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The Parasitophorous Vacuole Membrane of *Toxoplasma gondii* is Targeted for Disruption by Ubiquitin-like Conjugation Systems of Autophagy

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Summary

Autophagy is a lysosomal degradation pathway that is important in cellular homeostasis. Prior work showed a key role for the autophagy related 5 (Atg5) in resistance to *Toxoplasma gondii*. Here we show that the cassette of autophagy proteins involved in the conjugation of microtubule associated protein 1 light chain 3 (LC3) to phosphatidylethanolamine, including Atg7, Atg3, and the Atg12-Atg5-Atg16L1 complex play crucial roles in the control of *T. gondii* *in vitro* and *in vivo*. In contrast, pharmacologic modulation of the degradative autophagy pathway or genetic deletion of other essential autophagy genes had no substantial effects. Rather the conjugation system was required for targeting of LC3 and interferon- γ effectors onto the vacuolar membrane of *T. gondii* and its consequent disruption. These data suggest that the ubiquitin-like conjugation systems that reorganize intracellular membranes during canonical autophagy are necessary for proper targeting of immune effectors to the intracellular vacuole membranes utilized by pathogens.

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Introduction

Toxoplasma gondii is a protozoan parasite that infects a broad range of animals and causes zoonotic toxoplasmosis in humans (Sibley, 2011). Toxoplasmosis is a leading cause of reportable foodborne illness in the United States and contributes to congenital disease and opportunistic disease in immunocompromised persons throughout the world (Jones et al., 2010). Although 25–30% of the world's population are believed to carry persistent *T. gondii* infection for life, most people remain asymptomatic as long as the parasites are under the control of the immune system (Pappas et al., 2009). Therefore, understanding how the normal immune system controls the *T. gondii* infection may lead to better management of toxoplasmosis in patients, especially the immunocompromised.

The active invasion of host cells by *T. gondii* leads to the formation of the non-fusogenic parasitophorous vacuole (PV), a cytoplasmic membranous structure that envelops the invading *T. gondii* in the host cell (Sibley, 2011). Inside the PV, *T. gondii* replicates by endodyogeny while protected from the inimical host cytoplasm. To fight the *T. gondii* infection, interferon- γ (IFN γ) secretion by immune cells, its subsequent binding to host cell receptors and induction of cellular activation arms infected and naïve cells with effector molecules, such as the immunity related p47 GTPases (IRGs) and guanylate-binding proteins (GBPs) (Howard et al., 2011; Kim et al., 2012). These effectors rapidly accumulate on and around the PV membrane (PVM), leading to the disruption of PVM and subsequent death of the parasite. The proper loading of the effectors onto the PVM is known to require GTP-binding dependent oligomerization, which is controlled by regulatory interactions among the effectors (Howard et al., 2011). Moreover, we and others showed that the essential autophagy gene Atg5 (autophagy related 5) is required for the proper targeting of the effectors onto the PVM of *T. gondii* (Selleck et al., 2013; Zhao et al., 2009; 2008). Without Atg5, the effectors are induced by IFN γ normally, but form cytoplasmic aggregates instead of accumulating on the PVM. However, the mechanism of the Atg5-mediated targeting of the IFN γ effectors to the PVM of *T. gondii* is poorly understood (Howard et al., 2011).

Autophagy is an evolutionarily conserved intracellular degradation pathway that targets cellular constituents to lysosomes (Parzych and Klionsky, 2013). Among the different forms of autophagy, macroautophagy (henceforth, autophagy) is the pathway that sequesters cytoplasmic materials in double membrane bound autophagosomes and degrades the cargo through the fusion between the autophagosome and lysosome (Rubinsztein et al., 2012). Upon induction of autophagy by various signals, such as the inhibition of mammalian target of rapamycin (mTOR) kinase, the initiation complex of ULK1/2 (uncoordinated 51-like kinase 1/2)-Atg13-Atg101-FIP200 (focal adhesion kinase family-interacting protein of 200 kD) activates the phosphatidylinositol 3-kinase (PI3K) complex of Beclin1-Vps34-Vps15-Atg14L. This activation leads to the nucleation of isolation membrane formation and the elongation complex of Atg12-Atg5-Atg16L1 extends the membrane further by conjugating LC3 (microtubule associated protein 1 light chain 3) homologs to the phosphatidylethanolamine of the growing autophagosome (Rubinsztein et al., 2012).

The function of the autophagy pathway was initially identified as a starvation-induced homeostatic pathway for recycling of essential materials, but in recent years it has been expanded to include cellular remodeling, secretion, differentiation, and immune defense (Levine et al., 2011). Specifically, the role of autophagy in the selective recognition and subsequent lysosomal degradation of pathogens has been highlighted in the innate immune defense against intracellular pathogens (Levine et al., 2011). Furthermore, we showed that the autophagy elongation complex, but not the degradative autophagy pathway, plays an essential role in the control of murine norovirus (MNV) by IFN γ (Hwang et al., 2012). We also found that proximal components of the autophagy pathway including ULK1, Atg14L, and Beclin1 are required for replication of *Brucella abortus* in macrophages while distal components of the pathway such as Atg5, Atg7, and Atg16L1 are not (Starr et al., 2012). These observations open up the possibility that cassettes of autophagy proteins play a broad role in biology independent of the canonical degradation of cytoplasmic organelles and other constituents (Bestebroer et al., 2013; Subramani and Malhotra, 2013).

Here we show that the conjugation of LC3 through E1 Atg7, E2 Atg3, and E3 Atg12-Atg5-Atg16L1 complex is required to control *T. gondii* infection *in vitro* and *in vivo*. Using genetic and pharmacologic methods, we demonstrate that only the two ubiquitin-like conjugation systems of the autophagy pathway, but not the canonical degradative autophagy process nor the initiation-nucleation complex, are required for IFN γ to control *T. gondii* infection. These data suggest that the ubiquitin-like conjugation systems, which are normally involved in the reorganization of intracellular membranes in the canonical autophagy pathway, are also required for proper targeting of LC3 and IFN γ effectors toward the intracellular membrane structure of pathogens.

Results

Atg5, Atg7 and Atg16L1, but not Atg14L, are required to control *T. gondii* infection *in vivo*

Previously, we showed that Atg5 in myeloid lineage cells is required for resistance of mice to infection with *T. gondii* (Zhao et al., 2008). Although Atg5 was required for IFN γ induced control of *T. gondii* in primary macrophages, autophagosomes, the hallmark of canonical autophagy, are not visualized in this process. This finding led us to hypothesize that the role of Atg5 in intracellular immunity to *T. gondii* infection might be independent of its role in the elongation of autophagosomal membrane required for the canonical autophagy pathway (Zhao et al., 2008). To investigate the mechanism, we examined the role of other essential autophagy genes – Atg7, Atg14L, and Atg16L1 – during the *in vivo* infection of *T. gondii*. Atg7 is the E1 activating enzyme that is required for the activation of both ubiquitin-like molecules, LC3 and Atg12. Atg16L1 binds to the Atg12-conjugated Atg5 and form the autophagosome elongation complex, which is essential for the growth of autophagosome by functioning as E3 ligase for LC3 conjugation. Atg14L functions in endoplasmic reticulum targeting of PI3K complex for the nucleation of the autophagosomal membrane (Matsunaga et al., 2010; 2009).

