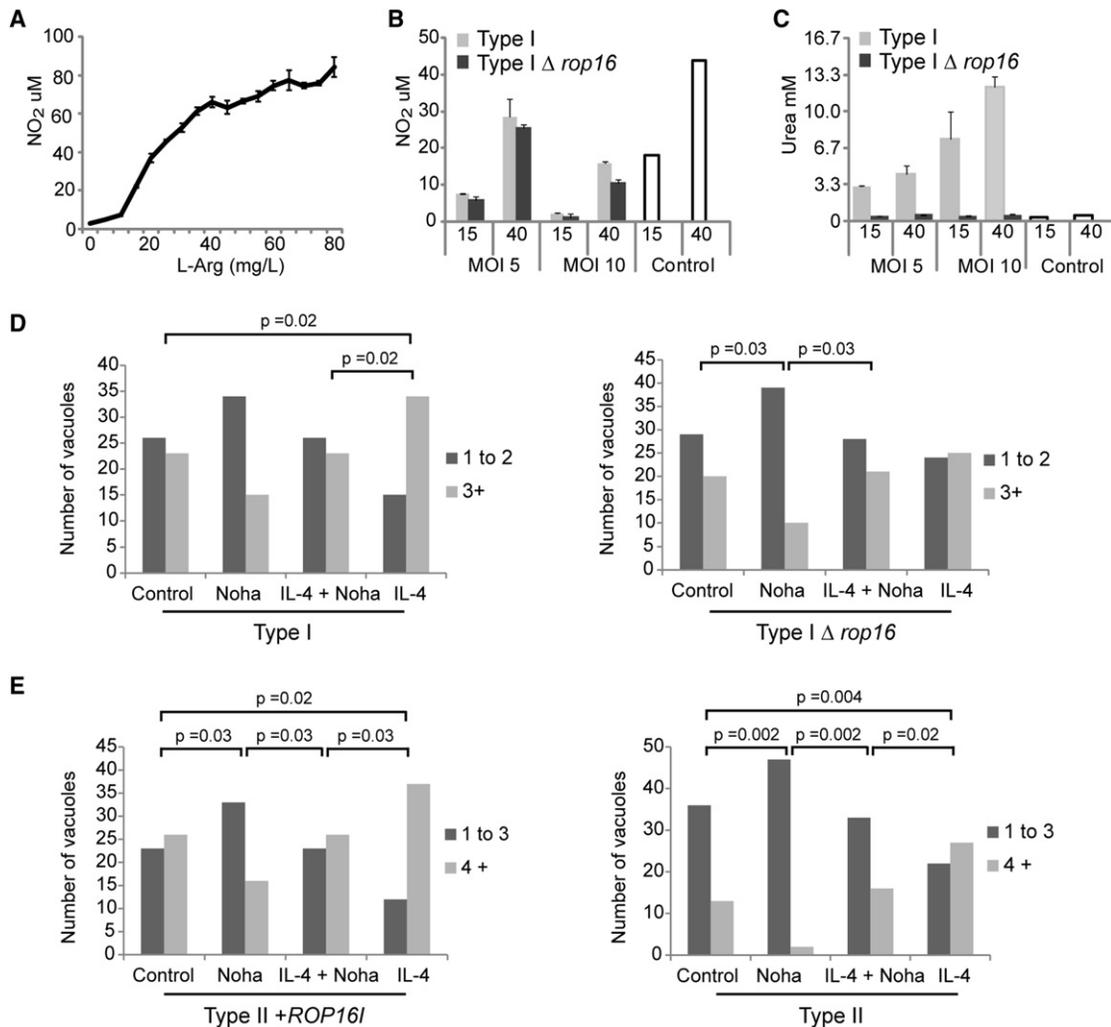


Figure 4. M2 Activation Promotes Parasite Growth, but Induction of Arginase by ROP16 Does Not Affect Nitric Oxide Production by Stimulated Mouse Macrophages

(A) RAW264.7 macrophages were stimulated with LPS (20 ng/ml) and IFN- γ (100U/ml) in medium containing different concentrations of L-arginine, and NO production was measured by determining the concentration of nitrite (NO₂⁻) in the medium.

