Toxoplasma gondii-Induced Activation of EGFR Prevents Autophagy Protein-Mediated Killing of the Parasite

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Abstract

Toxoplasma gondii resides in an intracellular compartment (parasitophorous vacuole) that excludes transmembrane molecules required for endosome - lysosome recruitment. Thus, the parasite survives by avoiding lysosomal degradation. However, autophagy can re-route the parasitophorous vacuole to the lysosomes and cause parasite killing. This raises the possibility that T. gondii may deploy a strategy to prevent autophagic targeting to maintain the non-fusogenic nature of the vacuole. We report that T. gondii activated EGFR in endothelial cells, retinal pigment epithelial cells and microglia. Blockade of EGFR or its downstream molecule, Akt, caused targeting of the parasite by LC3² structures, vacuole-lysosomal fusion, lysosomal degradation and killing of the parasite that were dependent on the autophagy proteins Atg7 and Beclin 1. Disassembly of GPCR or inhibition of metalloproteinases did not prevent EGFR-Akt activation. T. gondii micronemal proteins (MICs) containing EGF domains (EGF-MICs; MIC3 and MIC6) appeared to promote EGFR activation. Parasites defective in EGF-MICs (MIC1 ko, deficient in MIC1 and secretion of MIC6; MIC3 ko, deficient in MIC3; and MIC1-3 ko, deficient in MIC1, MIC3 and secretion of MIC6) caused impaired EGFR-Akt activation and recombinant EGF-MICs (MIC3 and MIC6) caused EGFR-Akt activation. In cells treated with autophagy stimulators (CD154, rapamycin) EGFR signaling inhibited LC3 accumulation around the parasite. Moreover, increased LC3 accumulation and parasite killing were noted in CD154-activated cells infected with MIC1-3 ko parasites. Finally, recombinant MIC3 and MIC6 inhibited parasite killing triggered by CD154 particularly against MIC1-3 ko parasites. Thus, our findings identified EGFR activation as a strategy used by T. gondii to maintain the non-fusogenic nature of the parasitophorous vacuole and suggest that EGF-MICs have a novel role in affecting signaling in host cells to promote parasite survival.

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects around a third of the human population worldwide. T. gondii is of clinical importance because it causes encephalitis in immunocompromised individuals and retino-chorioiditis in immunocompetent and immunosuppressed patients. T. gondii can also cause congenital infection that may result in cerebral and ocular disease. Tachyzoites of T. gondii infect virtually any nucleated cell through active invasion. This process is dependent on the parasite actin-myosin motor and sequential secretion of proteins from micronemes and rhoptries, specialized organelles present in the apical end of the parasite [1]. Once secreted, T. gondii micronemal proteins (MICs) are expressed at the parasite surface membrane and they interact with host cell receptors [2]. MICs contain adhesive domains such as type I chormbospodin repeats, apple domains, EGF repeats and integrin A domains [3,4]. The connection between transmembrane MICs to the actin-myosin motor (glideosome) of the parasite together with the binding of host cell receptors by MICs is considered to enable the organism to penetrate host cells [5,6]. Following the release of MICs, rhoptries secrete rhoptry neck proteins (RONs) that are critical for the formation of a structure called the moving junction (MJ) [7,8]. The MJ anchors the parasite to the host cell while the parasite penetrates it. The MJ is also believed to function as a sieve that excludes host type I transmembrane proteins from entering the PV membrane [PVM] [8,9]. The end result is the formation of a parasitophorous vacuole that is devoid of host proteins required for recruitment of endosomes and lysosomes [10].

T. gondii cannot withstand the lysosomal environment. Thus, the non-fusogenic nature of the PV is critical since it allows the parasite to survive and replicate. The immune system can deprive
Toxoplasma gondii resides in a parasitophorous vacuole that excludes transmembrane proteins required for recruitment of endosomes and lysosomes and thus, does not follow the path of classical lysosomal degradation. However, the non-fusogenic nature of the vacuole can be reverted when autophagy, a pathway to lysosomal degradation, is upregulated through the immune system or pharmacologically. Maintenance of the non-fusogenic nature of the vacuole is central to parasite survival. Thus, in addition to preventing degradation through a classical lysosomal pathway, T. gondii may also deploy strategies to prevent constitutive levels of autophagy from targeting the pathogen and causing its lysosomal degradation. We report that T. gondii accomplishes this task by causing EGFR activation in host cells. In cells that were not subjected to immune or pharmacologic upregulation of autophagy, blockade of EGFR resulted in parasite encasing by structures that expressed the autophagy protein LC3, vacuole-lysosomal fusion and autophagy protein-dependent killing of the parasite. Moreover, EGFR signaling also impaired targeting of the parasite by LC3+ structures in cells treated with stimulators of autophagy. Studies with T. gondii deficient in EGF domain containing-micronemal proteins (EGF-MICs) and recombinant EGF-MICs support the concept that these parasite adherins contribute to EGFR activation.

The highly successful nature of T. gondii as a pathogen together with evidence that the autophagy pathway can trigger lysosomal killing of the pathogen raise the possibility that T. gondii prevents autophagic targeting of the PV to maintain the non-fusogenic nature of the PV. Moreover, approximately 25–35% of various CD40+ cells subjected to CD40 ligation are unable to kill T. gondii further suggesting that the parasite may utilize mechanism(s) to prevent induction of autophagic killing. Here we report that maintenance of the non-fusogenic nature of the PV requires T. gondii-induced activation of EGFR-Akt, a signaling cascade that prevents autophagy protein-dependent vacuole-lysosomal fusion, lysosomal degradation and killing of the parasite. Blockade of EGFR-Akt may prove of therapeutic benefit for toxoplasmosis since it is sufficient to induce killing of the parasite without the need for immune-induced activation of host cells.

**Results**

T. gondii induces rapid Akt activation in non-hematopoietic cells through phosphatidylinositol 3-kinase (PI3K)

We determined whether Akt is quickly activated by T. gondii during infection of various non-hematopoietic cells. Activation of Akt is a multistep process where phosphorylation of Serine 473 results in full activation of the molecule [27]. Primary human brain microvascular endothelial cells (HBMEC) were infected with either type I (RH) or type II (ME49) strains of T. gondii under conditions that caused synchronized infection. T. gondii infection resulted in an enhanced phosphorylation of Akt Serine 473 as assessed by immunoblot (Figure 1A). Similar results were obtained with a mouse endothelial cell line mHEVc (Figure 1B). T. gondii also caused Akt phosphorylation in a human retinal pigment epithelial (RPE) cell line, an effect that decreased at later time points post-infection (Figure 1C). We assessed whether viable parasites are required to induce activation of Akt. HBMEC were challenged with live or killed parasites followed by determination of Akt activation. Viable but not killed tachyzoites induced Akt phosphorylation (Figure 1D). Activation of phosphatidylinositol 3-kinase (PI3K) with resulting production of phosphatidylinositol 3,4,5 trisphosphate (PIP3) production is a major trigger of Akt activation [28]. The amino-terminal pleckstrin homology (PH) domain of Akt mediates recruitment of this molecule to plasma membrane containing increased PI(3,4,5)P3 or PI(3,4)P2 [29]. Indeed, the PH domain of Akt fused to GFP (PH-Akt-GFP) has been used as a probe to examine sites of PIP3 accumulation [30]. HBMEC were transiently transfected with a plasmid encoding PH-Akt-GFP followed by challenge with RH T. gondii that express cytoplasmic RFP (T. gondii-RFP). T. gondii-infected cells exhibited accumulation of PH-Akt-GFP around the parasite (Figure 1E). To examine the role of PI3K in this process, HBMEC were incubated with or without LY294002, a specific PI3K inhibitor, followed by challenge with the parasite. LY294002 did not affect the percentage of infected cells (not shown). Accumulation of PH-Akt-GFP around T. gondii was ablated by LY294002 (p<0.01) (Figure 1E). Moreover, incubation with LY294002 impaired the upregulation of Akt phosphorylation induced by T. gondii, especially in the earlier time points post-infection (Figure 1F). Similarly, Akt phosphorylation during T. gondii infection was impaired in HBMEC transfected with siRNA against the PI3K catalytic subunit p110α (Figure 1G). Taken together, these findings indicate that T. gondii induces rapid Akt activation in non-hematopoietic cells in a manner that is dependent on PI3K.