Since full deletion of essential autophagy genes causes neonatal lethality, we took a conditional deletion approach (Mizushima and Levine, 2010). We infected *Atg7^{flox/flox}+LysMcre*, *Atg16L1^{flox/flox}+LysMcre*, *Atg14L^{flox/flox}+LysMcre* and their control

littermate mice with *T. gondii*. Similar to *Atg5^{flox/flox}+LysMcre* mice (Supplementary Figure S1A), both *Atg7^{flox/flox}+LysMcre* (Figure 1A) and *Atg16L1^{flox/flox}+LysMcre* (Figure 1B) mice were more susceptible to lethal infection with *T. gondii* than their controls. *Atg7^{flox/flox}+LysMcre* mice were less susceptible to *T. gondii* infection than *Atg5^{flox/flox}+LysMcre* and *Atg16L1^{flox/flox}+LysMcre* mice, possibly due to less efficient functional deletion of Atg7 by LysMcre (Figure S1B). In contrast, both *Atg14L^{flox/flox}* and *Atg14L^{flox/flox}+LysMcre* mice showed similar resistance to the infection of *T. gondii*, suggesting that Atg14L is not required to control *in vivo* infection of *T. gondii* (Figure 1C). This lack of a phenotype was not due to insufficient functional deletion of Atg14L *in vivo*, because macrophages directly isolated from *Atg14L^{flox/flox}+LysMcre* mice have undetectable Atg14L expression and a significant accumulation of p62 protein, which is normally removed through functional autophagy, compared to control (Figure 1D) without significant induction of p62 mRNA (Figure S1C). In contrast, the conversion of LC3-I (cytosolic) to LC3-II (lipidated and membrane-bound) was largely unaffected by the deletion of Atg14L, which is consistent with previous findings (Matsunaga et al., 2009; Zhong et al., 2009). These genetic data distinguish the role of autophagy proteins in the control of *in vivo* infection of *T. gondii* from their role in canonical degradative autophagy that requires Atg14L.

Canonical degradative autophagy pathway is not required for IFN γ to control *T. gondii* infection

To investigate the control of *T. gondii* by IFN γ in primary macrophages more efficiently than provided by previous microscopy based assays (Zhao et al., 2008), we developed a flow cytometry-based system to assess the level of *T. gondii* infection (Figure S2A). We compared the data of the immunofluorescence method to those of the flow cytometry method for the measurement of *T. gondii* infection, and found that these two assays performed comparably (as described below). Therefore, we utilized the flow cytometry based assay system for the rest of our study.

We first investigated the role of the degradative autophagy pathway in the control of *T. gondii* by IFN γ using pharmacologic approaches. Degradative autophagy was induced with treatment of an mTOR inhibitor (rapamycin) or starvation (Earle's Balanced Salt Solution, EBSS) and inhibited with treatment of PI3K inhibitors (wortmannin or LY294002). Late steps of degradative autophagy, the fusion of the autophagosome with lysosome and subsequent degradation of its contents within the autolysosome, were blocked with lysosomal acidification inhibitors (chloroquine or bafilomycin A₁) or lysosomal protease inhibitors (E64D and pepstatinA). Induction of autophagy is manifested as increased LC3-II and decreased p62 and its inhibition leads to decreased LC3-II and increased p62, although compensatory feedback can occur for prolonged treatment (Hwang et al., 2012; Mizushima et al., 2010). In contrast, lysosomal degradation blockers increase the level of both LC3-II and p62. The treatments with pharmacologic agents had significant and expected effects on degradative autophagy in control cells (Figure 2A), but none of the treatments significantly affected the control of *T. gondii* by IFN γ (Figure 2B). In fact, none of them, except starvation in EBSS medium, which caused significant death of the treated cells, significantly affected the infection level with *T. gondii* (Figure S2B).

We further tested the role of the canonical autophagy pathway in the control of *T. gondii* by IFN γ using genetic approaches. Consistent with the lack of a role for Atg14L in resistance of mice to *T. gondii* infection *in vivo* (Figure 1C), *T. gondii* infection in both control (*Atg14L*^{flox/flox}) and Atg14L-deficient macrophages (*Atg14L*^{flox/flox}+*LysMcre*) was similarly inhibited by IFN γ (Figure 2C). The ULK1 and ULK2 complex is essential in initiating autophagosome formation and deletion of both ULK1 and ULK2 causes perinatal lethality as observed for knockout of essential autophagy genes (McAlpine et al., 2013). Therefore, we utilized mouse embryonic fibroblasts (MEFs) from *Ulk1*^{-/-}*Ulk2*^{-/-} mice to investigate the role of the initiation complex in the control of *T. gondii* by IFN γ . Similar to the Atg14L-deficient cells, deletion of both ULK1 and ULK2 did not affect the control of *T. gondii* by IFN γ (Figure 2D). Therefore, the deletion of Atg14L in the nucleation complex (Figure 2E) and ULK1 and ULK2 in the initiation complex (Figure S2C) did not have any significant effect on the control of *T. gondii* by IFN γ . Collectively, pharmacologic and genetic modulation of the autophagy pathway showed that the canonical degradative autophagy pathway is not required for IFN γ to control *T. gondii* infection, which is consistent with *in vivo* data (Figure 1).

Atg12-Atg5-Atg16L1 complex is required for IFN γ to control *T. gondii* infection *in vitro*

To examine the role of Atg5 in the control of *T. gondii* by IFN γ , we reconstituted Atg5-deficient macrophages with wild type and mutants of Atg5 using lentiviral transduction. We first confirmed that *T. gondii* infection was significantly inhibited by IFN γ in control (*Atg5*^{flox/flox}) but not in Atg5-deficient (*Atg5*^{flox/flox}+*LysMcre*) macrophages transduced with control lentivirus, using both flow cytometry (Figure 3A) and immunofluorescence assays (Figure S3A). Transduction of wild type Atg5 into Atg5-deficient macrophage restored the control of *T. gondii* by IFN γ and basal autophagy in the transduced cells, as represented by the conversion of LC3-I to LC3-II and the decrease of p62 (Figures 3A and 3B). Atg5 functions as a complex with Atg12 and Atg16L1 in the degradative autophagy pathway. Thus, we further assessed the requirement for the Atg12-Atg5-Atg16L1 complex in the IFN γ -mediated control of *T. gondii* by utilizing Atg5 mutants, Atg7-deficient (*Atg7*^{flox/flox}+*LysMcre*) and Atg16L1-deficient (*Atg16L1*^{flox/flox}+*LysMcre*) macrophages. In contrast to wild type Atg5, the Atg12 conjugation defective mutant (Atg5-K130R) (Mizushima et al., 1998) and Atg16L1 binding defective mutants (partial defective Atg5-D88A and severely defective Atg5-G84A-D88A) of Atg5 (Hwang et al., 2012) were not able to restore the control of *T. gondii* by IFN γ (Figure 3A) or basal autophagy (Figure 3B) in the Atg5-deficient macrophages. These data suggest that the conjugation of Atg12 to Atg5 and the binding of Atg5 to Atg16L1 are required for IFN γ to control *T. gondii* infection.

