

Autophagosome-Independent Essential Function for the Autophagy Protein Atg5 in Cellular Immunity to Intracellular Pathogens

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SUMMARY

The physiologic importance of autophagy proteins for control of mammalian bacterial and parasitic infection in vivo is unknown. Using mice with granulocyte- and macrophage-specific deletion of the essential autophagy protein Atg5, we show that Atg5 is required for in vivo resistance to the intracellular pathogens *Listeria monocytogenes* and *Toxoplasma gondii*. In primary macrophages, Atg5 was required for interferon γ (IFN- γ)/LPS-induced damage to the *T. gondii* parasitophorous vacuole membrane and parasite clearance. While we did not detect classical hallmarks of autophagy, such as autophagosomes enveloping *T. gondii*, Atg5 was required for recruitment of IFN- γ -inducible p47 GTPase IIGP1 (Irga6) to the vacuole membrane, an event that mediates IFN- γ -mediated clearance of *T. gondii*. This work shows that Atg5 expression in phagocytic cells is essential for cellular immunity to intracellular pathogens in vivo, and that an autophagy protein can participate in immunity and intracellular killing of pathogens via autophagosome-independent processes such as GTPase trafficking.

INTRODUCTION

Classical cellular immunity to intracellular bacteria and parasites, first described by Mackaness more than 40 years ago (Mackaness, 1964), requires the activation of monocytes/macrophages by IFN- γ . The lysosomal system is critical for this type of cellular immunity via its role in killing pathogens and digesting pathogen corpses. The Gram-positive bacteria *Listeria monocytogenes* (*L. monocytogenes*), mycobacteria such as *Bacillus Calmette-Guerin* (BCG), and apicomplexan protozoa such as *Toxoplasma gondii* (*T. gondii*) are well-studied intracellular pathogens that survive in nonactivated macrophages utilizing different strate-

gies. *L. monocytogenes* escapes the phagolysosomal system into the cytoplasm (Edelson and Unanue, 2000). *T. gondii* survives in a specialized parasitophorous vacuole that resists fusion with lysosomes (Mordue and Sibley, 1997; Sibley, 2003). Mycobacteria inhibit phagosome acidification (Gutierrez et al., 2004; Sturgill-Koszycki et al., 1994). Activation of macrophages by IFN- γ or IFN- γ plus bacterial lipopolysaccharide (LPS) overcomes these pathogen survival mechanisms, resulting in blockade of pathogen replication, killing, and clearance of the pathogen from the cell. IFN- γ is essential for resistance of mice to infection with *L. monocytogenes* (Buchmeier and Schreiber, 1985), *T. gondii* (Suzuki et al., 1988), and mycobacteria (Flynn and Chan, 2001; Dalton et al., 1993; Cooper et al., 1993; Flynn et al., 1993). Resistance to acute *T. gondii* infection relies primarily on monocytes/macrophages (Robben et al., 2005) following activation by IFN- γ (Suzuki et al., 1988). However, the effector mechanisms responsible for IFN- γ -induced killing and clearance of intracellular pathogens from activated macrophages are not completely defined. Importantly, a series of IFN- γ -inducible p47 GTPases have been implicated in the control of a range of bacterial and parasitic infections, including *T. gondii* (Taylor et al., 2007, 2000; Ling et al., 2006; Butcher et al., 2005; Halonen et al., 2001).

Autophagy involves the concerted action of cytoplasmic proteins that generate curved isolation membranes to envelop cytoplasm and cytoplasmic organelles. In the canonical pathway, the resulting 0.5–1.5 μm double-membrane-bound vesicles fuse with lysosomes to deliver their cytoplasmic cargo for degradation and recycling (Levine and Kroemer, 2008). Autophagy requires the action of two Atg5-dependent, ubiquitin-like conjugation systems. One conjugation system generates Atg5-Atg12 conjugates, which complex with Atg16 to associate with the elongating isolation membrane (Mizushima et al., 2002). The second conjugation system modifies the free C-terminal glycine of Atg8/LC3 (LC3-I) with phosphatidylethanolamine, generating the lipidated LC3-II form of Atg8/LC3, which becomes associated with autophagosomes. Atg5 is essential for conversion of LC3-I to LC3-II and for localization of LC3-II to autophagosomes (Mizushima et al., 2002). LC3 can also be found associated with

other cellular structures, including aggregates of ubiquitinated proteins and newly forming phagosomes (Sanjuan et al., 2007; Kuma et al., 2007), raising the possibility that autophagy proteins may participate in cellular processes in addition to classical autophagy. For example, Atg5 may have autophagy-independent functions (Codogno and Meijer, 2006).