Blockade of Akt induces accumulation of the autophagy protein LC3 around the parasite, vacuole-lysosomal fusion and killing of T. gondii dependent on autophagy proteins

We performed studies to investigate whether blockade of Akt signaling promotes killing of T. gondii. HBMEC were incubated with or without Akt inhibitor IV followed by challenge with T. gondii. The percentage of infected cells at 2 hours and 24 hours post-challenge were determined. Akt inhibitor IV did not impair the percentage of infected cells at 2 h (Figure 2A). However, treatment with Akt inhibitor IV markedly reduced the percentage...
of infected cells at 24 h (p<0.01) (Figure 2A). Changes in the percentage of infected cells were not due to preferential cell loss in Akt inhibitor IV-treated cells since cell densities as determined with an eyepiece grid were similar in all experimental groups and inhibition of Akt did not induce a detectable increase in apoptosis of *T. gondii*-infected cells (not shown). Akt inhibitor IV not only induced a significant decrease in the numbers of parasites per 100 HBMEC at 24 h but it also caused a profound reduction in the numbers of *T. gondii*-containing vacuoles per 100 HBMEC (p<0.01) (Figure 2A, Figure S1A). Similar results were obtained...
Figure 2. Blockade of Akt induces accumulation of the autophagy protein LC3 around the parasite, vacuole-lysosome fusion and killing of *T. gondii* dependent on the autophagy proteins. A, HBMEC, mHEVC and human RPE cells were incubated with or without Akt inhibitor IV (1.25 µM) for 1 h prior to challenge with *T. gondii*. Monolayers were examined by light microscopy 2 h and 24 h post-challenge. B, HBMEC were transfected with control siRNA or Akt siRNA. Cells were then challenged with *T. gondii* 48 h after transfection. Monolayers were examined microscopically 24 h post-challenge. C, RAW 264.7 were incubated with or without Akt inhibitor IV for 1 h prior to challenge with *T. gondii*. Monolayers were examined by light microscopy 2 h and 24 h post-challenge. D, mHEVC-LC3-EGFP cells were incubated with or without Akt inhibitor IV followed by challenge with *T. gondii*-RFP. Monolayers were examined by fluorescence microscopy 5 h post-challenge. Arrowheads indicate accumulation of LC3 around the parasite. E, HBMEC were treated with or without Akt inhibitor IV for 1 h prior to challenge with *T. gondii* (T) and then
with mouse endothelial cells (mHEVc; Figure 2A, Figure S1A) and human RPE cells (p<0.01) (Figure 2A, Figure S1A). Not only pharmacologic inhibition of Akt but also Akt knockdown in HBMEC reduced the parasite load and the number of T. gondii-containing vacuoles (p<0.01) (Figure 2B, Figure S1A). The vacuoles that persisted after Akt knockdown had similar numbers of parasites as those from control cells (Figure S1B). These results indicate that blockade of Akt caused parasite killing. T. gondii infection causes Akt activation in macrophages [31]. Similar to endothelial and epithelial cells, treatment with Akt inhibitor IV caused anti-T. gondii activity in the mouse macrophage line RAW 264.7 and in mouse microglia line BV-2 (p<0.01) (Figure 2C and not shown). These findings revealed an important role of Akt activation in promoting survival of T. gondii within host cells.

T. gondii survives within mammalian cells by avoiding delivery of the lysosomal contents into the parasitophorous vacuole [32–34]. Akt is a negative regulator of autophagy [35], a cellular mechanism that results in lysosomal degradation and killing of T. gondii [13–15]. First, we examined T. gondii-infected cells after Akt inhibition to determine the distribution of LC3, a protein associated with the autophagosome membrane. mHEVc-LC3-EGFP cells were treated with or without Akt inhibitor IV and challenged with T. gondii-RFP. Akt inhibitor IV led to significant accumulation of LC3 around the parasite (p<0.01) (Figure 2D).

Electron microscopy studies were performed since a double membrane isolation membrane that encircles portions of cytoplasm or organelles is formed during autophagy [16]. Indeed, a double membrane structure was noted around the parasitophorous vacuole membrane in HBMEC treated with Akt inhibitor IV (Figure 2E). Next, we examined the effects of Akt inhibition on the distribution of the late endosomal/lysosomal molecule LAMP-1. Endothelial cells were incubated with or without Akt inhibitor IV, challenged with T. gondii-YFP followed by staining with anti-LAMP-1 mAb. Treatment with Akt inhibitor IV resulted in a remarkable increase in the percentage of parasites surrounded by LAMP-1 (p<0.01) (Figure 2F). To explore whether the killing of T. gondii during inhibition of Akt is dependent on the autophagy machinery, we examined the effects of knockdown of the autophagy proteins Beclin 1 or Atg7 on T. gondii survival. Transfection with Beclin 1 siRNA or Atg7 siRNA effectively diminished expression of Beclin 1 or Atg7 respectively (Figure 2G, H). Endothelial cells transfected with Beclin1 siRNA or Atg7 siRNA were incubated with or without Akt inhibitor and challenged with T. gondii. Cells transfected with Beclin1 siRNA (Figure 2G) or Atg7 siRNA (Figure 2H) were unable to control the parasite in the presence of the Akt inhibitor IV. Since autophagosomes deliver their contents to lysosomes for degradation, we examined the role of lysosomal degradation in killing of T. gondii utilizing the lysosomal protease inhibitors leupeptin and pepstatin. mHEVc and RPE cells were treated with or without Akt inhibitor IV and infected with T. gondii. 1 h post infection cells were treated with or without leupeptin plus pepstatin. Lysosomal protease inhibitors impaired the anti-T. gondii activity induced by Akt inhibition (p<0.05) (Figure 2I and not shown). Finally, the anti-T. gondii activity induced by Akt inhibitor IV in mouse microglia and human RPE cells was impaired by 3-methyl adenine, an inhibitor of autophagy (p<0.05) (Figure 2J and not shown). Together, these results indicate that T. gondii-induced Akt activation is critical to promote parasite survival because it prevents killing of T. gondii dependent on the autophagy pathway and lysosomal protease activity.

T. gondii infection induces EGFR activation in mammalian cells that prevents autophagy pathway dependent killing of the parasite

Akt activation classically occurs downstream of cell membrane receptors that include growth factor receptors, G protein-coupled receptor (GPCR) and TLR [36]. To examine the role of GPCR in Akt activation in non-hematopoietic cells, HBMEC were incubated with or without Pertussis toxin (PTx), an inhibitor of GPCR signaling, followed by challenge with T. gondii tachyzoites. PTx did not affect the initial percentage of infected cells (data not shown). Incubation with PTx decreased basal Akt phosphorylation. However, PTx did not prevent the increased Akt phosphorylation induced by T. gondii (Figure 3A) indicating that T. gondii can activate Akt independently of GPCR signaling. In contrast, PTx inhibited Akt activation induced by lysophosphatidic acid (LPA), a GPCR ligand [37] (Figure 3A). To examine the potential role of TLR signaling in Akt, MyD88 was knocked-down in HBMEC using siRNA. Knockdown of MyD88 did not affect T. gondii-induced Akt activation (Figure 3B). In contrast, as assessed by FACs, the ICAM-1 upregulation induced by LPS (1 μg/ml) in HBMEC was inhibited in cells transfected with MyD88 siRNA compared to those transfected with control siRNA (cMFI: Control siRNA = 10,682±1,053; MyD88 siRNA = 3,250±527; p<0.05). These studies indicate that GPCR and TLR are unlikely to play a major role in Akt phosphorylation induced by T. gondii in non-hematopoietic cells.

