Toxoplasma gondii inhibits mast cell degranulation by suppressing phospholipase Cγ-mediated Ca2+ mobilization

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INTRODUCTION

The apicomplexan Toxoplasma gondii has evolved to be an extremely successful obligate intracellular parasite. It parasitizes a multitude of mammalian and avian species as intermediate hosts. In felines, which serve as the definitive host, sexual reproduction results in shedding of highly infectious oocysts. The Center for Disease Control and Prevention estimates one fifth of the US. human population is latently infected with T. gondii. In most hosts, infection is long-lived but asymptomatic. Nevertheless, under certain conditions, such as in immuno-compromised individuals, acute toxoplasmosis poses serious health risks (Dubey, 1998).

T. gondii infects host cells through a process of active invasion and establishment of a parasitophorous vacuole that resists fusion with the phago-lysosomal system (Sibley, 2004). One of the probable reasons for the success of Toxoplasma as an intracellular pathogen is its development of immuno-modulatory mechanisms to evade and control the host response to infection (Laliberte and Carruthers, 2008; Leng et al., 2009a). In vivo infection results in a strong IFN-γ-mediated protective immune response that is necessary for host survival, and, as a result, for parasite survival (Lambert and Barragan, 2010). At the same time, infection actively suppresses production of many pro-inflammatory cytokines (Leng et al., 2009a). Virulence factors such as ROP16 and ROP18 are secreted from parasite rhoptries and act to directly modulate host cell signaling and interfere with host antimicrobial function (Butcher et al., 2005; Saeij et al., 2006; Taylor et al., 2006; Yamamoto et al., 2009, 2011). Ca2+ mobilization is a key regulator of many signaling pathways in immune cells, including those that control granule exocytosis, chemotaxis, and gene transcription and expression (Putney, 2009). A recent study demonstrated Toxoplasma alteration of Ca2+ signaling in neurons during chronic infections (Haroon et al., 2012). Studies of T. gondii invasion in the context of a well-established immune model in which Ca2+ signaling triggers a rapid response, such as mast cell degranulation, are useful to understand mechanisms by which Toxoplasma can modulate Ca2+ signaling.

While there is evidence that peritoneal mast cells mount an immune response to Toxoplasma infection (Ferreira et al., 2004; Sawesi et al., 2010, 2011), mast cells have not been determined to be reservoirs for T. gondii in vivo. However, other immune cell types, such as macrophages, dendritic cells, and neutrophils, are known targets of T. gondii infection (Bierly et al., 2008; Lambert and Barragan, 2010). In all of these cell types, Ca2+-dependent signaling is involved in crucial cellular functions. For example, Ca2+-mediated signaling pathways are involved...
in FcRy-mediated phagocytosis, inflammation, and nitric oxide synthesis in macrophages (Jongstra-Bilen et al., 2008; Braun et al., 2009; Huang et al., 2012), and C-type lectin signaling in dendritic cells relies on phospholipase C (PLC)γ2 (Xu et al., 2009). In response to N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), Ca2+ mobilization by neutrophils is activated via PLCβ (Andersson et al., 1986; Ferretti et al., 2001).

Mast cells express FcεRI, the high affinity receptor for IgE, and they are primary mediators of the allergic response (Metcalf et al., 1997). Crosslinking of IgE-FcεRI complexes on the cell surface by oligovalent antigen is the first step in the cascade of signaling events that results in the exocytosis of preformed mediators, such as histamine and serine proteases, with a time course of minutes (Metcalf et al., 1997). FcεRI belongs to the family of multichain immune recognition receptors (MIRRs) that also include B-cell and T-cell receptors (Cambier, 1995). Signal transduction through FcεRI has been extensively studied by us and others (Holowka et al., 2005; Rivera and Gilfillan, 2006) and involves PLCγ1 and PLCγ2 activation leading to Ca2+ mobilization and protein kinase C (PKC) activation, both of which are necessary for stimulated granule exocytosis (Ma and Beaven, 2009; Holowka et al., 2012).