T. gondii infection was not inhibited by IFN γ in Atg7-deficient macrophages, but transduction with wild type Atg7 restored the control of *T. gondii* by IFN γ as well as basal autophagy in Atg7-deficient macrophages (Figures 3C and 3D). However, an enzyme-null mutant of Atg7 (Atg7/C567A) (Fujita et al., 2008) was not able to restore either the control of *T. gondii* by IFN γ or basal autophagy (Figures 3C and 3D). It was recently shown that autophagic sequestration of invading bacteria and the subsequent degradation of the cargo (called xenophagy) can be targeted through the ubiquitination of the endosomes that contain

the invading bacteria (Fujita et al., 2013; Levine et al., 2011). The elongation complex localizes and directs LC3 to the ubiquitinated target because Atg16L1 recognizes the ubiquitinated substrates via three independent mechanisms. First, Atg16L1 directly binds to ubiquitin via the WD repeat domain at its C-terminus. Second, Atg16L1 binds to FIP200 in the initiation complex, which is recruited to the ubiquitinated target independently. This interaction is also required for the proper targeting of Atg16L1 to the site of autophagosome initiation for canonical autophagy (e.g. starvation-induced) (Gammoh et al., 2013; Nishimura et al., 2013). Third, amino acids 194–195 of Atg16L1 play a role through an unknown mechanism (Fujita et al., 2013). We therefore reconstituted Atg16L1-deficient macrophages with various mutants of Atg16L1 and investigated the IFN γ -mediated control of *T. gondii*. As observed for Atg5- and Atg7-deficient macrophages, *T. gondii* infection was not controlled by IFN γ in the absence of Atg16L1 (Figure 3E). Upon transduction of wild type Atg16L1 and its WD repeat deletion mutant (dWDR), the control of *T. gondii* by IFN γ and basal autophagy were restored (Figures S3B, 3E and 3F). Further, transduction of FIP200-binding defective derivative, dWDR(239–242)A, and a derivative carrying mutations in amino acids 194–195, dWDR(194–195)A, of WD repeat deletion mutant as well as triple mutant, dWDR(239–242)A(194–195)A (Fujita et al., 2013), restored the control of *T. gondii* by IFN γ significantly (Figure 3E). However, those mutants did not fully restore basal autophagy (no significant reduction in p62 level) while LC3-II conversion was restored (Figure 3F). These data suggest that the C-terminal WD repeat domain, binding to FIP200 and concomitant ubiquitin-binding activity of Atg16L1 are not required for IFN γ -mediated control of *T. gondii* and that the N-terminal conserved domain of Atg16L1, which includes a domain for Atg5-binding, is sufficient for its function. Taken together, all these data suggest that the whole elongation complex of Atg12-Atg5-Atg16L1 is required for IFN γ to control *T. gondii* infection *in vitro* and further indicated that the function of Atg12-Atg5-Atg16L1 complex in the control of *T. gondii* by IFN γ is different from its role in canonical autophagy and ubiquitin-dependent xenophagy.

Atg3 is required for IFN γ to control *T. gondii* infection

The only known function of the Atg12-Atg5-Atg16L1 complex so far is to provide an E3 ligase activity for the targeted conjugation of LC3 homologs to phosphatidylethanolamine on the growing autophagosome (Rubinsztein et al., 2012). Although the canonical autophagy pathway is not required for IFN γ to control *T. gondii* infection, it was still possible that the conjugation of LC3 homologs to membranes through the E3 ligase activity of the complex is required. To investigate the requirement for LC3 conjugation in the control of *T. gondii* by IFN γ , we utilized Atg4B- and Atg3-deficient macrophages because of the redundancy of LC3 family members in mammalian system (Shpilka et al., 2011). Atg4B is the dominant isoform of Atg4 in macrophages that is required for the proteolytic processing of LC3 homologs for both conjugation to and deconjugation from autophagosomal membranes (Hwang et al., 2012; Mariño et al., 2010). Atg3 is the essential E2 enzyme only for the conjugation of LC3 homologs (Sou et al., 2008), although Atg7 is required for conjugation of both Atg12 and LC3 homologs. In Atg4B-deficient macrophages, *T. gondii* infection was controlled by IFN γ significantly better than its control macrophages (Figure S3C). In contrast, in Atg3-deficient macrophages *T. gondii* infection was controlled by IFN γ significantly less than in control macrophages (Figure 4A). Since

the deletion of Atg3 by Cre recombinase in the macrophages was not complete (Figure 4B), we obtained MEFs with complete functional deficiency of Atg3 (*Atg3*^{-/-}, Figure 4C) and confirmed that *T. gondii* infection was not controlled by IFN γ (Figure 4D), which is consistent with the recent findings of others (Haldar et al., 2014). We further examined the physiological significance of Atg3 during the *in vivo* *T. gondii* infection by infecting *Atg3*^{flox/flox}+*LysMcre* and littermate control mice with *T. gondii*. Consistent with the *in vitro* data, mice with myeloid lineage specific deletion of Atg3 were significantly more susceptible to the lethal infection of *T. gondii* than control mice (Figure 4E). We further examined the importance of E2 enzyme activity of Atg3 in the IFN γ -mediated control of *T. gondii* by transducing *Atg3*^{-/-} MEF with wild type and enzyme-null mutant (C264S) of Atg3 (Figure 4F) (Sou et al., 2008). *T. gondii* infection was significantly controlled by IFN γ in *Atg3*^{-/-} MEF transduced with wild type Atg3 but not with the enzyme-null mutant, demonstrating that the E2 enzyme activity of Atg3 is required for the control of *T. gondii* by IFN γ (Figure 4G). Thus, E2 conjugating enzyme Atg3, in addition to E1 activating enzyme Atg7 and E3 ligase Atg12-Atg5-Atg16L1 complex, is required for the IFN γ -mediated control of *T. gondii* infection *in vitro* and *in vivo*.

Colocalization of LC3 with the parasitophorous vacuole membrane of *T. gondii*

We and others have showed that Atg5 is required for the proper targeting of the IFN γ -induced effectors to the PVM of *T. gondii* without the involvement of autophagosome (Selleck et al., 2013; Zhao et al., 2009; 2008). One plausible mechanism based on our data was that the conjugation of LC3 homologs plays a crucial role in the recruitment of the IFN γ effectors onto the PVM of *T. gondii* without the involvement of functional modules for autophagosome initiation or nucleation and lysosomal degradation. Therefore, we reasoned that LC3 homologs might be directly conjugated onto the PVM of *T. gondii*. However, it was previously shown that GFP-LC3, conventional marker for autophagosomal membrane, does not co-localize with the PVM of *T. gondii* (Martens et al., 2005). Since a discrepancy in cellular localization and function between the N-terminal GFP-tagged LC3 and endogenous LC3 has been reported (Reggiori et al., 2010), we examined the localization of endogenous LC3 with regard to invading *T. gondii* and IFN γ -induced effectors, such as immunity-related GTPase 6 (Irga6, a.k.a. interferon inducible GTPase 1, IIGP1). We first investigated the localization of LC3 in non-phagocytic cells, MEFs, because LC3 can be recruited to the phagosomal single membrane (Sanjuan et al., 2007). Live *T. gondii* enters cells by an active parasite-driven process that is independent of phagocytosis (Sibley, 2011). At 2 hour-post-infection (hpi) of IFN γ -treated MEFs, Irga6 localized on the PVM of *T. gondii* and the colocalization of endogenous LC3 with Irga6 was detected on the PVM (Figure 5). Therefore, endogenous LC3 can localize on or near the PVM of *T. gondii* and, considering the absence of double membrane bound autophagosome near the PVM (Zhao et al., 2008) and the necessity of LC3 conjugation machinery for the control of *T. gondii* by IFN γ , it is highly likely that LC3 is conjugated to the PVM of *T. gondii*.