(B and C) RAW264.7 macrophages cultured in medium with either 15 or 40 mg/L L-arginine were mock-infected (control) or infected (moi = 5 or 10) with type I or type I $\Delta rop16$ parasites and subsequently stimulated with LPS (20 ng/ml) and IFN- γ (100 U/ml). One day after infection NO production was measured by determining the concentration of nitrite in the medium, and (C) arginase activity was determined. Error bars, +SD.

(D) DC2.4 cells were infected with either type I or type I $\Delta rop16$ at an moi of 1 in medium supplemented with 35 mg/L L-arginine and stimulated with 50 ng/mL of IL-4 and/or treated with 100 μ M of the arginase inhibitor nor-NOHA. Twenty-four hours later, the number of parasites per vacuole was quantified by immunofluorescence microscopy. The bars represent the number of vacuoles containing 1 or 2 parasites (dark bars), or ≥ 3 parasites (light bars). Fisher's exact test one-tailed probabilities ≤ 0.03 are indicated.

(E) As in (D), but type II (PruA7 5-8b+, HXGPT+) and type II +ROP16; (2C4) parasites were assayed after 48 hr of infection. The bars depict the number of vacuoles containing 1–3 parasites (dark bars) or ≥ 4 parasites (light bars). Fisher's exact test one-tailed probabilities ≤ 0.03 are indicated (Fisher's exact test, $p \leq 0.002$, NOHA versus IL-4 for all infections in D and E, data not shown).

contains ~ 35 mg/L L-arginine). RAW264.7 macrophages were infected for 24 hr with type I or type I $\Delta rop16$ parasites and activated with LPS and IFN- γ for 24 hr. Although high arginase activity was induced only in macrophages infected with type I parasites, both strains inhibited NO production equally well at L-arginine concentrations of 15, 40, and 80 mg/L (data not shown). Since *Toxoplasma*-infected cells are refractory to IFN- γ signaling (Zimmermann et al., 2006), infection and stimulation were performed concurrently to bypass this refractory state. Indeed, NO produc-

tion was restored under these conditions. NO production by stimulated macrophages was dependent on the concentration of L-arginine and multiplicity of infection (moi); however, no significant difference in NO production was observed between cells infected with type I or type I $\Delta rop16$ parasites (Figure 4B), even though type I parasites induced high levels of arginase under these conditions (Figure 4C).

Arginase converts L-arginine to urea and ornithine, the latter of which can be utilized in a variety of metabolic pathways including

amino acid and polyamine synthesis. *Toxoplasma* lacks arginase activity and is a polyamine auxotroph (Cook et al., 2007). Induction of host arginase could therefore be a strategy to increase the availability of host polyamines to support *Toxoplasma* replication. Since DC2.4 cells are extremely responsive to induction of arginase-I (Figure 1C), we assayed parasite growth in these cells cultured in 35 mg/L L-arginine and either stimulated with IL-4 to induce arginase-I and/or treated with the arginase-I inhibitor nor-NOHA. In general, parasite replication for all strains was enhanced in IL-4-treated cells. The difference was apparent at 48 hr for the type II/III strains and at 24 hr for the type I strains (Figures 4D and 4E). Proliferative enhancement was through IL-4-induced host arginase, since IL-4-stimulated cells treated with nor-NOHA elicited a proliferative response that was similar in magnitude to parasite growth in nontreated cells (Figures 4D and 4E). Strains that express *ROP16_I* grew slightly faster (in particular type II +*ROP16_I*, compared to type II), and nor-NOHA inhibited parasite replication of all strains tested. These results suggest that *Toxoplasma* could use the arginase metabolic pathway to promote its own growth, possibly through the action of ROP16.