In the present study we demonstrate that Ca2+ responses are altered in Toxoplasma-infected primary neutrophils, and we utilize the well-established RBL mast cell model system to characterize the mechanism by which Toxoplasma rapidly modulates Ca2+ mediated immune cell signaling. We find that, within an hour of infection, parasites significantly inhibit antigen-mediated degranulation, primarily by inhibition of inositol 1,4,5-trisphosphate (IP3)-dependent Ca2+ mobilization. Additional experiments revealed that PLCγ activation by Syk tyrosine kinase is inhibited by Toxoplasma infection. Finally, we found that inhibition of degranulation prevails under conditions in which inhibition of actin polymerization prevents parasite invasion. Collectively, these results support a model in which T. gondii inhibits FcεRI receptor signaling during invasion by releasing a factor that inhibits Syk mediated activation of PLCγ, and thus interferes with hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to produce the second messengers IP3 and diacylglycerol (DAG) important for Ca2+ mobilization and degranulation.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Indo-1-AM and Fluo-4-AM were purchased from Invitrogen Corp. 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, cytochalasin D, FITC-dextran, and thapsigargin were purchased from Sigma-Aldrich. Unless otherwise noted, all other tissue culture reagents were purchased from Invitrogen, and all other chemicals were purchased from Sigma-Aldrich. Anti-DNP IgE was purified as described previously (Posner et al., 1992). Multivalent antigen, DNP-BSA, was prepared as described previously (Weetall et al., 1993).

CELLS AND PARASITES

RBL-2H3 mast cells

RBL-2H3 mast cells were maintained in monolayer culture through weekly passage as described previously (Gidwani et al., 2003). For stimulation, cells were sensitized with 1 μg/ml anti-DNP IgE for 4–24 h.

Mouse neutrophils

Female C57BL/6 mice (6–8 weeks of age) were purchased from either The Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (Germantown, NY) and were maintained in the Transgenic Mouse Core Facility at the Cornell University College of Veterinary Medicine, accredited by American Association of Accreditation of Laboratory Animal Care. Mouse neutrophils were isolated by percoll gradient purification as described previously (Abi Abdallah et al., 2011).

The experiments in this study were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at Cornell University (permit number 1995-0057). All efforts were made to minimize animal suffering during the course of these studies.

Parasites

RH, P TG, CTG, and RH-tomato strains of parasites were used in this study. The RH-tomato strain, stably expressing tomato fluorescent protein, was generated by Dr. B. Striepen [University of Georgia; kindly provided by Dr. E. Robey (University of California, Berkeley)]. All tachyzoites were maintained in vitro via passage through human foreskin fibroblast cultures in DMEM with FCS (1%), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Fibroblast media). For experiments, tachyzoites were harvested and passed through a 3 μm track etched membrane filter (Whatman) to remove fibroblast debris. Infections were performed at a multiplicity of infection (MOI) of 10:1 unless otherwise indicated and were synchronized by brief centrifugation (200 × g for 4 min).

DEGRANULATION

β-Hexosaminidase release

Cells were sensitized and plated in triplicate at a density of 5 × 105/well and incubated overnight. The next day, cells were washed with fibroblast media and parasites were introduced as described above. For some experiments, 1 μM cytochalasin D was added during infection to prevent invasion. Following infection, cells were washed three times with buffered saline solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5.6 mM glucose, 20 mM HEPES, pH 7.4, 1 mg/ml BSA) and β-hexosaminidase release in response to DNP-BSA was assessed as described previously (Naal et al., 2004).

Live cell degranulation imaging

Live cell degranulation imaging experiments were carried out as described previously (Cohen et al., 2012). Briefly, sensitized cells were plated overnight in 35 mm glass bottom dishes (MatTek) in the presence of FITC-dextran (1 mg/ml) and 5-HT serotonin (0.2 mM). The next day, cells were washed with fibroblast media and infected with RH-tomato tachyzoites as described above. Following 1 h of infection, cells were washed three times with BSS.
Imaging was conducted at 37°C on a Leica SP5 confocal microscope at an image acquisition rate of 1.7 Hz. Cells were monitored for 1 min prior to addition of multivalent antigen, DNP-BSA (10 ng/ml), then monitored for an additional 9 min.