Relevant to the possibility of activation of growth factor receptors during T. gondii-host cell interaction is the fact that host cell invasion by T. gondii requires the secretion of parasite micronemal proteins (MICs) with the potential to activate such receptors [38]. MICs exist as multiprotein complexes, the most important being MIC1/4/6, MIC3/8, MIC2/M2AP, and a complex of the microneme protein TgAMA1 with rhoptry neck proteins RON2/RON4/RON6/RON8 [39–41]. MIC3, MIC6 and MIC8 have multiple domains with homology to EGF [42] and are therefore termed EGF-MICs. As an initial experiment, we examined whether T. gondii induces autophosphorylation at 2 major tyrosine residues of EGFR (1068 and 1148). HBMEC were incubated with RH T. gondii tachyzoites followed by determination of EGFR phosphorylation by immunoblot. T. gondii induced activation of EGFR, as indicated by phosphorylation of tyrosine residue 1068 (Figure 4A). Moreover, the parasite caused phosphorylation of tyrosine residue 1148, a site that appears to be phosphorylated only by ligand binding to EGFR [43] (Figure 4A). Similar results were found using the ME49 strain of Toxoplasma, EGFR-Akt and Autophagy.
EGFR activation occurred in HBMEC upon challenge with viable but not killed parasites (Figure 4B). EGFR autophosphorylation was not only observed in endothelial cells but also in human RPE cells and mouse microglia incubated with *Toxoplasma* (Figure 4C, 4D). Thus, *Toxoplasma* causes EGFR activation in various mammalian cells.

Next, we examined whether EGFR signaling is involved in activation of Akt triggered by *Toxoplasma*. Endothelial cells were transiently transfected with a plasmid that encodes either control siRNA or EGFR siRNA followed by challenge with *Toxoplasma*. The efficiency of EGFR knockdown was confirmed by immunoblot (Figure 5A). EGFR knockdown ablated the ability of *Toxoplasma* to induce activation of Akt at all time points tested (Figure 5A). Next, we explored the role of EGFR signaling on Akt activation in professional phagocytes. Mouse microglia were treated with vehicle or AG1478, a pharmacological inhibitor of EGFR kinase activity, followed by challenge with *Toxoplasma*. Inhibition of EGFR kinase activity ablated parasite-induced Akt activation in mouse microglia (Figure 5B).

We assessed whether EGFR activation affects *Toxoplasma* survival within host cells. HBMEC were treated with vehicle or AG1478

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**Figure 3. Role of G protein coupled receptors and MyD88 in *T. gondii*-induced Akt phosphorylation.** A, HBMEC were treated with or without pertussis toxin (PTx; 100 ng/mL) for 4 h prior to challenge with *T. gondii*. Cell lysates were obtained at 5, 15 or 30 min post-incubation with *T. gondii* and used to examine total Akt and phospho-Akt serine 473 by immunoblot. Densitometry data represent means ± SEM of 3 experiments. A vertical line was inserted between densitometry data from control and PTx-treated cells to indicate that band densities from infected cells treated with or without PTx were compared to bands from their respective uninfected cells, which were given an arbitrary number of 1. HBMEC were also treated with or without LPA (10 μM) in the presence or absence of PTx. Cell lysates were obtained at 5 min and subjected to immunoblotting. B, HBMEC were transfected with MyD88 siRNA or control siRNA followed by challenge with *T. gondii* after 48 h. Cell lysates were used to examine total Akt and phospho-serine 473 Akt by immunoblot. Results shown are representative of 3 independent experiments.

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**Figure 4. *T. gondii* infection induces EGFR activation in mammalian cells.** A, HBMEC cells were challenged with RH *T. gondii* and cell lysates were obtained to probe for total EGFR, phospho-tyrosine 1068 EGFR and phospho-tyrosine 1148 EGFR by immunoblot. B, HBMEC were challenged with live vs killed tachyzoites. Total EGFR and phospho-tyrosine 1068 EGFR expression was examined by immunoblot. C, D, Human RPE cells (C) and mouse microglia (D) were challenged with RH *T. gondii* and total EGFR and phospho-tyrosine 1068 EGFR expression was examined by immunoblot. Results shown are representative of 3 independent experiments.

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followed by challenge with *T. gondii*. While AG1478 did not affect the percentage of infected cells at 2 h, AG1478 caused a marked reduction in the percentage of infected cells 24 h post-challenge (*p* < 0.05) (Figure 6A). In addition, there was a significant reduction in the numbers of parasites per 100 endothelial cells (*p* < 0.01) (Figure 6A). Similar results were obtained whether HBMEC or human retinal endothelial cells were infected with RH or ME49 strains of *T. gondii* (not shown). The role of EGFR in affecting parasite survival was confirmed with a genetic approach since knockdown of EGFR in human RPE cells resulted in enhanced killing of *T. gondii* (*p* < 0.01) (Figure 6B). Similar to the studies of blockade of Akt, inhibition of EGFR signaling not only reduced the percentages of infected cells but also caused a reduction in the numbers of vacuoles per 100 cells without affecting the numbers of parasites in the vacuoles that persisted after EGFR blockade (not shown). The effects of EGFR signaling inhibition were not restricted to non-hematopoietic cells since mouse bone marrow-derived macrophages also acquired anti-*T. gondii* activity when treated with AG1478 (*p* < 0.05) (Figure 6C). To further explore the role of EGFR in the survival of *T. gondii*, we took a reverse approach and infected parental CHO cells, known to be EGFR null [44], and CHO cells expressing human EGFR (CHO-EGFR). A reduction in the percentage of infected cells and a reduction in parasite load at 24 h were observed in parental CHO cells compared to CHO-EGFR cells (*p* < 0.05) (Figure 6D). These findings revealed an important role of EGFR in promoting Akt activation and *T. gondii* survival within host cells.

We investigated whether *T. gondii* killing induced by inhibition of EGFR is dependent on autophagy proteins. Knockdown of EGFR in mHEVc cells or treatment of these cells with AG1478 resulted in an enhanced accumulation of LC3 and LAMP-1 around the parasite (*p* < 0.05) (Figure 6E and 6F). Moreover, silencing of Beclin 1 or Atg7 prevented induction of anti-*T. gondii* activity in endothelial cells subjected to EGFR knock-down or treated with AG1478 (*p* < 0.01) (Figure 6G and 6H). Taken together, activation of EGFR signaling promoted survival of *T. gondii* within host cells by inhibiting autophagy protein-dependent killing of the parasite.

*T. gondii* MICs can induce phosphorylation of EGFR and Akt in host cells

EGFR ligands exist as precursors transmembrane proteins that are shed from the plasma membrane by members of the ADAM (a disintegrin and metalloprotease) family of zinc-dependent metalloproteases [45]. This results in an autocrine or paracrine EGFR activation, a phenomenon that explains how proteins such GPCR activate EGFR [45]. We explored whether EGFR activation triggered by *T. gondii* could be due to this mechanism of autocrine/paracrine signaling. HBMEC were treated with GM6001, a broad spectrum ADAM inhibitor, followed by challenge with *T. gondii*. GM6001 did not affect the percentage of infected cells (data not shown) and did not prevent the ability of *T. gondii* to induce EGFR activation (Figure 7A). Moreover, EGFR phosphorylation after *T. gondii* infection took place despite incubation with PTx (Figure 7B). These findings suggest that ADAM- and GPCR-dependent EGFR activation do not play a major role in EGFR phosphorylation induced by *T. gondii*.