Atg5 and Atg3, but not Atg14L, are required for the localization of LC3 and Irga6 on the PVM

To examine the genetic requirement of LC3 and Irga6 localization on the PVM of *T. gondii*, we further investigated their localization with regard to invading *T. gondii* in *Atg5*- and

Atg14L-deficient macrophages, as well as in *Atg3*^{-/-} MEFs. In IFN γ -treated control macrophages, Irga6 and LC3 localized on the PVMs of *T. gondii* both individually and concurrently (Figure 6A). Even in untreated macrophages, LC3 alone localized on the PVM of a small percentage of *T. gondii* in the absence of Irga6, suggesting that the localization of LC3 on the PVM of *T. gondii* is not dependent on induction or recruitment of IFN γ effectors. In contrast, in Atg5-deficient macrophages the localizations of LC3 and Irga6 on the PVM of *T. gondii* were significantly reduced and Irga6 formed cytoplasmic aggregates as shown previously (Zhao et al., 2008). These data demonstrate that Atg5 is required for the proper targeting of both LC3 and Irga6 to the PVM of *T. gondii* as well as for the control of *T. gondii* by IFN γ .

In contrast to Atg5-deficient macrophages, in Atg14L-deficient macrophages there was no reduction in the localization of LC3 and Irga6 to the PVM of *T. gondii* (Figure 6B) and Irga6 did not form aggregates, indicating that localization of LC3 and Irga6 onto the PVM as well as the control of *T. gondii* by IFN γ do not require a gene essential for canonical degradative autophagy. However, in *Atg3*^{-/-} MEFs, LC3 did not localize on the PVM of *T. gondii* and Irga6 formed cytoplasmic aggregates (Figure 6C). Again, LC3 was detected on the PVM of a small percentage of *T. gondii* even in the absence of IFN γ treatment, confirming that the localization of LC3 on the PVM requires functional Atg3 but not IFN γ . Collectively, our data demonstrated that Atg5 and Atg3, but not Atg14L, are required for the localization of LC3 and Irga6 on the PVM of *T. gondii* and that LC3 localizes on the PVM of *T. gondii* independent of IFN γ .

The ubiquitin-like conjugation systems are required for the localization of IFN γ effectors onto PVM

We confirmed that another member of the IRG family (Irgb6, Figure 7A) and GBPs (GBP1-5, Figure 7B) (Howard et al., 2011; Kim et al., 2012) were also recruited onto the PVM of *T. gondii* only in the presence of Atg3 and Atg7. Further, when we tested the control of endodyogenic replication of *T. gondii* within a parasitophorous vacuole by IFN γ , IFN γ also significantly reduced the replication of *T. gondii* in the vacuole, in an Atg3- and Atg7-dependent manner (Figure S4A). Lastly, to examine the necessity of the enzyme activity of the Atg3 and Atg7 in the recruitment of IFN γ effectors onto the PVM of *T. gondii*, we reconstituted *Atg3*^{-/-} and *Atg7*^{-/-} MEFs with wild type and catalytically inactive mutants of Atg3 (C264S) and Atg7 (C567A), respectively (Figures 4F and S4B). Consistent with the genetic data (Figures 3C and 4G), wild type Atg3 and Atg7 were able to restore the recruitment of Irga6 onto the PVM of *T. gondii* in *Atg3*^{-/-} and *Atg7*^{-/-} MEF, respectively (Figures 7C and 7D). However, catalytically inactive mutants of Atg3 and Atg7 were incapable of restoring the recruitment of the IFN γ effector onto the PVM. Collectively, these data demonstrate the essential role of the ubiquitin-like conjugation systems of the autophagy pathway in the recruitment of IFN γ effectors onto the PVM of *T. gondii* and subsequent control of *T. gondii* infection and replication.

Discussion

Degradative autophagy has been shown to play crucial roles in immune defense against intracellular pathogens as well as its conventional roles in recycling and remodeling (Levine et al., 2011). In addition, the evolutionarily conserved autophagy proteins have evolved and acquired unique functions in the immune system and myeloid cells, independent of their roles in the degradation pathway (Bestebroer et al., 2013; DeSelm et al., 2011; Hwang et al., 2012; Reggiori et al., 2010; Starr et al., 2012; Subramani and Malhotra, 2013). In this report, we extended these observations by demonstrating that the ubiquitin-like conjugation machinery of the autophagy pathway, E1 Atg7, E2 Atg3, and E3 Atg12-Atg5-Atg16L1, are required for the proper targeting of IFN γ effectors onto the PVM of *T. gondii* and subsequent control of *in vitro* and *in vivo* *T. gondii* infection. Although degradative autophagy has been proposed to play a role in the CD40 ligation-mediated defense against *T. gondii* in non-hematopoietic cells (Van Grol et al., 2013), the multiple aspects of the overall autophagy pathway including the ULK1 and ULK2 initiation complex, the Atg14L nucleation complex, and lysosomal degradation were not required for the control of *T. gondii* by IFN γ in MEFs or macrophages. We further found that LC3 localized on, and possibly was conjugated to, the PVM of *T. gondii*, and confirmed that the recruitment of IFN γ effectors to the PVM was dependent on the functional ubiquitin-like conjugation systems of the autophagy pathway. These data suggest that the ubiquitin-like conjugation systems utilized for the reorganization of intracellular membranes in the canonical autophagy pathway have evolved to target immune effectors toward the intracellular vacuole membrane structure of pathogens.

Although IRGs and GBPs are thought to participate in disrupting the PVM, the molecular mechanism of their recruitment to this interface through Atg5 is incompletely understood (Howard et al., 2011). The autophagosome itself is not directly involved in the control of *T. gondii per se* (Zhao et al., 2009; 2008). Nevertheless, it was possible that a functional degradative autophagy pathway may be required to remove prematurely activated IFN γ effectors, which tend to aggregate and subsequently inhibit the proper loading of the effectors on the target membrane (Selleck et al., 2013). In this setting, one might predict that the entire degradative autophagy pathway and all essential autophagy proteins are required for proper effector function. However, our data clearly demonstrated that the degradative autophagy pathway and the other functional cassettes of autophagy proteins were not required for efficient control of *T. gondii* by IFN γ . In fact, unlike the situation for Atg5-deficient cells, we did not detect any aggregation of the activated IFN γ effectors in Atg14L-deficient macrophages, even though canonical autophagy was significantly inhibited. Thus, our current data do not support the role of degradative autophagy for the proper maintenance of activated IFN γ effectors.

Independent of IFN γ effector targeting to *T. gondii*, LC3 localized on a small percentage of PVMs in a ubiquitin-like conjugation system dependent process. This finding is reminiscent of Atg16L1 localization on the membranous replication complex of MNV without IFN γ -treatment (Hwang et al., 2012). The Atg12-Atg5-Atg16L1 complex, particularly Atg16L1, can specify the lipidation site of LC3 for the membrane biogenesis in both nonselective autophagy (Fujita et al., 2008) and selective xenophagy (Fujita et al., 2013). Thus, it is

tempting to speculate that the Atg12-Atg5-Atg16L1 complex may function as a module to recognize and/or mark the “non-self” intracellular membrane structure of pathogens through the conjugation of LC3. The conjugated LC3 may recruit the effectors through direct interaction or indirectly through adaptor proteins in a similar way to its function in canonical autophagy (Shpilka et al., 2011). Alternatively, although the localization of LC3 on the PVM of *T. gondii* happened even in non-phagocytic cells, it is still possible that these events may represent parasites that were engulfed by cellular processes other than pathogen-driven invasion. Resolving these respective roles will take further studies on the kinetics of LC3 recruitment to PVMs in non-IFN γ -treated cells.