WESTERN BLOTTING
Sensitized, adherent cells were infected as described above. Following infection, cells were stimulated for 0–20 min, and whole cell western blotting samples were prepared and blotted as described previously (Young et al., 2005). Samples were run on Tris-glycine gels under reducing conditions. For assessment of phosphorylation, anti-phosphotyrosine (clone 4G10) (Millipore) was used, and pp72 was identified based on molecular weight. To assess sample loading, blots were reprobed with anti-actin (clone ACTN05; Neomarkers). Antibodies against PLCγ1 (Santa Cruz), PLCγ1-pY783, PLCγ2, and PLCγ2-pY1217 (Cell Signaling Technology) were used to assess PLCγ activity. To determine relative intensity, the normalized ratio of phosphorylated band intensity to loading control was calculated, and all values were then normalized as compared to the intensity of the control sample at 20 min post-stimulation.

INTRACELLULAR Ca2+ MEASUREMENTS
Fluorimetry
Measurement of intracellular Ca2+ mobilization in response to antigen (10 ng/ml DNP-BSA), 200 nM thapsigargin or 10 μM fMLP was carried out in tachyzoite infected cells using indo-1 as a Ca2+ indicator dye as described previously (Smith et al., 2010). Time-integrated responses were determined as the area under the stimulated time course minus the baseline over 400 s, normalized to the maximal response in Triton X-100 lysed cells (Field et al., 2000).

Live cell Ca2+ imaging
Single cell Ca2+ measurements in infected and control cells were conducted using Fluo-4 AM as described previously (Gadi et al., 2011) on the Leica SP5 confocal system with an image acquisition rate of 0.5 Hz. Analysis of Ca2+ oscillations in individual cells was performed using Matlab software (Mathworks). Briefly, code was written to track the location and average fluorescence intensity of the green channel (Fluo-4) within a circular region of interest (ROI) in the cytoplasm of each cell. These measurements were plotted with respect to time, and the number of oscillations for each ROI, reflected by increases in fluorescence intensity, were enumerated.

MEASUREMENTS OF PHOSPHOINOSITIDE (PIP2 AND PIP3) LOCALIZATION
Cells were sparsely plated (1 – 3 × 10^3/ml) on # 1.5 coverslips or in 35 mm glass bottom dishes (MatTek). After overnight culture, cells were transfected with either PH-PLCδ-EGFP (Varnai and Balla, 1998) or PH-Akt-EGFP (Srinivasan et al., 2003) using 2 μg DNA and 8 μl Fugene HD (Roche Diagnostics) in 1 ml OptiMEM for 1 h before addition of 1 ng/ml phorbol 12,13-dibutyrate for 3–5 h to enhance DNA uptake (Gosse et al., 2005). Samples were then washed into full media and cultured for 16–24 h to allow for protein expression.

Transfected cells were infected for 1–2 h with RH-tomato parasites followed by fixation with 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffered saline (PBS) for 10 min at room temperature. Excess fixative was quenched by 10 mg/ml BSA in PBS with 0.01% sodium azide. Fixed cells were imaged on a Leica SP2 confocal system. Line scan analysis of equatorial cross sections using ImageJ (NIH) was performed using average fluorescence values to determine the ratio of the PH domain at the plasma membrane to that in the cytoplasm (Smith et al., 2010).

STATISTICAL ANALYSES
Statistical analysis was performed with Prism software (Graphpad). All bar graphs display mean ± SEM unless otherwise noted. Statistical significance was determined by One-Way ANOVA (Analysis of Variance) followed by Tukey’s post test. Level of significance is denoted as follows: *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

RESULTS
Ca2+ RESPONSES ARE REDUCED IN T. gondii-INFECTED NEUTROPHILS
T. gondii is known to actively modulate signaling in the immune cells it infects (Laliberte and Carruthers, 2008). Ca2+ mobilization is central to many aspects of immune signaling, so we examined the effects of parasite infection on neutrophil Ca2+ responses to the bacterial chemotactic factor, fMLP. Freshly purified mouse neutrophils were isolated, purified and labeled with indo-1 to monitor Ca2+ mobilization stimulated by fMLP following infection by T. gondii. As shown in Figure 1, we found that the neutrophil response to fMLP was inhibited by infection with all types of T. gondii. These data provided initial evidence that Ca2+ signaling in an immune cell type that is an established host target for T. gondii is altered by parasite infection.