As stated above, MIC3, MIC6, MIC8 have multiple domains with homology to EGF [42]. MIC7 and MIC9 also express EGF-like domains but these MICs have poor or no expression in tachyzoites [42]. We examined the effect of deficiency of MICs on the ability to induce activation of EGFR and Akt. HBMEC were infected with wild type (WT), MIC1 ko (lacks MIC1, resulting in deficient secretion of MIC6 [46]), MIC3 ko (lacks MIC3), MIC1-3 ko (lacks MIC6 secretion and MIC3) parasites followed by determination of EGFR and Akt activation. These MIC ko parasites still express MIC8 (MIC8 deficiency results in parasites that are unable to infect mammalian cells). The multiplicity of infection was adjusted so that the initial percentages of infected HBMEC were similar for all strains of the parasite (Figure 8A). Compared to WT *T. gondii*, MIC1 ko and MIC3 ko parasites caused a partial reduction in EGFR and Akt phosphorylation (*p* < 0.05) (Figure 8B, 8C). MIC1-3 ko parasites caused further decrease in EGFR and Akt phosphorylation compared to MIC1 ko and MIC3 ko parasites (*p* < 0.05) (Figure 8B, 8C). However, even in cells infected with MIC1-3 ko parasites the reduction in EGFR and Akt phosphorylation was not complete. MIC1-3 ko parasites still express MIC8, a molecule that has EGF-like...
domains. We used conditional MIC8 knockout *T. gondii* previously generated using a tetracycline-inducible system to explore the potential role of MIC8 in signal activation [47]. Incubation of these parasites with anhydrotetracycline (ATc) results in almost complete ablation of MIC8 [47]. Parasites previously grown in the absence or presence of ATc were incubated with HBMEC. We could not detect an appreciable decrease in Akt phosphorylation in cells exposed to MIC8 deficient parasites (Figure S2). To further explore the role of MICs in the activation of EGFR and Akt, HBMEC were incubated with *Pichia pastoris*-derived MIC3. Although the EGF-like domains alone do not appear to promote the adhesion of MIC3 to mammalian cells [48], it was still possible that MIC3 could cause EGFR and Akt activation. Indeed, compared to recombinant MIC4 (a control that does not express EGF-like domains) incubation with recombinant MIC3 caused enhanced phosphorylation of EGFR and Akt in HBMEC (Figure 8D, 8E). Moreover, incubation with *E. coli*-derived MIC6 but not M2AP caused EGFR-Akt phosphorylation (Figure 8D, 8E). This response was unlikely to be mediated by LPS since M2AP and MIC6 preparations had similar concentrations of LPS (12 ng/ml and 12.4 ng/ml respectively). In addition, LPS at concentrations between 10–1,000 ng/ml failed to induce EGFR phosphorylation in HBMEC (not shown). Taken together, EGF-MICs (MIC3 and MIC6) can induce EGFR-Akt activation and parasites deficient on these MICs have diminished capacity to activate EGFR and Akt.

Figure 6. Blockade of EGFR induces accumulation of the autophagy protein LC3 around the parasite, vacuole-lysosome fusion and killing of *T. gondii* dependent on the autophagy proteins. A, HBMEC were incubated with AG1478 (1 μM) 1 h prior to challenge with *T. gondii*. Monolayers were examined by light microscopy at 2 and 24 h. B, Human RPE cells transfected with either EGFR or control siRNA were challenged with *T. gondii* followed by examination of monolayers by light microscopy at 24 h. C, Mouse bone marrow-derived macrophages were incubated with AG1478 and challenged with *T. gondii*. Monolayers were examined by light microscopy at 2 and 24 h. D, Parental CHO and CHO-EGFR cells were challenged with RH *T. gondii*. Monolayers were examined microscopically 2 h or 24 h post-challenge. E, mHEVC-LC3-EGFP cells transfected with control siRNA or EGFR siRNA were challenged with *T. gondii*-RFP. Monolayers were examined by fluorescence microscopy 5 h post-challenge to determine the percentage of endothelial cells with LC3 accumulation around the parasite. F, HBMEC cells treated with or without AG1478 were challenged with either *T. gondii*-YFP. Expression of LAMP-1 was examined by fluorescent microscopy 8 h post-challenge. The percentages of endothelial cells with LAMP-1 accumulation of around the parasite were determined. G, H, mHEVC cells transfected with Beclin1 siRNA (G) or Atg7 siRNA (H) were transfected with EGFR siRNA or treated with or without AG1478 followed by challenge with *T. gondii*. Monolayers were examined by light microscopy 24 h post-challenge. Results are shown as the mean ± SEM and are representative of 3 independent experiments.

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EGFR signaling and MICs impair autophagic killing of *T. gondii*

Cells stimulated with CD154 (CD40 ligand) exhibit accumulation of LC3 around *T. gondii* and killing that is dependent on autophagy proteins [13–15]. We examined whether targeting of the parasite by LC3+ structures in CD154-treated cells can be affected by EGFR signaling. Endothelial cells were treated with or without CD154 followed by challenge with *T. gondii* in the presence or absence of EGF. EGF did not affect the initial percentage of infected cells (not shown). As previously reported [15], CD154 caused accumulation of LC3 around *T. gondii* (Figure 9A). Targeting of parasites by LC3+ structures was inhibited in cells that were exposed to EGF (p<0.05) (Figure 9A). The effect of EGF was specific since addition of AG1478 to cells treated with EGF restored LC3 accumulation around *T. gondii* (Figure 9A). Similar results were obtained using rapamycin, a well-described stimulator of autophagy (Figure 9B).

Next, we explored the role of MICs on the distribution of LC3+ structures in *T. gondii* micronemal proteins appear to induce EGFR and Akt activation.

Figure 7. Role of metalloproteinases and G protein coupled receptors in *T. gondii*-induced EGFR phosphorylation. HBMEC were treated with or without GM6001 (10 μM) for 1 h prior to challenge with *T. gondii* (A) or with Pertussis Toxin (PTx; 100 ng/ml) for 4 h prior to parasite challenge (B). Cell lysates were used to examine total EGFR and phospho-tyrosine 1068 EGFR by immunoblot. Densitometry data represent means ± SEM of 3 experiments. A vertical line was inserted between densitometry data from control and GM6001- or PTX-treated cells to indicate that band densities from infected cells treated with or without these inhibitors are compared to bands from their respective uninfected cells, which were given an arbitrary number of 1. Results shown are representative of 3 independent experiments.

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Figure 8. *T. gondii* micronemal proteins appear to induce EGFR and Akt activation. A, B, C HBMEC were challenged with ΔHx (WT), MIC1 ko, MIC3 ko, MIC1-3 ko *T. gondii* at MOIs that yielded similar percentages of infected cells (A). Cell lysates were obtained and used to examine total EGFR and phospho-tyrosine 1068 EGFR (B) or total Akt and phospho-Ser 473 Akt (C) by immunoblot. HBMEC were incubated with 10 nM of recombinant MIC4, MIC3, M2AP or MIC6 for 15 minutes followed by examination of total EGFR and phospho-tyrosine 1068 EGFR (D) or total Akt and phospho-Ser473 (E) by immunoblot. Densitometry data represent means ± SEM of 4 experiments. Band densities from infected cells or cells treated with MICs were compared to bands from uninfected or untreated cells (Un), which were given an arbitrary number of 1. Results shown are representative of 4 independent experiments.

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endothelial cells treated with CD154. Endothelial cells were treated with or without CD154 followed by challenge with WT, MIC1 ko, MIC3 ko, MIC1-3 ko and their respective complemented parasites. Infection with MIC1 ko, MIC3 ko or MIC1-3 ko parasites induces a partial decrease in EGFR-Akt activation (see Figures 8B, 9C). Indeed, in control endothelial cells (no CD154 treatment) there were no differences in the low level LC3 accumulation around the parasites (Figure 9C). After treatment with CD154, enhanced accumulation of LC3 around the parasites was similar in endothelial cells infected with WT, MIC1 ko or MIC3 ko parasites (Figure 9C). In contrast, cells infected with MIC1-3 ko parasites (the strain that was the weakest inducer of EGFR-Akt activation) exhibited a significant further increase in LC3 accumulation (p < 0.05) (Figure 9C). These results are specific because the phenotype was lost in the complemented strain of *T. gondii* (MIC1-3 ko+MIC1-3) (Figure 9C). Examination of the parasite load revealed that the loads of MIC1 ko, MIC3 ko and MIC1-3 ko parasites were not significantly different from those of WT parasites in control endothelial cells (no CD154 treatment) (Figure 9D). When cells were treated with CD154, MIC1-3 ko *T. gondii* displayed increased susceptibility to CD154-induced anti-*T. gondii* activity (p < 0.05) (Figure 9D). Similar to the studies of LC3 expression, the phenotype of MIC1-3 ko parasites was lost in the complement strain (MIC1-3 ko+MIC1-3) (Figure 9D). Next, we examined whether increased killing of MIC1-3 ko parasites was observed in cells treated with another autophagy inducer (rapamycin) or in cells treated with IFN-γ, a cytokine that triggers anti-*T. gondii* activity independently of autophagic degradation [13–15]. Similar to CD154-stimulated cells, MIC1-3 ko parasites were more susceptible to rapamycin-induced killing (p < 0.05) (Figure 9E). Moreover, in contrast to the results obtained with CD154-stimulation, anti-*T. gondii* activity induced by IFN-γ/TNF-α was similar in all parasite strains tested including MIC1-3 ko *T. gondii* (Figure 9F). Finally, we explored the effects of recombinant MICs on CD154-induced killing of MIC1-3 ko *T. gondii*. In initial experiments, recombinant MICs did not affect the load of *T. gondii* in non-activated (control) endothelial cells or cells treated with IFN-γ, a cytokine that triggers anti-*T. gondii* activity independently of autophagic degradation [13–15]. Similar to CD154-stimulated cells, MIC1-3 ko parasites were more susceptible to rapamycin-induced killing (p < 0.05) (Figure 9E). Moreover, in contrast to the results obtained with CD154-stimulation, anti-*T. gondii* activity induced by IFN-γ/TNF-α was similar in all parasite strains tested including MIC1-3 ko *T. gondii* (Figure 9F). Finally, we explored the effects of recombinant MICs on CD154-induced killing of MIC1-3 ko *T. gondii*. In initial experiments, recombinant MICs did not affect the load of *T. gondii* in non-activated (control) endothelial cells or cells treated with IFN-γ/TNF-α (not shown). Next, control or CD154-activated endothelial cells were challenged with WT or MIC1-3 ko parasites in the presence of absence of recombinant MICs. Whereas treatment of endothelial cells with MIC4 and M2AP did not affect the load of WT or MIC1-3 ko parasites in CD154-activated cells, treatment with MIC3 or MIC6 inhibited CD154-induced *T. gondii* activity (p < 0.05) (Figure 9G). Moreover, the phenotype of MIC1-3 ko parasites of increased susceptibility to CD154-mediated anti-*T. gondii* activity was lost in the presence of either MIC3 or MIC6 since the loads of WT and MIC1-3 ko parasites were no longer different in cells treated with these EGF-MICs (Figure 9G). Taken together, our findings indicate that EGFR, MIC3 and MIC6 negatively regulate autophagic killing of *T. gondii*.