Interestingly, *T. gondii* infection was controlled by IFN γ more efficiently in Atg4B-deficient cells than in control cells, which also happened in the control of MNV by IFN γ (Hwang et al., 2012). A major function of Atg4 family proteins is to cleave the membrane bound LC3 as well as to proteolytically process LC3 for membrane conjugation. Thus, in the absence of Atg4B, the deconjugation or delipidation of LC3 from the membrane is slowed down, which may lead to the more recruitment of the IFN γ effectors and thus more efficient control of pathogens by IFN γ . In this regard, it is interesting to note that Atg4B can be recruited onto the membrane structure of pathogens by one of the IFN γ effectors, GBP7 (Kim et al., 2011). The recruitment of Atg4B to the membrane by the IFN γ effectors and subsequent cleavage of LC3 from the membrane might be a negative feedback mechanism for proper activity of IFN γ effectors.

Although both LC3 and the IFN γ effector Irga6 localized on the PVM of *T. gondii*, there was relatively little concurrent colocalization between LC3 and Irga6 on the PVM. These data suggest that LC3 and the IFN γ effectors may not work simultaneously and their recruitment to the PVM of *T. gondii* may be spatially and temporally regulated for efficient targeting and disruption of the membrane structure of the pathogens. Alternatively, the ubiquitin-like conjugation system of the autophagy pathway might be required for some yet undefined role that is necessary for proper homeostasis of the IFN γ effectors, since they form aggregates when these autophagy proteins are absent. Indeed, it was previously proposed that Atg5 may be required for the normal function of regulatory IRGs (GMS subfamily, e.g. Irgm1, Irgm3) to demarcate endosomal membranes and special pathogen-oriented membranes and/or to regulate nucleotide exchange and activation of the effector IRGs (GKS subfamily, e.g. Irga6, Irgb6) (Haldar et al., 2013; Hunn et al., 2008). This explanation is not mutually exclusive with the mechanisms proposed here. Further studies are required to elucidate how the conjugation of LC3 homologs by the ubiquitin-like conjugation systems determines the recruitment of the IFN γ effectors onto the PVM of *T. gondii*.

It is interesting to note that a similar dependence on Atg5 and Atg7 and targeting of LC3 to single membranes has been observed for the secretion of lysosomal proteins by osteoclasts and mucin by colonic goblet cells, LC3-associated phagocytosis (LAP) and entotic cell engulfment (DeSelm et al., 2011; Florey et al., 2011; Martinez et al., 2011; Patel et al., 2013). Particularly, considering the enhanced killing of phagocytosed microbes through LAP (Sanjuan et al., 2007), it will be important to further study the shared and differentiated mechanism between LAP and the process described here that targets intracellular membrane

structures in host immune defense. Notably, the parasitophorous vacuole of *T. gondii* is actively made by the pathogen itself even in non-phagocytic cells, suggesting that, even if the same machinery is required by both LAP and destruction of intracellular *T. gondii*, the membrane events involved may differ topologically.

Our findings confirm and extend recently published findings showing the critical role of Atg3, Atg7, and Atg16L1, but not Atg9a and Atg14, for the control of *T. gondii* by IFN γ effectors (Haldar et al., 2014; Ohshima et al., 2014). The data we provide here show *in vivo* relevance and molecular proof of the specific functions of the required autophagy proteins (e.g. enzymatic activity of Atg3 and Atg7, binding of Atg5 to Atg16L1) in intracellular killing of *T. gondii*. Importantly, we also show here the functions of the autophagy proteins in macrophages, primary cells that are involved in resistance to *T. gondii*. Thus, our study firmly demonstrated the nondegradative function of ubiquitin-like conjugation systems of the autophagy pathway in cell-autonomous immune defense system. Together, these studies establish the paradigm that autophagy proteins function in intracellular immunity in ways that do not require the entire degradative autophagy pathway.

In summary, we found that the ubiquitin-like conjugation machinery of the autophagy pathway, but not the degradative autophagy pathway itself, plays crucial roles in the anti-parasitic activity of IFN γ in that it is required for the proper targeting of the IFN γ effectors to the vacuole membrane structure of the parasites. Elucidating such nondegradative roles of autophagy proteins in sensing and inducing the destruction of pathogenic membrane structures in the cytoplasm may lead to novel therapeutic and/or prophylactic treatments for the infectious diseases caused by these pathogens as well as to a greater appreciation and understanding of fundamental biological processes of the genes in the autophagy pathway. The evolutionary conservation of autophagy from the earliest stages of metazoan evolution may have favored the diversification of autophagy proteins to contribute differentially to host defense and thereby to play key roles both through degradative functions of autophagy and through other, perhaps as yet undiscovered, mechanisms of intracellular immunity.

Experimental Procedures

Mice and Cells

Atg5^{flox/flox} +/- LysMcre, *Atg7^{flox/flox} +/- LysMcre*, *Atg16L1^{flox/flox} +/- LysMcre* mice were previously described (Hwang et al., 2012). *Atg14L^{flox/flox} +/- LysMcre* mice were kindly provided by Dr. Shizuo Akira, Osaka University, Japan. *Atg3^{flox/flox} +/- LysMcre* mice were derived from *Atg3^{flox/flox}* mice kindly provided by Dr. You-Wen He, Duke University, U.S.A. (Jia and He, 2011). All mice were housed and bred at Washington University in St. Louis, St. Louis MO and University of Chicago, Chicago IL under specific-pathogen-free conditions in accordance with federal and university guidelines. Bone marrow derived macrophages (BMDMs), MEFs, and 293T cells were used for *T. gondii* infection and lentiviral transduction. See the Supplemental Experimental Procedures for the details.

Infection with *T. gondii*

T. gondii (type-II strains) expressing luciferase (ME49) and green fluorescent protein (PTG) were maintained in HFF cells (Zhao et al., 2008). At the time of infection, an inoculum of infectious *T. gondii* tachyzoites was prepared by disrupting the infected HFF cells using 26G needle and syringe, centrifuging the lysate at 400 x g for 10 minutes, and resuspending the precipitate in culture media (*in vitro*) or serum free DMEM (*in vivo*). For *in vitro* experiments, cells were pretreated with recombinant murine IFN γ at the indicated doses (e.g. 100 U/ml = 50 ng/ml) for 24 hours, infected with *T. gondii* tachyzoites at the multiplicity of infection (MOI) of 1. At 24 hr-post-infection (hpi), the infected cells were fixed with 2% formaldehyde (Ted Pella; 18505) in PBS for 10–20 minutes at room temperature. The fixed cells were permeabilized with PBS + 0.1% Triton X-100 (PBS-T) overnight at 4°C and *T. gondii* infection was assessed by indirect immunofluorescence or flow cytometry. For *in vivo* experiments of *Atg5*^{flox/flox}_{+/-}*LysMcre*, *Atg7*^{flox/flox}_{+/-}*LysMcre*, *Atg14L*^{flox/flox}_{+/-}*LysMcre*, and *Atg16L1*^{flox/flox}_{+/-}*LysMcre* mice, 9~11 week old mice were inoculated intraperitoneally with 200 (male) or 100 (female) of *T. gondii* tachyzoites per mouse (Zhao et al., 2008) and the survival of the infected mice was monitored for 30 days in accordance with the guideline of Washington University in St. Louis. No significant phenotypic difference was observed between male and female mice. The *T. gondii* infection study of *Atg3*^{flox/flox}_{+/-}*LysMcre* mice were performed in the presence of 0.6 ug/ml buprenorphine under the guideline of University of Chicago. Two or three independent experiments were done for each *in vivo* infection study.