ROP16 Promotes Host Survival and Quells *Toxoplasma*-Induced Ileitis in C57BL/6 Mice

Following natural peroral infection with the type II strain, C57BL/6 mice rapidly die during the acute phase of infection, which correlates with severe intestinal pathology (Liesenfeld et al., 1996). *Toxoplasma*-induced ileitis can be cured by removing a variety of proinflammatory cytokines including IFN γ (Liesenfeld et al., 1996), IL-23, and IL-22 (Munoz et al., 2009). Given the anti-inflammatory capacity of the Jak-STAT6 and -STAT3 signaling pathways to regulate these mediators, we decided to explore the role of ROP16 in inflammatory modulation at the site of infection.

To this end, B6 mice were perorally challenged with type II +*ROP16_I*, or +*ROP16_{III}* parasites, or with the type II or III strains. With high-dose infection (800 cysts), the majority of mice survived infection with the type II *ROP16_{III}* strains, and those that died did so at a much later time point than mice infected with the type II strain (Figure 5A). Survival following type II +*ROP16_I* infection correlated with reduced intestinal inflammation across the entire length of the intestine (Figure 5D). By day 8, cellular infiltrate into the lamina propria, villi blunting, and necrosis were nearly absent in type II +*ROP16_I*-infected mice (Figures 5B and 5D). Protection also correlated with reduced submucosa thickening (type II, 72 μ M; versus type II +*ROP16_I*, 56 μ M, average over 400 measurements $p < 10^{-7}$, data not shown). Whereas the type II strain elicited a significant influx of granulocytes into the villi and Peyer's patches, as detected by Gr-1 and myeloperoxidase (MPO) staining, granulocyte recruitment was significantly reduced in type II +*ROP16_I*-infected animals (Figure 5E). Villus expression of iNOS (NOS2) in type II +*ROP16_I*-infected mice was reduced but not significantly different from type II infection. Lymphocytes isolated from the Peyer's patches of type II +*ROP16_I*-infected animals produced less IL-22 and IFN- γ when stimulated with plate-bound anti-CD3 and anti-CD28 antibodies compared to lymphocytes from type II infected mice (Figure 5C), while lamina propria lymphocytes from type II +*ROP16_I*-infected

animals produced less IL-22 but similar amounts of IFN- γ compared to lymphocytes from type II infected animals. Thus, type I/III ROP16 expression correlates with a general dampening of *Toxoplasma*-induced ileitis after oral infection in B6 mice.

DISCUSSION

In this report, we have demonstrated that *Toxoplasma* type I and type III strains can induce the M2 phenotype, while the type II strain induces M1-like macrophages. The alternative activation of macrophages is dependent in large part on the *Toxoplasma* polymorphic protein kinase ROP16, while the classical activation of macrophages by the type II strain is due to the unique ability of its GRA15 protein to activate the NF- κ B pathway and elicit proinflammatory cytokines. In total, polymorphisms in these two factors account for ~25%–50% of the gene expression differences between type II- or III infected cells (data not shown).

Interestingly, ROP16 and GRA15 seem to affect signaling pathways that are themselves differentially “wired” between different hosts. For example, there is considerable mouse strain variation in the ability to generate M1 or M2 cells (Mills et al., 2000). One hypothesis is that parasite effectors from different *Toxoplasma* strains evolved to work optimally in hosts predisposed to certain types of immune responses, such as those along the Th1/Th2/Th17 or M1/M2 axes. Conversely, ending up in the wrong host might lead to severe disease and failure to establish chronic infection. Evidence surrounding this idea include (1) C57BL/6, but not BALB/c, mice challenged orally with type II strains, but not type III strains (data not shown), can develop severe immune pathology caused by high levels of proinflammatory cytokines in the small intestine (ileitis) (Liesenfeld et al., 1996); (2) chronic infections with type II strains, but not other strains, can cause severe pathology in the brain (encephalitis) of susceptible mice (Suzuki and Joh, 1994); (3) type I or type I/III recombinant strains are more often sampled in patients that present severe ocular toxoplasmosis (Grigg et al., 2001); and (4) although the type I strain is lethal in laboratory mice, rats are extremely resistant to *Toxoplasma* infection in general (Sibley and Ajioaka, 2008). Whether the type II or other strains are uniquely suited to spread in nature is an unresolved question; however, the type II strain is the most abundant strain identified in livestock and in humans in North America and Europe.