**Discussion**

Avoidance of lysosomal degradation is pivotal for the survival of numerous intracellular pathogens including *T. gondii*. Our studies indicate that, in addition to exclusion of type I transmembrane proteins from the PVM, *T. gondii* also activates EGFR-Akt signaling in the host cell to prevent targeting of the parasite by LC3 structures and pathogen killing that is dependent on autophagy proteins and lysosomal protease activity. Thus, these studies identified EGFR-Akt signaling as a pathway critical for pathogen survival. In addition, they suggest that EGF-MICs may be involved in pathogen virulence not only by allowing parasite invasion of host cells but also by activating host cell signaling that counter-regulates autophagy.

Various bacteria and viruses encode virulence factors that impair the function of autophagy proteins and as a result, avoid their degradation via the autophagy pathway [18–24]. It has been suggested that HIV-1 and *M. tuberculosis* may prevent autophagic degradation by affecting signaling cascades that regulate the autophagy pathway [25,26]. Our studies indicate that indeed a pathogen can act at the level of a regulatory pathway to avoid its degradation by the autophagy machinery. Relevant to our findings is the report that HIV-1 tat impairs autophagy by stimulating counter-regulatory cascades (Akt and STAT3), although these studies did not examine whether these pathways would prevent lysosomal degradation of the virus [49].

Our studies indicate that *T. gondii*-induced EGFR activation is a major event upstream of Akt phosphorylation in endothelial and RPE cells, a finding consistent with the important role of EGFR and other growth factor receptors as activators of Akt signaling [36,50]. PI3K is a classical link between growth factor receptors and Akt activation. However, in contrast inhibition of EGFR signaling, the effect of PI3K inhibition on Akt activation appeared to be more transient. These findings may be explained by the fact that, besides PI3K, there are additional activators of Akt that might be engaged by growth factor receptors [51]. *T. gondii* has been reported to activate Akt in macrophages, a phenomenon that was inhibited by PTx [31]. Our studies indicate that EGFR also contributes to Akt activation in macrophages/microglia since the parasite caused EGFR autophosphorylation and inhibition of EGFR signaling impaired parasite-induced Akt activation. Moreover, not only activation of Akt but also activation of EGFR in endothelial cells, RPE cells and macrophages/microglia prevented killing of *T. gondii* dependent on autophagy proteins and lysosomal enzymes. The fact that Akt activation has been linked to inhibition of apoptosis of *T. gondii*-infected cells [31] raises the possibility that parasite-induced EGFR - Akt signaling may not only promote parasite survival by preserving the non-fusogenic nature of the PV but also by avoiding death of infected cells subjected to pro-apoptotic signals. While EGFR is a central mediator of Akt activation in the early stages after *T. gondii*, Akt phosphorylation has recently been reported at 24 h post-infection with the parasite [52]. This raises the possibility that *T. gondii* may also activate Akt through additional mechanisms besides parasite engagement of EGFR.

Although *T. gondii* causes EGFR - Akt activation and these signaling molecules have been shown to inhibit autophagy [35,53,54], *T. gondii* does not appear to prevent autophagosome formation in infected cells. Indeed, large LC3 structures were readily detected within infected cells during early stages post-infection (see Figure 2D), a finding previously reported in host cells at 24 h post-infection [55]. Moreover, there is no decrease in the levels of LC3 II (the lipidated form of LC3 that associates with the autophagosome membrane) during the early stages of infection (Muniz-Feliciano and Subauste, unpublished observations). In fact, *T. gondii* has been reported to increase LC3 II levels and autophagosome formation in host cells at 24 h post-infection, presumably as an attempt to gain access to nutrients [55]. Our studies indicate that while global autophagy did not appear to be inhibited by *T. gondii*, engagement of EGFR impaired targeting of the PV by LC3 structures. Future studies that identify how autophagosomes target the PV will likely shed light on the molecular mechanism by which EGFR - Akt diminish autophagic targeting of the parasite.

Various pathogens can target EGFR. *Pseudomonas aeruginosa* and *Helicobacter pylori* can cause EGFR phosphorylation that is
mediated by the release of membrane-bound EGF ligands and transactivation of EGFR [56,57]. *Klebsiella pneumoniae* causes EGFR
activation that appears to be dependent on bacterial capsule polysaccharide engagement of TLR4 and subsequent Src-dependent EGFR activation [58]. In addition, proteins from oncogenic viruses activate EGFR to mediate transformation [59]. Much less is known on whether microbial products can directly engage and activate EGFR. It has been suggested that *H. influenzae* may activate EGFR through the presence of bacterial-derived molecules with EGF-like properties [60]. Uptake of Influenza A virus causes EGFR activation, a process that may be dependent on multivalent binding of hemagglutinin to sialic acids present on EGFR or ganglioside GM1 leading to aggregation of rafts, clustering of EGFR and its activation [61]. Our studies suggest that EGF-MICs play a role in mediating EGFR-Akt activation of host cells and prevention of parasite killing since: recombinant EGF-MICs (MIC3 and MIC6) induce EGFR-Akt activation while MICs that lack EGF domains do not cause appreciable phosphorylation of EGFR and Akt; EGFR signaling inhibits LC3 accumulation around *T. gondii* parasites deficient in 2 EGF-MICs (MIC3 and MIC6; MIC1-3 ko parasites) cause markedly impaired EGFR-Akt activation and exhibit increased encasement by LC3+ structures as well as killing in cells treated with autophagy stimulators; MIC3 and MIC6 impair parasite killing mediated by the autophagy pathway.

It was interesting to note that MIC1-3 ko parasites are not targeted by LC3+ structures and are not more likely to be killed in unstimulated cells despite the markedly weakened EGFR-Akt signaling. MIC1-3 ko parasites only display increased susceptibility to autophagic targeting and killing when autophagy is stimulated by CD154 or rapamycin.