Live microscopy is a powerful tool to probe the mechanistic aspects of Ca2+ responses, but our attempts to use this approach in neutrophils was hampered by their relatively short lifespan.
ex vivo and our findings that neutrophil adhesion on poly-L-lysine-coated glass, necessary for imaging, triggered spontaneous activation, including neutrophil extracellular trap formation (Abi Abdallah, unpublished observations; Abi Abdallah et al., 2012). To overcome these technical limitations, we utilized RBL mast cells as a model system more amenable for investigation of signaling mechanisms important for immune cell-mediated Ca²⁺ responses in the context of Toxoplasma infection. Mast cell signaling through the IgE receptor, FcεRI, is a well-studied immune signaling process that occurs on the time scale of minutes. An end result of this signaling is granule fusion and release of histamine, serine proteases and proteoglycans in a process termed degranulation (Blank and Rivera, 2004).

**T. gondii INFECTION RAPIDLY INHIBITS ANTIGEN-MEDIATED MAST CELL DEGRANULATION**

We infected RBL-2H3 mast cells with Toxoplasma type I (RH), II (PTG), or III (CTG or VEG) tachyzoites. After 1 h we assessed the capacity of the infected cells to degranulate in response to multivalent antigen, DNP-BSA. In a bulk assay we found that acute *T. gondii* infection reduced degranulation by approximately 50%, irrespective of the genotype of parasite used (Figure 2A). Under these conditions, infection rates were between 50 and 70% and FcεRI receptor expression on the mast cell surface remained unchanged (N.L. Smith, data not shown). This inhibition directly correlated with multiplicity of infection (MOI), suggesting that inhibition is dependent on infection levels (Figure 2B).

![Figure 2](image-url)
We used RH-tomato parasites in live imaging experiments to assess degranulation by FITC-dextran release (Figure 2C, movie M1). In these experiments, FITC-dextran is taken up into the granules of RBL mast cells. FITC fluorescence is pH sensitive and remains quenched in the acidic environment of the granules. Upon stimulation, granules fuse with the plasma membrane, their contents are exposed to a higher pH and local bursts of FITC-dextran fluorescence are detected (Cohen et al., 2012). We found that infected cells are defective in their response to antigen crosslinking and showed delayed exocytosis. Neighboring uninfected cells responded robustly to antigen as seen by bursts of FITC-dextran fluorescence from granules (Figure 2C, arrow heads) and apparent flattening and ruffling of the cells (movie M1). Taken together, these results indicate that *T. gondii* inhibits Ca$^{2+}$ signaling.

**FIGURE 3 | *T. gondii* infection reduces antigen-mediated Ca$^{2+}$ responses in RBL mast cells.** (A) Representative indo-1 fluorescence measurements of Ca$^{2+}$ responses to multivalent antigen (10 ng/ml DNP-BSA) in control uninfected (black), Type I-infected (red), Type II-infected (blue) or Type III-infected (green) infected cells (MOI 10:1). (B) Integrated Ca$^{2+}$ responses over 300 s of stimulation as a percentage of control Ca$^{2+}$ response. Histogram shows averages for 3–5 independent experiments. (C) Live Fluo-4 (green) Ca$^{2+}$ imaging in uninfected or RH-tomato (red) infected RBL-cells. (see supporting movies M2 and M3). Quantification of the percentage of responding cells (D) or average number of Ca$^{2+}$ oscillations per cell in 10 min (E) in live Ca$^{2+}$ imaging experiments (*n* = 174 cells over 3 experiments). Error bars represent SEM (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 relative to uninfected cells).
mast cell degranulation, and this inhibition directly correlates with infection.