The fact that type I and III parasites initially induce M2 macrophages seems to contradict the well-established fact that *Toxoplasma* infections with the type I strain induce a strong Th1 response (Mordue et al., 2001). We favor the hypothesis that early alternative activation of macrophages, decreased macrophage IL-12 secretion (Robben et al., 2004), and reduced dendritic cell expansion following type I infection (Tait et al., 2010) contribute to a lower IFN- γ and cytotoxic host response against the parasite. Later during infection, the higher type I parasite burden could lead to increased levels of proinflammatory cytokines (Mordue et al., 2001). This could be due to “danger signals” released from lysing parasites such as profilin that can directly stimulate dendritic cells to produce high levels of IL-12 (Yarovinsky et al., 2005).

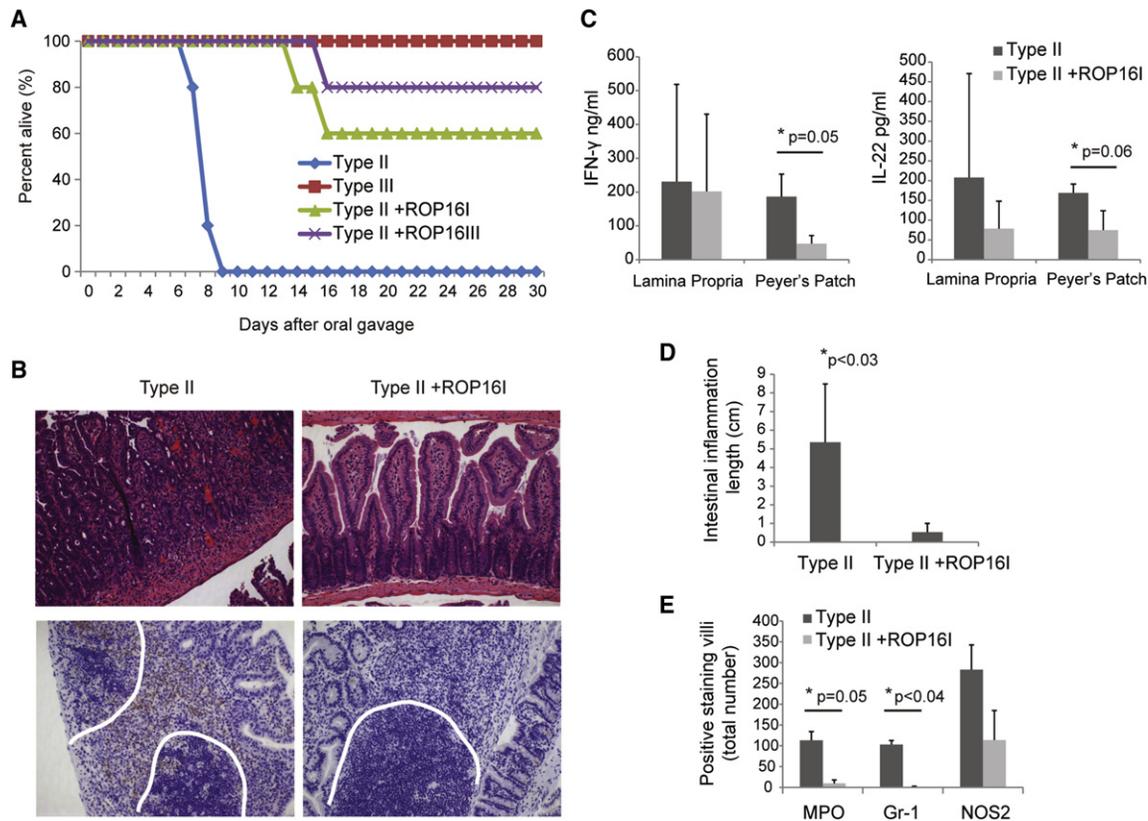


Figure 5. ROP16 Prevents *Toxoplasma*-Induced Ileitis

(A) Susceptible C57BL/6 mice were orally infected by gavage with 800 cysts of the type II, type II + ROP16, type II + ROP16_{III} (Pru background), or type III (CEP) strains, and survival was monitored. The combined results of two experiments are shown (n = 10).