Our studies also support the existence of signaling thresholds that need to be achieved in order for autophagy to take place [62,63]. For example, in *Drosophila* both the Ret-like receptor tyrosine kinase Stitcher (Stit) and insulin receptor (InR) are required for cell growth and proliferation through the PI3K-I/TORC1 pathway in the wing disc [63]. A decrease in either Stit or InR signaling diminishes TORC1 activity and suppresses growth [63]. However, this decrease in TORC1 activity is not sufficient to trigger autophagy in the wing [63]. Autophagy only takes place when...
both Stit and InR are impaired [63]. It was proposed that the simultaneous inactivation of Stit and InR reduces PI3K-I activity and TORC1 signaling below a critically low level at which autophagy in the wing can no longer be prevented [63]. Given that the EGFR-Akt pathway inhibits autophagy by regulating TORC1 activity, a similar phenomenon could be at play in the case of T. gondii infection. The reduction in EGFR-Akt observed in cells infected with MIC1 ko or MIC5 ko parasites does not translate in increased autophagic killing of these parasites either in unstimulated cells or in cells treated with stimulators of autophagy. The further reduction in EGFR-Akt signaling observed in cells infected with MIC1-3 ko may still be sufficient to prevent autophagic killing in unstimulated cells but results in enhanced killing in cells treated with autophagy stimulators. Finally, further inhibition of EGFR-Akt signaling (by genetic or pharmacological approaches) triggers autophagic targeting of T. gondii even in unstimulated cells. Thus, our studies suggest that the effects of MIC deficiency on the levels of EGFR-Akt activation likely explain the differences in outcome observed after infection. Taken together, in addition to being key for invasion of host cells, EGFR-MICs (MIC3 and MIC6) contribute to the induction of a signaling cascade within these cells that is required to avoid lysosomal degradation of the parasite.

While MIC1-3 ko parasite exhibited a marked defect in EGFR-Akt activation in host cells, phosphorylation of these molecules still took place. Although we cannot rule out a role of MIC8 in activation of this cascade, it appears that the residual ability of MIC1-3 ko parasites to activate EGFR-Akt may not be explained by their expression of MIC8 (an EGF-MIC). Conditional MIC8 ko parasites did not exhibit a noticeable defect in signal activation in host cells. These findings are likely explained by the fact that MIC8 ko parasites do not exhibit defects in attachment to host cells and they secrete MICs [47]. The presence of an additional mechanism of EGFR-Akt activation that normally cooperates with MIC-dependent EGFR signaling may explain why MIC1-3 ko T. gondii have residual capacity to activate the EGFR-Akt pathways.

T. gondii is very successful as a pathogen and utilizes various strategies to manipulate host cell signaling to ensure its survival [64–67]. Here we report that the parasite activates EGFR-Akt to maintain the non-fusogenic nature of PV a process that appears to be dependent at least in part on EGF-MICs. These findings may be of therapeutic relevance since various inhibitors of EGFR are being used for treatment of cancer. The fact that EGFR inhibition induced parasite killing in cells not treated with immune activators, raises the possibility that this approach may be effective even in immunocompromised hosts.

Materials and Methods

Mammalian cells

Primary human brain microvascular endothelial cells (HBMEC) were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured in fibronectin-coated tissue culture flasks and basal medium supplemented with Endothelial Cell Growth Supplement (ECGS) and 5% fetal bovine serum (FBS) from ScienCell. The mouse high endothelial venule cell line (mHEVc) (gift from Joan Cook-Mills, Northwestern University, Chicago, IL) and mHEVc cells stably expressing LC3-EGFP construct (mHEVc-LC3-EGFP) or hCD40 plus LC3-EGFP (hCD40 plus mHEVc-LC3-EGFP) [15] were cultured in DMEM plus 10% FBS (HyClone; Logan, UT). A human RPE cell line (ARPE-19; American Type Culture Collection, Manassas, VA), a mouse macrophage line (RAW 264.7) and mouse microglia line (BV-2) were cultured in DMEM plus 10% FBS. Mouse bone marrow-derived macrophages were obtained as described and cultured in DMEM plus 30% L929-conditioned medium, 10% FBS and 5% horse serum [60]. Parental Chinese Hamster Ovary (CHO) cells and CHO cells expressing human EGFR (CHO-EGFR) were cultured in MEM plus 10% FBS.

T. gondii and infection

Experiments were conducted using tachyzoites of the RH strain of T. gondii (Type I strain), RH that express cytoplasmic YFP [69] or cytoplasmic DsRed (RPF) [69], tachyzoites of the ME49 (Type II strain), transgenic parasites deficient in micronemal proteins MIC1 ko, MIC3 ko, MIC1-3 ko and the complemented strains (MIC1ko+MIC1, MIC3 ko+MIC3 and MIC1-3 ko+MIC1-3; gift from Maryse Lebrun, Université de Montpellier 2, France) [39], as well as conditional MIC8 ko parasites (gift from Markus Meissner, University of Glasgow). Parasites were maintained in human foreskin fibroblasts following standard procedures [70]. In order to deplete MIC8, conditional MIC8 ko parasites were cultured in HFF in the presence of anhydrotertracycline (1 μg/ml) for 48 h. T. gondii tachyzoites were killed by incubation in 1% paraformaldehyde in PBS.

A potassium buffer shift was used to synchronize T. gondii invasion of serum-starved (0.1% FBS) mammalian cells as described [71]. Briefly, freshly egressed tachyzoites were resuspended in Endo buffer and incubated with cells for 20 minutes at 37°C. The Endo buffer was replaced for a low-potassium permissive medium to allow parasite invasion. In certain experiments, mammalian cells were incubated with Akt inhibitor IV (1.25 μM; EMD Millipore, Billerica, MA), PI3K inhibitor LY294002 (20 μM; Sigma–Aldrich; St. Louis, MO), EGFR inhibitor (AG1478; 1 μM; EMD Millipore), a broad spectrum ADAM inhibitor (GM6001; 10 μM; EMD Millipore) (all 1 h prior to challenge with T. gondii), Pertussis Toxin (PTx; 100 ng/ml; EMD Millipore; 4 h prior to challenge), leupeptin (10 μM; EMD Millipore) and pepstatin (10 μM; EMD Millipore; both 1 h after challenge with T. gondii), 3-methyl adenine (3MA; 10 mM; Sigma Chemical) and rapamycin (1 μM; EMD Milipore; both 2 h after challenge with T. gondii) or vehicle. To induce CD40 signaling, mHEVc cells were treated with cell-free supernatants containing either multiemeric human CD154 or a non-functional CD154 mutant [72] (T147N; both obtained from Dr. Richard Kornbluth, Univ. of California San Diego, current address Multimeric Biotherapeutics Inc., La Jolla, CA) for 18 h at 37°C as previously described [73] prior to challenge with parasites. Monolayers were fixed at indicated time points and stained with Diff-Quick (Dade Diagnostics, Aguada, Puerto Rico). The percentage of infected cells, the numbers of tachyzoites and vacuoles per 100 cells as well as the numbers of parasites per vacuole were determined by light microscopy by counting at least 200 cells or 200 vacuoles per monolayer as previously described [15].

T. gondii proteins

For expression of MIC3 and MIC4 in P. pastoris, amplified DNA fragments were cloned into a pPICZa.A vector (Invitrogen; Carlsbad, CA). The pPICZa.A vector contains the S. cerevisiae a-factor secretion signal that allows for the secretion of folded proteins from P. pastoris. Cells were grown in BMGY media, washed and resuspended in BBMMY media for an initial OD600 of 20–40. The culture was then incubated in a 28°C incubator with vigorous shaking. The culture was then grown for 1–5 days depending on the optimal period of expression. Inhibition of glycosylation during culture required the addition of 20 μg/ml of tunicamycin. The supernatant is then passed through a HiTrap Q, HF Column (GE Healthcare; Little Chalfont, UK). The eluted fraction was buffer exchanged into nickel-column binding buffer
Transfection
Cells were transiently transfected with a plasmid that encodes Akt-PH-GFP [76], human PI3K p110α siRNA [77], human Akt siRNA [78], mouse Beclin1 siRNA [79], mouse Arg7 siRNA [79], human MyD88 siRNA [80], human EGFR siRNA [81] or control siRNA (Dharmacon) using Lipofectamine 2000 (Invitrogen) or an Amaxa nucleofector as described [13,15], siRNA against mouse EGFR was synthesized using siRNA construction kit (Ambion) following manufacturer’s recommendation and used for mouse EGFR knock-down after transfection using Lipofectamine 2000.