**CA2+ MOBILIZATION IN RESPONSE TO ANTIGEN IS REDUCED IN Toxoplasma-INFECTED RBL-2H3 MAST CELLS**

Ca2+ release from ER stores is mediated by binding of IP3 to its receptors in the ER membrane. This event triggers coupling of the ER-localized Ca2+ sensor, STIM1, and the plasma membrane Ca2+ channel Orai1, resulting in additional Ca2+ influx from extracellular space, a process known as store operated Ca2+ entry (SOCE). These events are important downstream steps in the pathway that leads to degranulation in mast cells (Di Capite and Parekh, 2009; Holowka et al., 2012). Measurement of intracellular Ca2+ transients reveals that infection by *T. gondii* significantly reduces the Ca2+ response to antigen (Figure 3A). Integration of Ca2+ responses over 5 min shows a 39% reduction in Ca2+ mobilization in 3–5 independent experiments, averaged over all parasite types (Figure 3B).

In these bulk population measurements, it is difficult to evaluate what percent of infected cells are inhibited. We directly addressed this question by conducting live cell imaging experiments to evaluate Ca2+ responses in individual cells. Figure 3C, top panel, and movie M2 show that control, uninfected cells exhibit robust Ca2+ signaling in response to antigen, with 94% of the cells responding with an average of 12 oscillations during a 10-min period. In contrast, as shown in Figure 3C, bottom, and movie M3, the RH-tomato infected cells are much less responsive, such that only 58% of cells containing one or more parasites respond, typically with a slower onset and with an average of 6 oscillations over the same time period. These parameters are compared in Figures 3D,E.

It is known that intracellular parasites interact extensively with the ER (Sinai and Joiner, 1997), and one possibility is that these interactions somehow block the exit of Ca2+ from the ER during antigen-stimulated, IP3-dependent depletion of ER Ca2+. Receptor-mediated, IP3-dependent release of Ca2+ from ER stores can be bypassed by treating cells with thapsigargin, a SERCA pump inhibitor, resulting in passive leakage of Ca2+ from the ER that activates SOCE. Under these conditions, cells infected with all types of *T. gondii* have Ca2+ responses comparable to uninfected cells (Figures 4A,B), showing that *T. gondii* is not directly blocking SOCE. These results contrast with those of Haroon and colleagues, who showed reduced thapsigargin-mediated Ca2+ responses in Toxoplasma-infected mouse neurons (Haroon et al., 2012). Collectively, our results indicate that a common factor, shared by the three genotypes of parasite, inhibits granule exocytosis via a mechanism that inhibits Ca2+ mobilization upstream of SOCE.

**PLCγ ACTIVATION IS REDUCED IN Toxoplasma-INFECTED RBL-2H3 CELLS**

Antigen stimulation of FcεRI activates phospholipase Cγ (PLCγ), resulting in hydrolysis of PIP2 to DAG and IP3. This, in turn, triggers Ca2+ release from ER stores via IP3 receptors at the ER. One possible mechanism of inhibition of this process is that intracellular parasites might sequester PIP2, such that it is no longer available as a substrate for PLCγ. To address this possibility, we assessed whether infection by *Toxoplasma* alters the plasma membrane association of GFP-tagged PH-PLCδ that is highly specific for PIP2 (Ferguson et al., 1995). We found no appreciable differences in the abundance of PIP2 availability at the plasma membrane under these conditions (Figures A1A,B).

As phosphoinositide availability at the plasma membrane does not appear to be significantly changed in infected RBL cells, we next asked whether the parasite-mediated inhibition is due to a defect in PLCγ activation. For PLCγ to enzymatically cleave PIP2, it must be recruited to the plasma membrane and phosphorylated at specific tyrosine residues. Western blotting with anti-pY1217-PLCγ2 shows that antigen-stimulated phosphorylation at this residue is reduced in all infected samples (Figure 5A). At 10 min post-stimulation in the presence of types I, II and III
parasites, Y1217-PLCγ2 phosphorylation is reduced by >65% in at least 3 independent experiments (Figure 5B). PLCγ1, like PLCγ2, shows reduced phosphorylation at its activating tyrosine, Y783 (N.L. Smith, data not shown).

To further assess the activity of PLCγ, we asked if antigen-mediated IP3 generation was reduced in T. gondii infected RBL cells. Specifically, we examined Ca2+ mobilization in the absence of extracellular Ca2+ as a measure of PLCγ-dependent Ca2+ release from ER stores. Figure 5C is a representative experiment that shows all parasite types significantly reduce the amount of Ca2+ released from ER stores in response to antigen. Over multiple experiments, parasite infection reduced the Ca2+ release from ER stores by 37, 50, and 49% for Types I, II, and III, respectively (Figure 5D).