(B) On day 8 of infection, the entire length of the small intestine was fixed, sectioned, and stained with hematoxylin and eosin dyes (top panels), or with antibodies to Gr-1 (Ly6C/G) (RB6-8C5, brown staining) and hematoxylin (lower panels). Representative pictures of the villi (top panels) or Peyer's patches (lower panels) from the intestines of mice infected with either the type II or type II + ROP16_I strains are shown at 20× magnification. The border of the Peyer's patch is outlined in white.

(C) On day 8 of infection, lymphocytes were harvested from the Peyer's patch or lamina propria and stimulated for 20 hr in vitro with plate-bound anti-CD3 ϵ and anti-CD28 antibodies. IFN- γ and IL-22 was detected in the supernatant by ELISA. The average of three biological replicates and the standard deviation are plotted. Student's t test, p \leq 0.06 are indicated.

(D) For each mouse intestine (n = 3), severe inflammation was quantified along the entire length of the intestine by microscopy of the tissue sections. If a region met the following criteria—(1) increased cellular influx, (2) villi necrosis or villi blunting, and (3) mucosal thickening—then that region was considered severely inflamed and the length of that region was measured. The sum of all regions was tallied per mouse intestine, and the average of the biological replicates and standard deviation is plotted. The Student's t test p value is indicated.

(E) For each intestine, the number of villi or villi remnant that stained positive for iNOS (NOS2), Gr-1 (Ly6C/G), or MPO was quantified, and the average of the biological replicates and standard deviation is plotted. Student's t test p values \leq 0.05 are indicated.

If both the type I and III strains induce alternative macrophage activation, why then are type III strains avirulent? The major *Toxoplasma* locus responsible for the difference in virulence between type I and III strains encodes another rhopty protein kinase, ROP18 (Saeij et al., 2006; Taylor et al., 2006), which can phosphorylate and thereby inactivate mouse p47 GTPases, which are crucial for IFN- γ mediated killing of *Toxoplasma* (Fentress et al., 2010; Steinfeldt et al., 2010). Type III strains express extremely low levels of ROP18 due to an insertion in its promoter (Saeij et al., 2006), and the addition of the type I ROP18 locus to a type III strain causes this strain to be as virulent as type I strains (Taylor et al., 2006). In contrast, we recently demonstrated that the virulence differences in mice between type II and III strains is multifactorial, with five *Toxoplasma* loci being involved, including ROP18, ROP16 (Saeij et al., 2006), ROP5 (Reese

et al., 2011), and most likely GRA15 (Rosowski et al., 2011). With the exception of ROP5, evidence now exists that these virulence factors can directly manipulate macrophage function, and the likelihood that other *Toxoplasma* effectors, polymorphic or not, would do likewise seems probable, especially considering the findings presented here.

We recently reported that following i.p. infection with type II Δ gra15 parasites, a lower host production of IL-12p70 and IFN- γ at 1–2 days of infection preceded a higher parasite burden at day 5 compared to the type II strain (Rosowski et al., 2011). It is possible that M1 activation by GRA15_{II} helps drive IFN- γ production to keep parasite numbers low, thus facilitating chronic infection. The induction of IL-12 by GRA15_{II} may be especially important in hosts that do not express TLR11, as in humans (Roach et al., 2005). With respect to the in vivo effects of

ROP16, we previously observed that the type II +ROP16_i or +ROP16_{iii} strains were less virulent following i.p. infection compared to the parental type II parasites (Saeji et al., 2006). We have extended this analysis to the oral model, and found that ROP16_i prevents *Toxoplasma*-induced ileitis. The mechanism by which ROP16 promotes host survival is currently under investigation, but it is unlikely that ROP16 mediates its effect by inhibiting macrophage NO production. L-arginine depletion by arginase-1 also inhibits T cell responses (Pesce et al., 2009). Thus, ROP16-induced arginase activity, suppression of IL-12 family members, and its induction of the T cell coinhibitory molecules B7-DC and B7-H1 might all feed into a general dampening of the CD4 T cell response, which is implicated in driving *Toxoplasma*-induced intestinal inflammation.