Many studies have demonstrated colocalization of LC3 to structures either induced by or containing bacteria or parasites such as *T. gondii* (Andrade et al., 2006; Martens et al., 2005; Chercroun et al., 2006; Amer and Swanson, 2005; Birmingham et al., 2006; Ogawa et al., 2005; Gutierrez et al., 2004; Nakagawa et al., 2004; Py et al., 2007; Gutierrez et al., 2005; Romano et al., 2007; Schnaith et al., 2007), and this has suggested a role for autophagy in these processes. Given that LC3 can colocalize with various types of structures inside the cell, the physiologic meaning of colocalization per se is not clear (Sanjuan et al., 2007; Kuma et al., 2007; Klionsky et al., 2008). For example, Atg5 and autophagy play no role in coronavirus replication in primary macrophages, despite the colocalization of LC3 with viral replication compartments in cell lines (Zhao et al., 2007b). However, autophagy may be an important pathogen control mechanism (Levine and Kroemer, 2008; Levine and Deretic, 2007) since, in cultured cells, the presence of Atg5 delays the growth of *L. monocytogenes* by about 2 hr (Py et al., 2007), decreases the number of viable *Streptococcus pyogenes* by about 3-fold at 4 hr after infection (Nakagawa et al., 2004), and decreases intracellular *Salmonella typhimurium* about 2-fold at 8 hr after infection (Birmingham et al., 2006). Furthermore, the induction of autophagy by starvation or treatment with rapamycin decreased mycobacterial viability 40%–70% 3 hr after infection (Gutierrez et al., 2004). One study in transformed fibroblasts suggests a role for Atg5 in IFN- γ -mediated control of *T. gondii* (Konen-Waisman and Howard, 2007). Indeed, studies in *Drosophila* clearly indicate that autophagy has a role in the control of *L. monocytogenes* infection in primary hemocytes (Yano et al., 2008). Such studies have not yet been performed in mammals.

In contrast to the potential protective role for autophagy against bacterial and parasitic infection, other studies suggest that both bacteria and parasites may subvert the autophagic process or autophagy proteins for their own benefit, resulting in enhanced replication in cultured cells (Schnaith et al., 2007; Swanson and Isberg, 1995; Romano et al., 2007). In addition, it is clear that some pathogens, such as HSV-1, have evolved elegant mechanisms for inhibiting both signaling processes that induce autophagy and autophagy effector mechanisms (Orvedahl et al., 2007). Such mechanisms may contribute to the apparent lack of a role for autophagy in a specific situation. Thus, autophagy and autophagy proteins may play complex roles in vivo, indicating the importance of studies in intact animals and primary cells to determine the physiologic importance of autophagy and individual autophagy proteins in vivo. A role for an autophagy protein in a specific process in immunity may reflect a role for classical autophagosomes in control of infection (Yano et al., 2008). However, it is also possible that these proteins play cellular roles in addition to their role in the generation of classical autophagosomes.

T. gondii provides a unique opportunity to define mechanisms of cellular immunity, since elimination of parasites in activated