**SYK KINASE ACTIVITY IS REDUCED BY T. gondii INFECTION**

Following crosslinking of IgE/FcεRI complexes by multivalent antigen, tyrosine residues within ITAMs are phosphorylated in the cytoplasmic segments of the β and γ subunits of FcεRI. This, in turn, recruits and activates Syk tyrosine kinase. Syk kinase has a number of downstream targets, including PLCγ. Therefore, we asked if antigen stimulation of the phosphorylation of additional Syk substrates, detected as pp72 (Benhamou et al., 1993), is altered in infected cells. As we saw for PLCγ, stimulated phosphorylation of Syk substrate pp72 is also reduced in infected cells (Figures 6A,B). These results suggest that inhibition of PLCγ-mediated hydrolysis of PIP2 is due to reduction in the activation of Syk kinase.
FIGURE 6 | Tyrosine phosphorylation of pp72 Syk substrate is reduced in T. gondii infected cells. IgE-sensitized RBL cells were infected for 1 h with Type I, II or III tachyzoites as indicated. Antigen-stimulated cells were lysed at 0, 5, or 10 min after addition of multivalent antigen (10 ng/ml DNP-BSA). (A) Representative blot: Top panel shows phosphorylation of pp72 Syk substrate. Bottom panel shows loading control (α-tubulin) (B) Quantification of Syk substrate band intensity. Error bars represent SD of 4 independent experiments (*P < 0.05, **P < 0.01, and ****P < 0.0001 relative to uninfected cells).

INHIBITION OF MAST CELL SIGNALING BY T. gondii REQUIRES PARASITE ATTACHMENT, BUT NOT ENTRY

Our single cell Ca^{2+} and degranulation measurements indicate that the inhibitory action of T. gondii requires direct contact between the host cell and parasite and possibly parasite entry. To more directly address these issues, we compared degranulation responses in RBL cells infected with the Type I parasites to responses to cells that were incubated with the supernatant from an equivalent number of parasites (Type I supernatant). As shown in Figure 7A, RH supernatant did not inhibit antigen-stimulated degranulation under conditions in which infection by intact parasites was effective (+ Type I vs. Type I supernatant). Additionally, degranulation is not inhibited by heat-killed parasites, fixed parasites or by supernatants from infected fibroblasts (N.L. Smith, data not shown).

Parasite entry depends primarily on the parasite actin cytoskeleton (Dobrowolski and Sibley, 1996; Hakansson et al., 2001). Therefore, infections carried out in the presence of the inhibitor of actin polymerization, cytochalasin D, result in a frustrated state where parasites attach and secrete proteins into the host cell but do not complete invasion (Hakansson et al., 2001). Cytochalasin D is known to enhance degranulation responses of RBL cells to antigen (Frigeri and Apgar, 1999), and, as expected, we see robust stimulated degranulation under these conditions (Figure 7A, Control + Cyto D). However, degranulation in the presence of cytochalasin D and parasites is still significantly reduced compared to cytochalasin D treatment alone (Figure 7A). The average inhibition over three independent experiments is 32%. Microscopic observations under these conditions confirmed that infection rates in the presence of cytochalasin D were extremely low: 4% in cytochalasin D treated samples compared to approximately 70% under control conditions (Figure 7B). These results indicate that inhibition under these conditions is due to attached, but not intracellular parasites. Although we cannot rule out the possibility that the attached parasite directly inhibits the host’s tyrosine kinase activity without entering the cell, it is more likely that the agent responsible for mediating inhibition of mast cell signaling is secreted into the cell at the initiation of the invasion process.

DISCUSSION

Based on its global prevalence and its capacity to infect a multitude of hosts, T. gondii is regarded as one of the world’s most successful parasites. It now appears that the success of this parasite...
effector cells, including neutrophils, macrophages and dendritic cells. Accordingly, we found that this infection suppresses receptor-mediated Ca²⁺ mobilization and continues over several tens of minutes. Intracellular Ca²⁺ levels are tightly regulated, and elevation of intracellular Ca²⁺ in granule exocytosis and cytokine production (Holowka et al., 2006). MAPK activity leading to IL-12 production (Masek et al., 2006).