In conclusion, *Toxoplasma* appears unique in that strains of the same species can elicit polar opposite responses in infected macrophages. The rationale for alternative activation by *Toxoplasma* might be 2-fold: (1) to limit inflammatory damage in the infected host by quelling the Th1 response aimed at parasite elimination, and (2) to scavenge polyamines or other metabolites for energy consumption. On the other hand, the promotion of M1 cells could assist the host to develop a better Th1 response required for parasite clearance, and paradoxically allow the establishment of chronic infection. It appears that the survival of both the host and the parasite hangs in the delicate balance between opposing pro- and anti-inflammatory responses. Understanding how *Toxoplasma* regulates these immune decisions may provide important clues to their widespread success in establishing chronic infection.

EXPERIMENTAL PROCEDURES

Mice, Parasites, and Cells

Six- to ten-week-old female BALB/cJ, C57BL/6J, or B6.129S2(C)-Stat6^{tm1Gru}/J (Jackson Laboratories) mice were used in all experiments. All mice were maintained in specific pathogen-free conditions in accordance with institutional and federal regulations. The *Toxoplasma* strains used in this study are described in the Supplemental Experimental Procedures. RAW264.7, J774, and DC2.4 cell lines were maintained in the same medium as HFFs with an additional 10 mM HEPES (GIBCO Invitrogen). BMDMs were generated by culturing bone marrow cells in 20% L929 cell-conditioned medium, as previously described (Rosowski et al., 2011). This method yielded a highly pure population of CD11b⁺ F480⁺ macrophages (>99%) by FACS. All parasite strains and cell lines were routinely checked for mycoplasma contamination, and it was never detected.

Ex Vivo Phospho-STAT6 Assay and FACS Analysis

BALB/c mice were infected i.p. with 3×10^6 parasites; 21 hr later, PECs were harvested, and all manipulations were done in the presence of Phosphatase Inhibitor Cocktail 2 (Sigma) and 1 × Roche protease inhibitor (Roche). PECs were blocked with 4% FBS, FcBlock (BD PharMingen), and normal rat and mouse serums (Caltag Laboratories) and stained with α -CD11b M1/70 PE or Pacific Blue (eBioscience), washed, fixed in 4% paraformaldehyde, and permeabilized with 0.5% saponin in blocking solution, stained with rabbit α -SAG-1, α -CD206 MR5D3 biotin or PE (Serotec), and α -pSTAT6 pY641 J71-773.58.11 Alexa Fluor 647 (BD PharMingen), washed and detected with goat α -rabbit Alexa Fluor 488 (Invitrogen) and streptavidin PerCP (BD PharMingen) all in permeabilization solution. For the staining of infected BMDMs (moi = 0.5), cells were blocked with 10% normal mouse and hamster serums (Jackson ImmunoResearch) and FcBlock and stained with PE-labeled antibodies to CD86 (eBioscience), B7-H1, B7-DC (BD PharMingen), Dectin-1, or a RatIgG2a isotype control (R&D Systems), followed by washing. Cells were fixed with 2% formaldehyde and washed before FACS analysis.

Arginase and Nitric Oxide Assays

Parasites (moi of 20, 10, 5, or 1) were added to cells (10^5 cells/well) grown in 96-well plates, and arginase activity was measured 20–24 hr after infection as described (Corraliza et al., 1994). Wells with similar numbers of viable parasites were compared between parasite strains as inferred from a plaque assay. To measure the NO response, RAW264.7 cells were grown in DMEM without L-arginine complemented with 10% dialyzed serum and defined concentrations of L-arginine (MP Biomedicals). Nitrite was measured by using the Griess reaction.