macrophages is well studied (Ling et al., 2006; Taylor et al., 2000; Zhao et al., 2007a; Mordue and Sibley, 1997; Sibley, 2003; Sibley et al., 1991). Two mechanisms of macrophage activation result in killing and clearance of *T. gondii* in cultured cells: one dependent on IFN- γ /LPS, and the other on ligation of CD40. These two pathways are completely independent (Zhao et al., 2007a; Subauste and Wessendarp, 2006; Andrade et al., 2005, 2006). Mice lacking CD40 signaling fail to control chronic *T. gondii* infection, dying more than 50 days after infection (Reichmann et al., 2000). Mice lacking IFN- γ succumb within 10 days of infection (Suzuki et al., 1988; Scharton-Kersten et al., 1996), indicating the greater importance of the IFN- γ -mediated pathway for control of acute infection. In macrophages or astrocytes activated by IFN- γ , *T. gondii* succumbs to damage to the parasitophorous vacuole membrane, followed by stripping of the vacuolar membrane, killing of the parasite, and clearance of parasite corpses (Ling et al., 2006; Martens et al., 2005). For CD40-dependent killing, a role for autophagy is supported by the demonstration that inhibition of expression of the essential autophagy protein Atg6/beclin1 inhibits killing (Andrade et al., 2006). The role of autophagy proteins in IFN- γ -dependent killing and clearance of *T. gondii* is less clear; experiments to date indicate that IFN- γ -mediated killing is dependent on p47 GTPases (Ling et al., 2006; Martens et al., 2005; Taylor et al., 2007) and independent of beclin 1/Atg6 (Andrade et al., 2006). Importantly, in astrocytes, the GTPase IIGP1 localizes to the membrane of the parasitophorous vacuole very early after infection of IFN- γ -activated cells, overexpression of IIGP1 increases damage to the parasitophorous vacuole membrane, and expression of a dominant negative form of IIGP1 inhibits IFN- γ -mediated killing of *T. gondii* (Martens et al., 2005). Thus, IIGP1 is an important component of the cellular machinery that results in control of *T. gondii* infection.

Together, these emerging data on the role of autophagy in control of bacterial and parasitic infection in cultured cells and on the activation of autophagy by IFN- γ (Gutierrez et al., 2004; Singh et al., 2006) beg fundamentally important questions: How important are autophagy proteins for cellular immunity during infection of a living mammalian host? If autophagy proteins are important, do they act downstream of IFN- γ to control infection with intracellular pathogens in primary cells, and if so, how? Is the role of autophagy proteins in the control of intracellular pathogens reflective of a role for autophagosomal envelopment of pathogens, or is some other function of autophagy proteins involved? In this paper we address these questions using infection of mice and studies of infection in primary macrophages.

RESULTS AND DISCUSSION

Role of Atg5 in Autophagy in Primary Macrophages

Mice lacking Atg5 entirely die immediately after birth due to developmental defects (Kuma et al., 2004). We therefore deleted the *ATG5* gene from monocytes/macrophages and granulocytes (Kuma et al., 2004; Hara et al., 2006; Zhao et al., 2007b) by breeding *ATG5^{fllox/fllox}* mice (hereafter referred to as control mice) (Hara et al., 2006) with mice expressing the Cre recombinase from the endogenous lysozyme M locus to generate *ATG5^{fllox/fllox}-Lyz-Cre* mice (Clausen et al., 1999; Steed et al., 2007). Deletion of the *ATG5* gene in these cells resulted in