In vivo, T. gondii is known to preferentially infect immune effector cells, including neutrophils, macrophages and dendritic cells (Denkers and Butcher, 2005; Bierly et al., 2008). Accordingly, we found that this infection suppresses receptor-mediated Ca²⁺ mobilization in murine neutrophils (Figure 1). To further characterize the mechanism of such effects, we chose the well-studied immune signaling model, FcεRI in mast cells, to address the molecular basis of T. gondii effects on acute immune signaling events that rely on Ca²⁺ signaling. FcεRI-triggered degranulation in mast cells begins within minutes of stimulation and continues over several tens of minutes. Intracellular Ca²⁺ levels are tightly regulated, and elevation of intracellular Ca²⁺ is critical in immune responses in multiple cell types and contexts (Andersson et al., 1986; Penner and Neher, 1988; Putney, 2009). Host Ca²⁺ responses in macrophages have been implicated in regulating the initial recognition of T. gondii that results in MAPK activity leading to IL-12 production (Masek et al., 2006). Furthermore, intracellular parasites are sensitive to host Ca²⁺ responses, and exogenous stimulation Ca²⁺ influx by treatment with Ca²⁺ ionophore triggers tachyzoite egress (Caldas et al., 2007).

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Ca²⁺ mobilization is a central and well-studied aspect of IgE/FcεRI-mediated signaling in mast cells, including its role in granule exocytosis and cytokine production (Holowka et al., 2012). Our initial experiments revealed that parasite infection suppresses degranulation responses in RBL cells (Figure 2), and thus its inhibition of Ca²⁺ mobilization was a likely suspect. We showed that parasite infection blocks antigen-triggered, but not thapsigargin-triggered Ca²⁺ elevation (Figures 3, 4), indicating that infection acts upstream of Ca²⁺ release from stores. Inhibition of Ca²⁺ mobilization by T. gondii in the absence of extracellular Ca²⁺ further supports this explanation (Figure 5). Hydrolysis of PIP2 mediated by PLCγ to produce IP3 and DAG is critically important in Ca²⁺ mobilization by antigen in mast cells. One possible mechanism to account for decreased Ca²⁺ store release in response to antigen is that the parasite alters the amount or availability of PLCγ’s substrate, PIP2, at the plasma membrane. However, our results are inconsistent with this explanation, as we detect no significant change in PIP2 levels at the plasma membrane due to infection by T. gondii. We do, however, observe a reduction in the level of activating tyrosine phosphorylation of PLCγ during infection (Figure 5), suggesting that Toxoplasma is reducing the host’s capacity to hydrolyze PIP2.
PLCγ1 activity in mast cells is regulated by PI3-kinase-mediated production of PIP3 (Barker et al., 1998), but our results indicate that the parasite does not significantly change the level of PIP3 in infected cells (Figures A1C,D). Rather, our findings that phosphorylation of Syk substrates pp72, as well as PLCγ, are reduced in infected RBL cells, point to inhibition of the tyrosine kinase signaling cascade that culminates in PIP2 hydrolysis as the most immediate consequence of T. gondii infection. The earliest target in this cascade is not yet clear, as T. gondii infection caused some reduction in FceRI ITAM phosphorylation by Lyn kinase that was not statistically significant (N.L. Smith, unpublished observations). Our data collectively suggest a model in which one or more T. gondii-derived proteins act directly to reduce the activity of PLCγ by inhibiting Syk activation, thereby reducing the levels of IP3 and inhibiting subsequent signaling steps (Figure 8).