Chemicals

Rapamycin (sc-3504, 5 mM in DMSO), wortmannin (sc-3505, 5 mM in DMSO), bafilomycin A₁ (sc-201550, 100 uM in DMSO), E64D (sc-201280, 5 mg/ml in DMSO), pepstatin A (sc-45036, 5 mg/ml in DMSO) from Santa Cruz Biotechnology; chloroquine (C6628, 20 mM in water) from Sigma; LY294002 (#9901, 10 mM in DMSO) from Cell Signaling; mouse IFN γ (485MI/CF, 200 U/ul (100 ng/ul) in PBS/0.1%BSA) from R&D Systems.

Protein analysis by western blot

Total cellular proteins were harvested and proteins were analyzed as previously described (Hwang et al., 2012). Peritoneal exudate cells (PECs) were obtained by lavage, incubated at 37°C for 4 hours on tissue culture treated plates, and washed to purify adherent macrophages. Cell lysates were then harvested and analyzed by western blot. Commercial antibodies used in this study are as follows: 2A (Millipore, ABS31), Atg3 (MBL, M133-3), Atg7 (Sigma, A2856), Atg14L (Sigma, A6358), Atg16L1 (Sigma, A7356), Beta-actin (Sigma, A5316), LC3B (Sigma, L7543), and p62 (Sigma, P0067).

Immunofluorescence and Flow cytometry

Fixed and permeabilized cells were analyzed for *T. gondii* infection by immunofluorescence and flow cytometry using rabbit polyclonal anti-GRA7 (dense granule protein 7) (Selleck et al., 2013) or GFP-expressing reporter stain (PTG). See the Supplemental Experimental Procedures for the details.

Statistical analysis

All data were analyzed with Prism software (GraphPad). Unless otherwise stated, all experiments were performed at least three times and the data were combined for presentation as average \pm SEM. All differences not specifically indicated to be significant were not significant (n.s., $p > 0.05$). *: $p < 0.05$, **: $p < 0.01$, *** $p < 0.001$, ****: $p < 0.0001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A cassette of Atg7, Atg3, Atg12-Atg5-Atg16L1 mediates IFN γ control of *T. gondii*.
- The degradative pathway of autophagy is not required for IFN γ control of *T. gondii*.
- Autophagy proteins mark membrane structures by pathogens for intracellular immunity.
- Autophagy proteins are required to direct IFN γ effectors to membranes of pathogens.

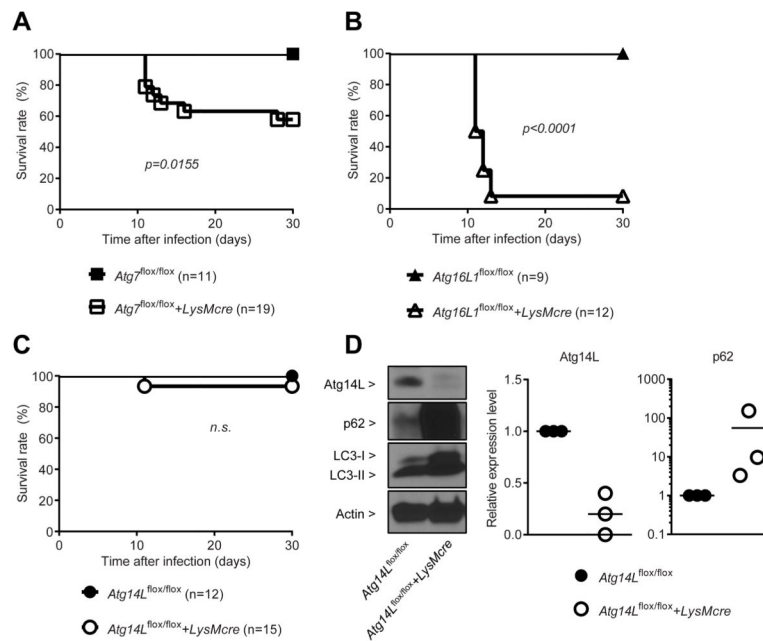


Figure 1. Atg7 and Atg16L1, but not Atg14L, are required to control *T. gondii* infection *in vivo* Survival curves after intraperitoneal inoculation with 200 (male) or 100 (female) of *T. gondii* per mouse. Number of mice in parentheses. (A) *Atg7^{fllox/fllox}+/-LysMcre*, (B) *Atg16L1^{fllox/fllox}+/-LysMcre*, (C) *Atg14L^{fllox/fllox}+/-LysMcre*. Statistical analysis by Log-rank (Mantel-Cox) test. (D) A representative protein blot and quantitative analyses of Atg14L and p62 in the peritoneal exudate macrophages. Combined data as each data point with average. See also Figure S1.

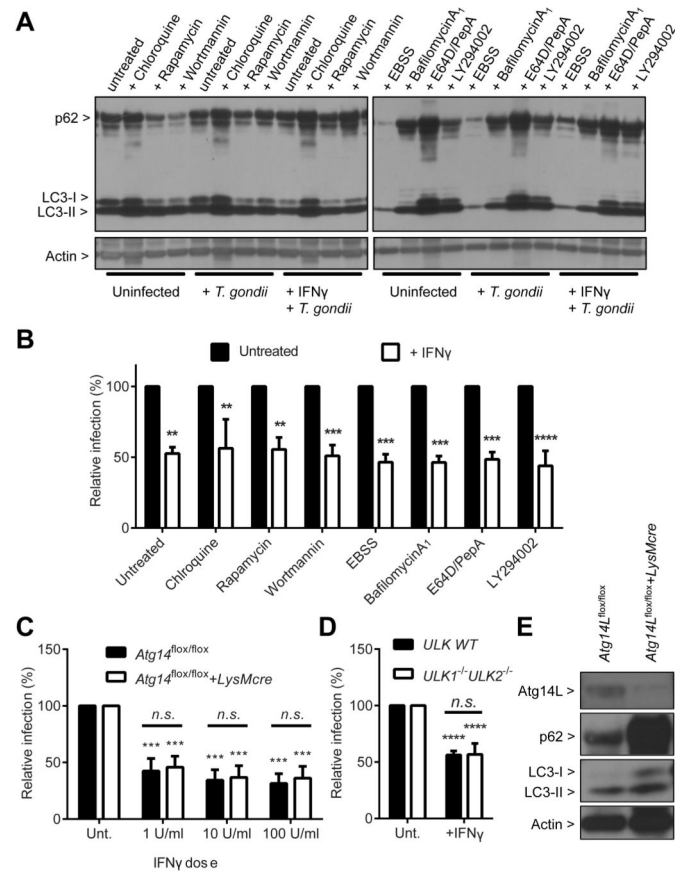


Figure 2. Degradative autophagy pathway is not required for IFN γ to control *T. gondii* infection (A) Protein blot for the autophagy status of the samples as shown in (B). Samples harvested at 24 hpi. (B) Flow cytometry analysis for the relative infection (%) of *T. gondii* at 24 hpi (MOI=1) in BMDMs from C57BL/6 mice treated with chemicals as indicated. Cells were treated with the chemicals for 24 hrs +/- 100 U/ml (50 ng/ml) of IFN γ before infection and treated again during the infection (total 48 hrs of treatment). Autophagy inducers - rapamycin, starvation (EBSS); autophagy inhibitors - wortmannin, LY294002; degradation blockers - chloroquine, bafilomycin A₁, E64D, pepstatinA. (C) Same analysis as shown in (B) for *Atg14L*^{flox/flox}+/-*LysMcre* BMDMs. Cells were pretreated with IFN γ for 24 hrs at the indicated doses (D) Same analysis as shown in (B) for WT and *Ulk1*^{-/-}*Ulk2*^{-/-} MEFs. (E) Protein blot for untreated/uninfected *Atg14L*^{flox/flox}+/-*LysMcre* BMDMs. Statistical analysis by one-way analysis of variance (1-ANOVA) with Tukey post test. n.s.: not significant (p>0.05), **: p<0.01, ***: p<0.001, ****: p<0.0001. Combined data as average \pm SEM. See also Figure S2.