Fluorescent microscopy
To assess for LC3 accumulation around the parasite, mHEVc-LC3-EGFP cells were cultured with or without Akt inhibitor IV or transfected with either control siRNA or EGFR siRNA or treated with or without EGF (50 ng/ml; PeproTech). Monolayers were challenged with RH T. gondii that express cytoplasmic RFP (T. gondii-RFP). Five hours post-challenge, monolayers were fixed with 4% paraformaldehyde, slides were mounted using Fluoromount G and assessed for LC3-EGFP accumulation around T. gondii as described [13,15]. In certain experiments, hmcCD40 mHEVc-LC3-EGFP cells treated with or without CD154 were infected with WT, MIC1 ko, MIC1 ko+MIC1, MIC3 ko, MIC3 ko+MIC3, MIC3 ko+MIC3, MIC3-3 ko or MIC3-3 ko+MIC3-3 tachyzoites. Monolayers were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and incubated with rabbit anti-T. gondii Ab (BioGenex; San Ramon, CA) for 30 minutes. Monolayers were then washed with PBS and incubated for 1 h at room temperature with goat anti-rabbit Alexa 568-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA).

Electron microscopy
Endothelial cells were seeded onto a sterilized Aclar Embedding Film (Electron Microscopy Sciences, PA) and incubated with or without T. gondii tachyzoites in the presence of Akt inhibitor IV or vehicle. At 5 h post-challenge, the Aclar sheets with their attached cells were fixed as described [83]. After a soak in acidified uranyl acetate, the specimen was dehydrated in ethanol, passed through propylene oxide, and embedded in Poly/Bed (Polysciences, PA). Sections were cut in a horizontal plane parallel to that of the Aclar film to provide panoramic views of the endothelial cells. Thin sections were stained with acidified uranyl acetate in 50% methanol followed by triple lead stain of Sato. These sections were examined in a JEOL 1200 EX electron microscope (Tokyo, Japan).

Immunoblot
Cells were lysed in buffer supplemented with protease and phosphatase inhibitors (Cell Signaling). Equal amounts of protein were subjected to either 7.5% or 10% SDS-PAGE (Bio-Rad) and transferred to PVDF membranes. Membranes were probed with either antibody to total Akt (Cell Signaling), phospho-Ser473 Akt (Cell Signaling), total EGFR (Santa Cruz Biotechnology), phospho-tyrosine 1068 EGFR (Invitrogen), phospho-tyrosine 1148 EGFR (Cell Signaling), Akt7 (Cell Signaling), Beclin 1 (BD Biosciences), PI3K p110α (Cell Signaling) or MyD88 (Cell Signaling) followed by incubation with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Bands were visualized by using enhanced chemiluminescence kit (Fierce Bioscience). Intensities of phospho-Akt and phospho-EGFR were calculated using ImageJ (NIH) and normalized against total Akt and total EGFR respectively.

Statistics
Results from pooled experiments were analyzed for statistical significance using 2-tailed Student’s t test and ANOVA (InStat version 3.0, GraphPad; LaJolla, CA). Differences were considered statistically significant when P was <0.05.

Supporting Information
Figure S1 Effects of Akt blockade on the numbers of T. gondii-containing vacuoles and the numbers of parasites per vacuole. A, HBMEC, mHEVc and human RPE cells were incubated with or without Akt inhibitor IV (1.25 µM) for 1 h prior to challenge with T. gondii. In addition, HBMEC were transfected with control siRNA or Akt siRNA. Cells were then challenged with T. gondii 48 h after transfection. Monolayers were examined by light microscopy 24 h post-challenge to determine the numbers of parasite containing vacuoles. B, HBMEC were transfected with control siRNA or Akt siRNA and were then challenged with T. gondii. Monolayers were examined by light microscopy 24 h post-challenge to determine the numbers of parasites per vacuole. Results are shown as the mean ± SEM and are representative of at least 3 independent experiments. (TIF)

Figure S2 Effects of MIC8 depletion on Akt activation. Conditional MIC8 ko parasites were cultured in HFF with or without ATc (1 µg/ml) for 48 h. Tachyzoites were harvested and antibody alone. Slides were analyzed using a Leica DMI 6000 B automated microscope equipped for epifluorescence microscopy. Experimental groups had triplicate samples and at least 100 cells per sample were counted.
incubated with HBMEC for the indicated time points. Cell lysates were used to probe for total Akt and phospho-Ser473 Akt by immunoblot. Results shown are representative of 3 independent experiments. (TH)

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Author Contributions

Conceived and designed the experiments: LMF, JVG, CSS. Performed the experiments: LMF, JVG, CSS. Analyzed the data: LMF, JVG, CSS. Contributed reagents/materials/analysis tools: CRC, BL, LM, SM, VBC. Wrote the paper: LMF, CSS.

References

1. Carruthers VB, Boothroyd JC (2007) Pulling together: an integrated model of Toxoplasma gondii cell invasion. Curr Opin Microbiol 10: 83–89.

2. Carruthers VB, Tonley FM (2008) Receptor-ligand interaction and invasion: microneme proteins in apicomplexans. Subcell Biochem 47: 33–45.

3. Anantharaman V, Iyer LM, Balaji S, Aravind L (2007) Adhesion molecules and other secreted host-interaction determinants in Apicomplexa: insights from comparative genomics. Int Rev Cytol 262: 1–74.

4. Tonley FM, Soldati DS (2001) Mix and match modules: structure and function of microneme proteins in apicomplexan parasites. Trends Parasitol 17: 81–85.

5. Sibley LD (2011) Invasion and intracellular survival by protozoan parasites. Immunol Rev 240: 72–91.

6. Soldati-Favre D (2008) Molecular dissolution of host cell invasion by the apicomplexans: the glideosome. Parasitol 135: 197–205.

7. Boothroyd JC, Dubremetz JF (2008) Kiss and quit: the dual roles of Toxoplasma rhoptries. Nat Rev Microbiol 6: 79–88.

8. Besterre S, Dubremetz JF, Lebrun M (2011) The moving junction of apicomplexan parasites: a key structure for invasion. Cell Microbiol 13: 797–805.

9. Mordue DG, Niesman I, Slesinger LD (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.

10. Mordue DG, Hakansson N, Niesman I, Slesinger LD (1999) Toxoplasma gondii resides in a vacuole that avoids fusion with host cell endocytic and exocytic vesicular pathways. Exp Parasitol 92: 87–99.

11. Martens S, Pancorbo I, Zerahn J, Griffiths G, Schell G, et al. (2005) Disruption of Toxoplasma gondii parasitophorous vacuoles by the mouse p47<sup>−/−</sup> resistance GTPathes. PLoS Pathogens 1: 187–201.

12. Zhao Z, Fux B, Goodwin M, Dunay IR, Strong D, et al. (2008) Autophagosome-independent essential function for the autophagy protein Atg9 in cellular immunity to intracellular parasites. Cell Host Microbe 4: 458–469.

13. Andrade RM, Wessendrop M, Gubbel HJ, Striepen B, Subauste CS (2006) Cd40 induces macrophage anti-Toxoplasma gondii activity by triggering autophagy-dependent fusion of pathogen-containing vacuoles and lysosomes. J Clin Invest 116: 2366–2377.

14. Meissner M, Reiss M, Viebig N, Carruthers VB, Toursel C, et al. (2002) A novel receptor recruits p47<sup>−/−</sup> macrophages. J Cell Biol 152: 563–578.

15. Sheiner L, Santos JM, Klages N, Parussini F, Jemmely N, et al. (2010) The role of phosphatidylinositol-3,4,5-triphosphate in the activation of protein kinase B. Science 329: 654–655.

16. Huynh M-H, Carruthers VB (2006) Toxoplasma MIC2 is a major determinant of invasion and virulence. PLoS Pathogens 2: e84.

17. Tigyi G, Parrill A (2003) Molecular mechanisms of lysophosphatidic acid action. Prog Lipid Res 42: 498–526.

18. Carruthers VB (2002) Host cell invasion by the opportunistic pathogen Toxoplasma gondii. Acta Tropica 81: 111–122.

19. Ceredo O, Dubremetz JF, Soe, M, Dedede D, Vial H, et al. (2005) Synergistic role of micronemal proteins in Toxoplasma gondii virulence. J Exp Med 201: 453–465.

20. Huyhn M-H, Carruthers VB (2006) Toxoplasma MIC2 is a major determinant of invasion and virulence. PLoS Pathogens 2: e94.