Microarray Analysis

Mouse macrophage (RAW264.7 and J774) and dendritic (DC2.4) cell lines were grown in a T75 to 80% confluency and were infected with the Me49 or CEP strains (moi = 7). BMDMs were plated at 3×10^6 cells per well (6-well plate) infected (moi = 3) for 18 hr, after which RNA was isolated using TRIzol (Invitrogen). RNA was labeled and hybridized to a mouse Affymetrix array (Mouse 430 2.0 or Mouse 430A 2) and analyzed as described (Rosowski et al., 2011).

Immunofluorescence Assay

Cells were fixed with 3% formaldehyde, treated with methanol, blocked, and permeabilized with 3% bovine serum albumin, 5% goat serum, 0.2% Triton X-100 in PBS. Coverslips were incubated in permeabilization solution with antibodies specific for Mrc1/CD206 sc-58987, mMgl sc-56109, or polyclonal antibodies against p-STAT6 sc-11762 (Santa Cruz Biotechnology) and a mouse monoclonal antibody against the surface antigen SAG-1, DG52. Alexa Fluor 488 or 594 (Molecular Probes)-coupled secondary antibodies and Hoechst dye were used for antigen and DNA visualization with a fluorescence microscope.

Western Blot

Lysates were prepared from infected (moi = 10 for 24 hr) macrophages grown in a 6-well plate. Western blots were performed as described (Rosowski et al., 2011) using antibodies against GAPDH sc-32233 (Santa Cruz Biotechnology), p-STAT6 558241 (BD PharMingen), mMgl AF-4297 (R&D systems), and SAG-1.

Parasite Growth Assay in DC2.4 Cells

DC2.4 cells were cultured in DMEM containing 10% dialyzed serum and 35 mg/ml L-arginine and infected with an moi of ~1 and simultaneously treated with 50 ng/ml mouse IL-4 (PeproTech) and/or 100 μ M nor-NOHA (N ω -hydroxy-nor-Arginine) (Cayman Chemical) to induce or inhibit arginase, respectively. After 24–48 hr, cells were fixed, blocked, permeabilized, and stained as described above. The number of parasites per vacuole for 50 vacuoles was counted per each condition.

Oral Infection, Tissue Sections, and Ex Vivo Cytokine Analysis

Brain homogenate of chronically infected mice was stained with dolichos biflorus-FITC (Vector Laboratories), and cysts were enumerated by microscopy. B6 mice were orally gavaged with 800 cysts and monitored for survival. Compared to the ME49 strain, the Pru strain is less virulent, and higher cyst numbers are needed to cause intestinal inflammation. For tissue sectioning, 8 days after infection the entire length of the small intestine (duodenum to ileum) was cut longitudinally, fixed in 10% buffered formalin, rolled into a “jelly roll,” sliced in two, mounted into cassettes, and processed for sectioning and hematoxylin and eosin staining. Antibodies to Gr-1 (Ly6C/G) RB6-8C5 (BD), MPO Ab-1 RB-373-A (Neo Marker), and iNOS sc-650 (Santa Cruz Biotechnology) were detected with HRP-linked secondary antibodies (Dako, BD). For ex vivo cytokine analysis, the Peyer’s patches were dissected, and lymphocytes were obtained by crushing. Pieces of the small intestine were then incubated in 5 mM EDTA in HBSS, washed, and digested with 1.25 mg/ml collagenase V and 50 U/ml DNase, and lamina propria lymphocytes were purified over a 40%/80% Percoll (GE) gradient. Cells were washed, and 3×10^5 cells were plated in 96-well V bottom plates (Costar) coated with plate-bound α -CD3 ϵ and α -CD28 (5 μ g/ml) in RPMI medium with 10% FBS, supplements, and antibiotics. Twenty hours later, supernatant was analyzed by ELISA for mouse IFN- γ or IL-22 (eBioscience).

ACCESSION NUMBERS

The microarray data for BMDMs infected with the engineered type II strains have been deposited in the NCBI's Gene Expression Omnibus under the GEO Series accession number GSE29404. Similarly, microarray data for BMDMs infected with type I *Δrop16* parasites, as well as data for macrophage and dendritic cell lines infected with type II and III strains, have been deposited under the GEO Series accession numbers GSE29582 and GSE29584, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.chom.2011.04.015.

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