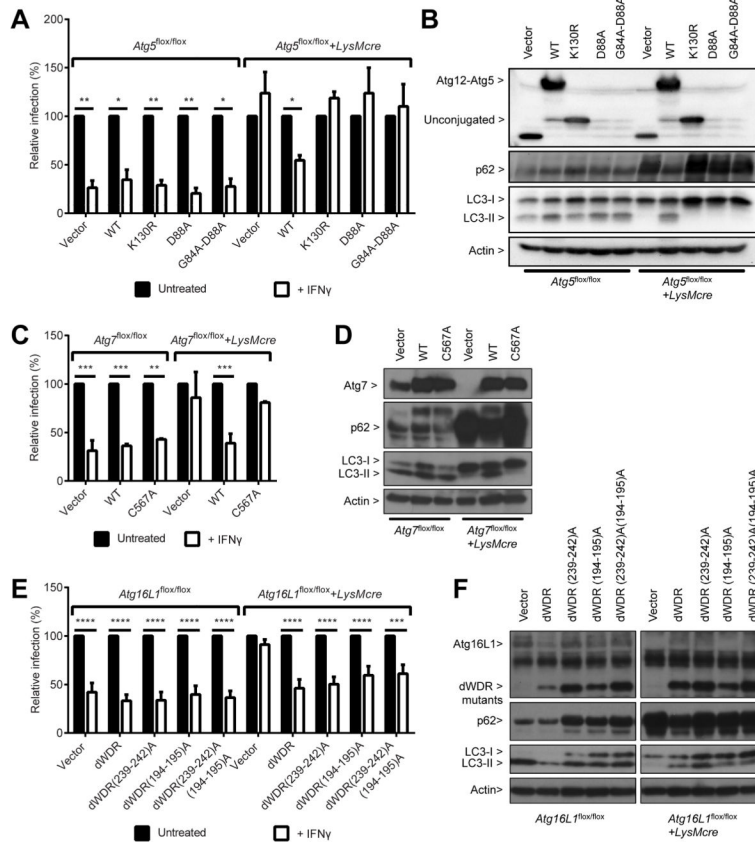


Figure 3. Atg12-Atg5-Atg16L1 complex formation via Atg7 is required for IFN γ to control *T. gondii* infection
 (A) Flow cytometry analysis for *T. gondii* infection +/- 24 hr pre-treatment of 100 U/ml of IFN γ at 24 hpi (MOI=1) in *Atg5*^{flox/flox} +/- *LysMcre* BMDMs transduced with control, WT Atg5 or mutants: K130R - defective in Atg12 conjugation, D88A and G84A/D88A - defective in Atg16L1 binding. (B) Protein blot for untreated or uninfected samples as shown in (A). (C) Same analysis as shown in (A) for *Atg7*^{flox/flox} +/- *LysMcre* BMDMs transduced with control, WT Atg7, or enzyme-null mutant (C567A). (D) Protein blot for untreated or uninfected samples as shown in (C). (E) Same analysis as shown in (A) for *Atg16L1*^{flox/flox} +/- *LysMcre* BMDMs transduced with control, dWDR (WD repeat deletion), dWDR(239-242)A (+FIP200 binding defective), dWDR(194-195)A (+ mutated 194th-195th), and dWDR(239-242)A(194-195)A. (F) Protein blot for untreated/uninfected samples as shown in (E). Statistical analysis by 1-ANOVA with Tukey post test. *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. Combined data as average \pm SEM. See also Figure S3.

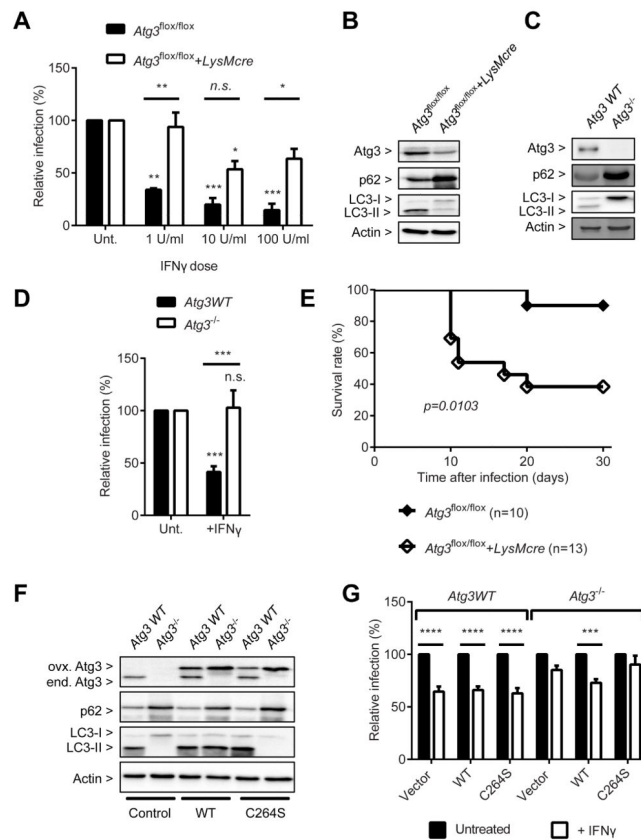


Figure 4. Atg3 is required for the control of *T. gondii* infection

(A) Flow cytometry analysis for *T. gondii* infection at 24 hpi (MOI=1) in *Atg3*^{flox/flox}/_{+/-} *LysMcre* BMDMs +/- 24 hr pre-treatment of IFN γ at the indicated doses. (B) Protein blot for untreated or uninfected *Atg3*^{flox/flox}/_{+/-} *LysMcre* BMDMs. (C) Protein blot for WT and *Atg3*^{-/-} MEF. (D) Same analysis as shown in (A) for the WT and *Atg3*^{-/-} MEF. (E) Survival curves of *Atg3*^{flox/flox}/_{+/-} *LysMcre* mice after intraperitoneal inoculation with *T. gondii*. (F) Protein blot for untreated or uninfected samples as shown in (G). (G) Same analysis as shown in (A) for WT and *Atg3*^{-/-} MEFs transduced with control, WT Atg3, or its enzyme-null mutant (C264S) +/- 24 hr pre-treatment of 100 U/ml of IFN γ . Statistical analysis by 1-ANOVA with Tukey post test or Log-rank (Mantel-Cox) test. n.s.: not significant ($p > 0.05$), **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. Combined data as average \pm SEM. See also Figure S3.