Furthermore, our results suggest that a secreted product from T. gondii mediates this inhibition at an early step during the invasion process. Previous work determined that invasion is a multi-step process (Carruthers and Boothroyd, 2007), and, in early steps, parasites attach to the host plasma membrane and release the contents of the rhoptries into the cell (Hakansson et al., 2001). Quantitative trait locus analysis revealed that rhoptry proteins, including ROP16 and ROP18, which are secreted during invasion, are key virulence factors in T. gondii infection (Saeij et al., 2006; Taylor et al., 2006). Recently, ROP16 has been shown to directly phosphorylate STAT molecules (Yamamoto et al., 2009; Ong et al., 2010). However, lack of parasite strain specificity argues against a role for ROP16 and ROP18 in the effects reported here, as there are documented differences in the activity of these ROP proteins in the three types of parasites evaluated (Saeij et al., 2006; Taylor et al., 2006; Boyle et al., 2008). Furthermore, we tested ROP16 null parasites and found them equally capable of blocking Ca\(^{2+}\) responses (N.L. Smith, unpublished observations). Nevertheless, ROP protein early release, relation to virulence, and immunomodulatory capabilities make these proteins attractive candidates for the Toxoplasma secreted factor responsible for the reduction in Ca\(^{2+}\) mobilization (Ong et al., 2010; Butcher et al., 2011). Future work will focus on identifying which parasite-derived protein(s) are responsible for the inhibition we observe.

We also note that while our results indicate that Toxoplasma inhibits mast cell immune responses, this is not contradictory to reports that mast cell responses contribute to the primary host response to Toxoplasma in vivo (Ferreira et al., 2004; Sawesi et al., 2010). Our data show that PLCγ-mediated responses are reduced by Toxoplasma infection in vitro. Furthermore, our findings that PLCβ-mediated neutrophil responses are inhibited by Toxoplasma infection (Figure 1), as are voltage-gated neuronal Ca\(^{2+}\) responses (Haroon et al., 2012), indicate that there are likely multiple mechanisms employed by T. gondii to subvert normal Ca\(^{2+}\) signaling. In future studies it will be important to assess whether inhibition of Ca\(^{2+}\) signaling is manifest in other immune cells infected by T. gondii, as well as the mechanisms utilized. Collectively, our results indicate that T. gondii targets Syk-dependent PLCγ activation as one mechanism to interfere with immune signaling that depends on Ca\(^{2+}\) mobilization.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Microbial_Immunology/10.3389/fmicb.2013.00179/abstract

Movie M1 | Live Imaging of RBL cell degranulation shows reduced responses in RH-tomato (red) infected cells compared to neighboring uninfected cells. Images were acquired at a rate of 1.7 Hz. Multivalent antigen (10 ng/ml DNP-BSA) was added when indicated in movie and degranulation events are observed as FITC-dextran bursts (green). Movie plays at 10 × speed.

Movie M2 | Live Ca\(^{2+}\) imaging in uninfected RBL cells shows robust response to antigen. RBL cells were loaded with fluo-4 (green) and imaged at a rate of 0.5 Hz. Multivalent antigen (10 ng/ml DNP-BSA) was added when indicated in movie. Movie plays at 12 × speed.

Movie M3 | Live Ca\(^{2+}\) imaging in RH-tomato (red) infected RBL cells show reduced response to antigen. RBL cells were loaded with fluo-4 (green) and imaged at a rate of 0.5 Hz. Multivalent antigen (10 ng/ml DNP-BSA) was added when indicated in movie. Movie plays at 12 × speed.

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APPENDIX

FIGURE A1 | PIP$_2$ and PIP$_3$ levels at the plasma membrane are not altered by *T. gondii* infection. (A) Representative images showing uninfected (top) or RH-tomato infected (bottom) RBL cells transiently transfected with PH-PLC$\delta$-EGFP. Accompanying fluorescence intensity profiles are taken along the white lines indicated in each image. (B) Ratio of plasma membrane to cytoplasmic PH-PLC$\delta$-EGFP fluorescence [given by intensity profiles as shown in (A)] as a relative measure of PIP$_2$ at the plasma membrane. (C) Representative images showing uninfected (top) or RH-tomato infected (bottom) RBL cells transiently transfected with PH-Akt-EGFP. Accompanying fluorescence intensity profiles are taken along the white lines indicated in each image. (D) Ratio of plasma membrane to cytoplasmic PH-Akt-EGFP fluorescence as a relative measure of PIP$_3$ at the plasma membrane. Data in (B) and (D) represent at least 30 cells for each condition collected over 3 experiments. Error bars indicate SEM.