21. Carruthers VB, Lebrun M, Wangs M, Mordue DG, Sibley LD, et al. (1999) Identification and characterization of an escorter for two secretory adhesins in Toxoplasma gondii. Acta Tropica 81: 111–122.

22. Ceredo O, Dubremetz JF, Soe, M, Dedede D, Vial H, et al. (2005) Synergistic role of micronemal proteins in Toxoplasma gondii virulence. J Exp Med 201: 453–465.

23. Reiss M, Viebig N, Brecht S, Fourmaux MN, Soete M, et al. (2001) Toxoplasma gondii transmembrane microneme proteins and their modular design. Mol Microbiol 77: 912–929.

24. Meissner M, Reiss M, Viebig N, Carruthers VB, Toursel C, et al. (2005) A family of transmembrane microneme proteins of Toxoplasma gondii contain EGF-like domains and function as escorters. J Cell Sci 115: 563–574.

25. Moro L, Dolce L, Cabodi S, E B, Boeri Erba E, et al. (2002) Integrin-induces activity in non-hematopoietic cells. PLoS Pathogens 2: e94.
54. Maynard AA, Dvorak K, Khailova L, Dobrenen H, Arganbright KM, et al. (2009) IGF-I, EGF, and sex steroids regulate autophagy in bovine mammary epithelial cells via the mTOR pathway. Endocrinology 150: 6873–6883.

55. Wang Y, Weiss LM, Orlofsky A (2009) Host cell autophagy is induced by Toxoplasma gondii proliferation require down-regulation of host Nox1 expression via activation of PI3 kinase/Akt signaling pathway. PLoS One 8: e66306.

56. Sobolewska A, Gajewska M, Zarzynska J, Gajkowska B, Motyl T (2009) IGF-I, EGF, and reactive nitrogen intermediates. Infect Immun 77: 4359–4366.

57. Zhou W, Quan J-H, Lee Y-H, Shin D-W, Cha G-H (2013) Toxoplasma gondii infection independently of IFN-γ. Infect Immun 81: 5456–5465.

58. Frank CG, Reguerlo V, Rother M, Moranta D, Maeurer AP, et al. (2013) Establishment of Toxoplasma gondii infection as a model for cytokine-induced autophagy. PLoS One 8: e66306.

59. Kung C-P, Meckes DG, Raab-Traub N (2011) Epstein-Barr virus LMP1 expression reduced autophagy in intestinal epithelium and in the rat model of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 299: G614–G622.

60. Wang Y, Weiss LM, Orlofsky A (2009) Host cell autophagy is induced by Toxoplasma gondii and contributes to parasitie growth. J Biol Chem 284: 1694–1701.

61. Koff JL, Shao MXG, Kim S, Ueki IF, Nadel JA (2010) Pseudomonas aeruginosa lipopolysaccharide accelerates wound repair via activation of a novel epithelial cell signaling cascade. J Immunol 184: 8009–8019.

62. Kates S, Sengodan S, Kates AC, Zhao D, Pek RMJ, et al. (2001) cagA+ Helicobacter pylori induce transactivation of the epidermal growth factor receptor in AGS epithelial cells. J Biol Chem 276: 40127–40134.

63. Frank CG, Reguerlo V, Rother M, Moranta D, Maeurer AP, et al. (2013) Miyobacteria pneumoniae targets an EGF receptor-dependent pathway to subvert inflammation. Cell Microbiol 15: 1212–33.

64. Kung C-P, Meckes DG, Raab-Traub N (2011) Epstein-Barr virus LMP1 activates EGF, STAT3, and ERK1/2 through effects on Fcγ receptor γ. J Biol Chem 286: 4399–4408.

65. Mikiwa F, Gu H, Jono H, Andalali A, Kai H, et al. (2005) Epidermal growth factor receptor acts as a negative regulator for bacterium notontyptable Haemophilus influenzae induced Toll-like receptor 2 expression via an Src-dependent p38 mitogen-activated protein kinase signaling pathway. J Biol Chem 280: 36195–36194.

66. Eierzoff T, Hricinou ER, Recher U, Ludwig S, Ehrhardt C (2010) The epidermal growth factor receptor (EGFR) promotes uptake of Influenza A viruses (IAV) into host cells. PLoS Pathog 6: e1001099.

67. Chhipa RR, Wu Y, Ip C (2011) AMPK-mediated autophagy is a survival mechanism in androgen-dependent prostate cancer cells subjected to androgen deprivation and hypoxia. Cell Signal 23: 1466–1472.

68. O’Farrell F, Wang S, Katheder N, Ruster T, Kudroski C (2012) Two-tiered control of epithelial growth and autophagy by the insulin receptor and the Ret-like receptor, Snchir. PLoS Biol 11: e1001612.

69. Boothroyd JC, Dubremetz JF (2000) Kiss and spit: the dual roles of Toxoplasma rhoptries. Nat Rev Microbiol 69: 79–88.

70. Lahbiri J, Caruthers VB (2008) Host cell manipulation by the human pathogen Toxoplasma gondii. Cell Mol Life Sci 65: 1900–1915.

71. Mahajan K, Mahajan NP (2012) PI3K-independent AKT activation in cancers: a treasure trove for novel therapeutics. J Cell Physiol 227: 3178–3194.

72. Bajorath J, Seyama K, Nonoyama S, Ochs HD, Aruffo A (1996) Classification of mutations in the human CD40 ligand, gp39, that are associated with X-linked hyper-IgM syndrome. Proc Natl Acad Sci U S A 93: 53–59.

73. Portillo J-AC, Van Groj J, Zheng L, Okunke G, Gentil K, et al. (2008) CD40 mediates retinal inflammation and neuro-vascular degeneration. J Immunol 181: 6719–8726.

74. Liu B, Sawmynaden K, Marchant J, Simpson P, Matthews S (2009) Complete gene expression assignments for the MiC2 associated protein from Toxoplasma gondii. Bioinformatics 25: 813–821.

75. Saouros S, Edwards-Jones B, Reiss M, Sawmynaden K, Kota E, et al. (2005) A novel galectin-like domain from Toxoplasma gondii micronemal protein 1 assists the folding, assembly, and transport of a cell adhesion complex. J Biol Chem 280: 38583–38591.

76. Kwon Y, Hofmann T, Montell C (2007) Integration of phosphoinositide- and Ca2+-dependent signaling of epithelial growth and autophagy by the insulin receptor and the Ret-like receptor, Snchir. PLoS Biol 11: e1001612.

77. Meng Q, Xia C, Fang J, Rojanasakul Y, Jiang B-H (2006) Role of PI3K and Src-Akt and STAT3. PLoS One: e11733.

78. Yu L, Alva A, Hu D, Dutt P, Freundt E, et al. (2004) Regulation of an autophagy protein, beclin 1, by PI3K and reactive nitrogen intermediates. Infect Immun 72: 989–998.

79. Kang C-S, Zhang Z-Y, Jia Z-F, Wang G-X, Qiu M-Z, et al. (2006) Suppression of an autophagy protein, beclin 1, by PI3K and reactive nitrogen intermediates. Infect Immun 72: 989–998.

80. Kuo PL, Hsu YL, Cho CY (2006) Plumbagin induces G2-M arrest and autophagy by inhibiting the Akt/mammalian target of rapamycin pathway in breast cancer cells. Mol Cancer Ther 5: 3209–3221.

81. Meng Q, Xia C, Fang J, Rojanasakul Y, Jiang B-H (2006) Role of PI3K and AKT specific isoforms in ovarian cancer cell migration, invasion and proliferation through the p70S6K1 pathway. Cell Signal 18: 2262–2271.

82. Kim S-E, Choi K-Y (2007) EGF receptor is involved in WNT3a-mediated proliferation and motility of NIH3T3 cells via ERK pathway activation. Cellular Signaling 19: 1554–1564.

83. Kim S-E, Choi K-Y (2007) EGF receptor is involved in WNT3a-mediated proliferation and motility of NIH3T3 cells via ERK pathway activation. Cellular Signaling 19: 1554–1564.

84. Fujikawa H, Tandler B, Consolo MC, Karnik P (2013) Division of mitochondria in cultured human fibroblasts. Microse Res Tech [Epub ahead of print].