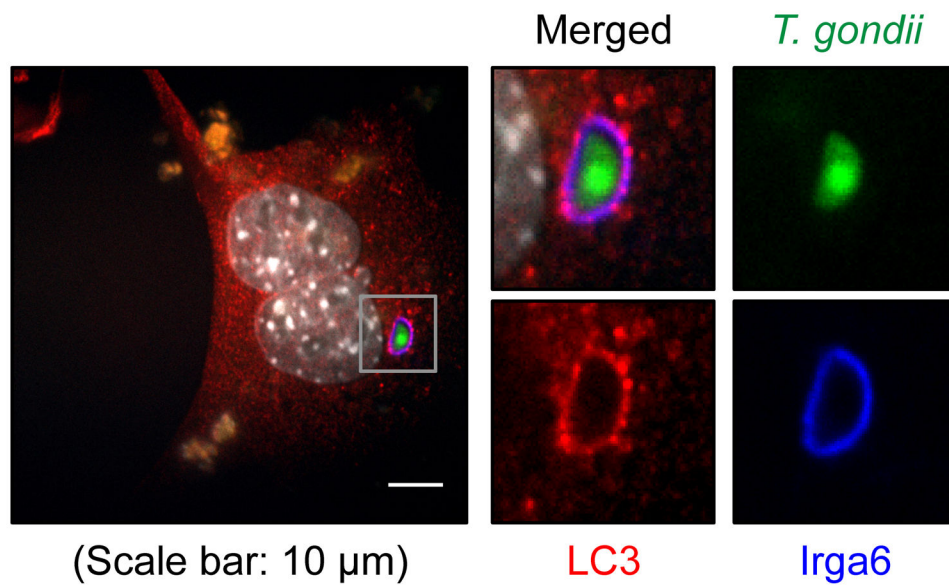


Figure 5. LC3 and Irga6 localize on the PVM of *T. gondii*
(A) A representative image of immunofluorescence for WT MEF at 2 hpi (MOI=1) of *T. gondii* infection with 24 hr pre-treatment of 100 U/ml of IFN γ . Experiments were performed more than thrice.

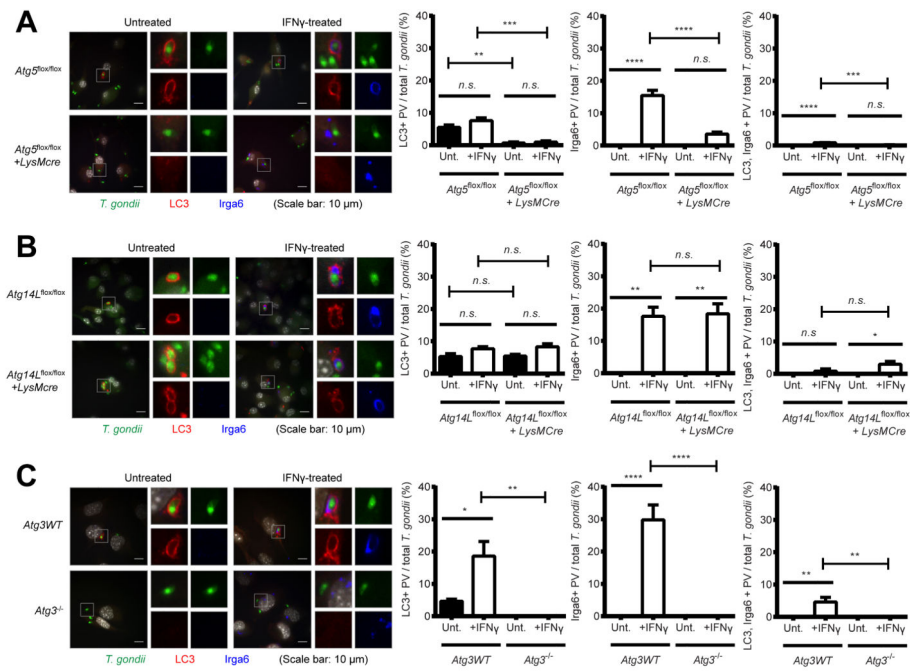


Figure 6. *Atg5* and *Atg3*, but not *Atg14L*, are required for the localization of LC3 and Irga6 on the PVM of *T. gondii*

Representative images (left) and quantitation (right) of immunofluorescence for *T. gondii*, LC3, and Irga6 in (A) *Atg5*^{flx/flx} +/− *LysMCre* and (B) *Atg14L*^{flx/flx} +/− *LysMCre* BMDMs and (C) *Atg3* WT and *Atg3*^{−/−} MEFs at 2 hpi (MOI=1) of *T. gondii* infection +/− 24 hr pre-treatment of 100 U/ml of IFN γ . At least 100 cells infected *T. gondii* were analyzed for quantitation. Statistical analysis by 1-ANOVA with Tukey post test. n.s.: not significant ($p > 0.05$), **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. Combined data as average \pm SEM.

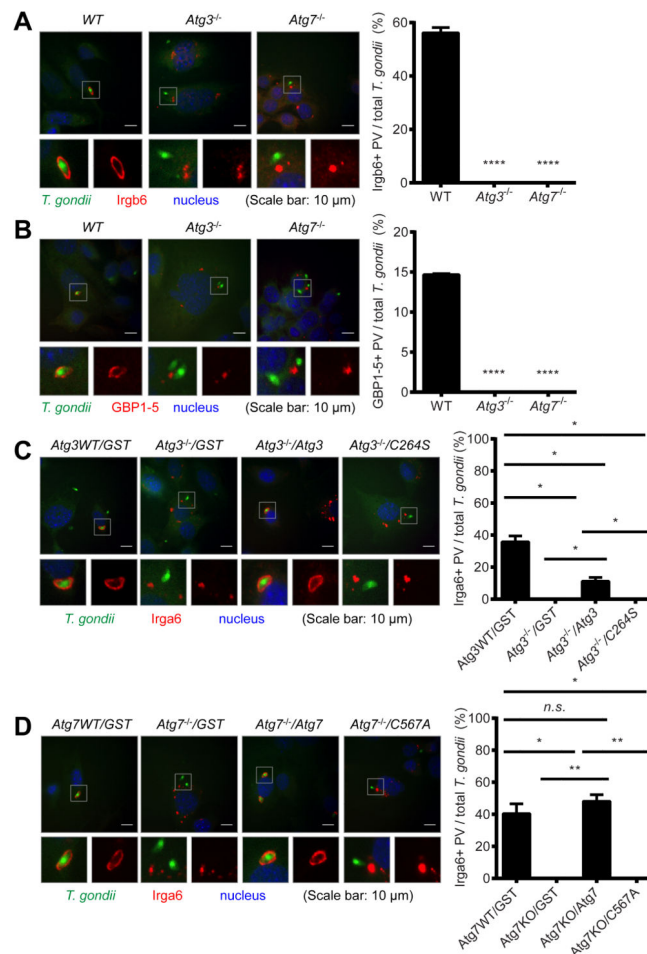


Figure 7. The ubiquitin-like conjugation systems of the autophagy pathway are required for the localization of IFN γ effectors onto the PVM of *T. gondii* infection
 Immunofluorescence of MEFs at 2 hpi (MOI=1) of *T. gondii* infection with 24 hr pre-treatment of 100 U/ml of IFN γ . Representative images (left) and quantitation (right) of immunofluorescence. Irgb6 (A) and GBP1-5 (B) in WT, *Atg3*^{-/-} and *Atg7*^{-/-} MEFs. At least 50 of infected *T. gondii* were analyzed for quantitation. Statistical analysis by 1-ANOVA with Tukey post test. ****: $p < 0.0001$. Experiments were performed twice. Combined data as average \pm SEM. (C) *T. gondii* and Irga6 in *Atg3* WT and *Atg3*^{-/-} MEFs transduced with control (GST, Glutathione-S-transferase), WT *Atg3*, or enzyme-null mutant (C264S). (D) *T. gondii* and Irga6 in *Atg7* WT and *Atg7*^{-/-} MEFs transduced with control (GST), WT *Atg7*, or enzyme-null mutant (C567A). At least 100 of infected *T. gondii* were analyzed for quantitation. Statistical analysis by unpaired t-test. *n.s.*: not significant. *: $p < 0.05$, **: $p < 0.01$. Experiments were performed twice. Combined data as average \pm SEM. See also Figure S4.