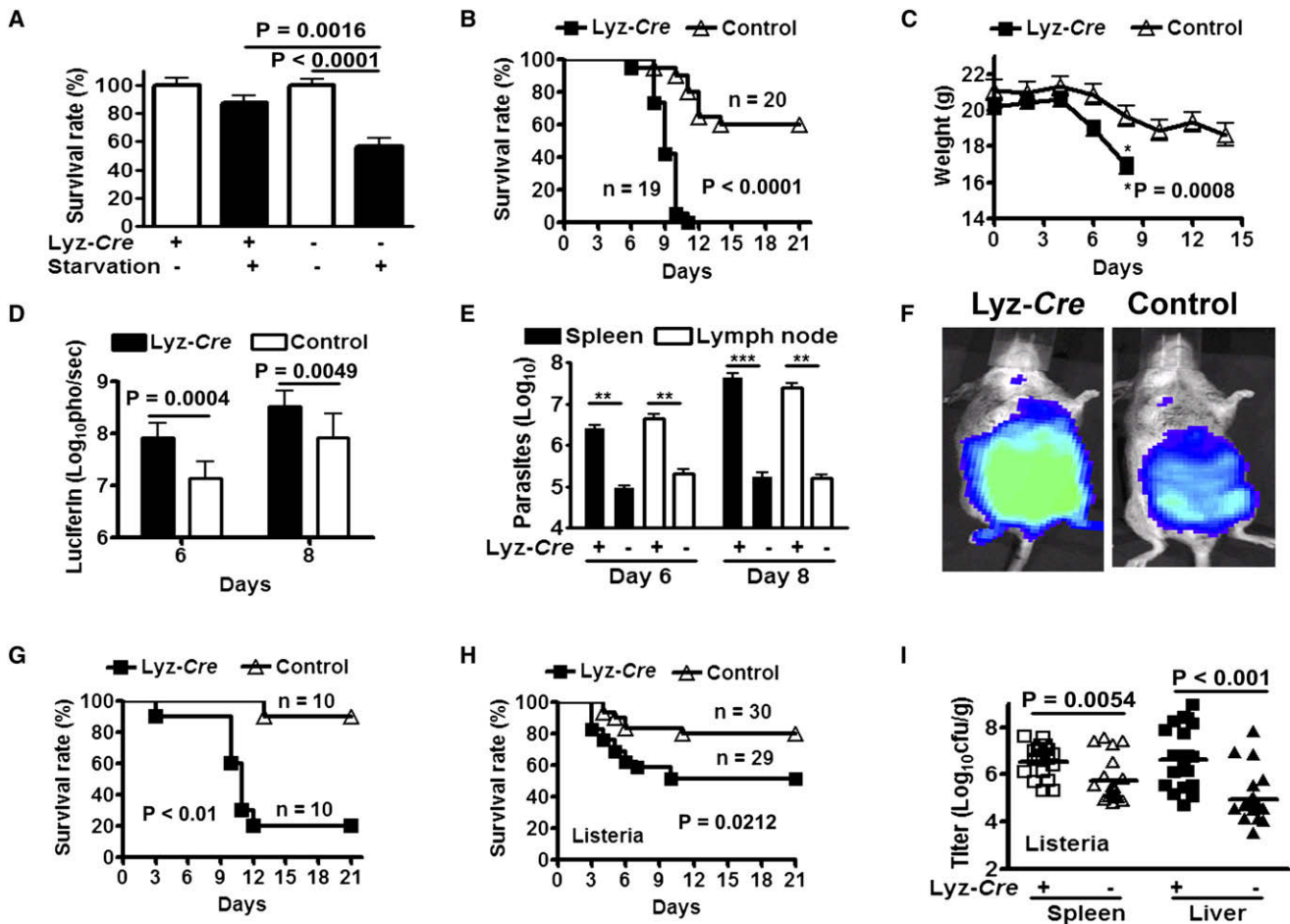


Figure 1. Atg5 Is Required for Cellular Immunity to *Toxoplasma gondii* and *L. monocytogenes* In Vivo

(A) Survival of BCG in Atg5-deficient and control macrophages after starvation. Results are pooled from three independent experiments. Data are presented as mean \pm SEM.
 (B) Survival of female mice after i.p. infection with 100 *T. gondii* parasites expressing luciferase. These data were pooled from four independent experiments.
 (C) Weight of mice in (B) over the course of *T. gondii* infection. Data are presented as mean \pm SEM.
 (D) Light emission from mice in (B) after injection of luciferin. Data are presented as mean \pm SEM.
 (E) Quantification of parasites in the indicated tissues using the methods and standard curve in Figure S3. These data were pooled from two independent experiments and presented as mean \pm SEM; ** $P < 0.001$, *** $P < 0.0001$.
 (F) Representative images of mice 8 days after infection with *T. gondii*. The full data set of which these are representatives is provided in Figure S2.
 (G) Survival of male mice after i.p. infection with 200 *T. gondii* parasites. This dose is higher than that used in female mice in (B).
 (H) Survival of mice after inoculation with 2×10^5 CFUs of *L. monocytogenes*.
 (I) *L. monocytogenes* colony forming units in spleen or liver 3 days after infection. These data were pooled from at least three independent experiments (20 control mice and 19 *ATG5^{flox/flox}-Lyz-Cre* mice).

a deficit in autophagy. Peritoneal macrophages and bone-marrow-derived macrophages from these mice lack Atg5 and fail to efficiently convert LC3-I to LC3-II (Zhao et al., 2007b) (Figure S1A). To confirm that the autophagy deficiency in primary macrophages from *ATG5^{flox/flox}-Lyz-Cre* mice is sufficient to alter control of infection with an intracellular pathogen in vitro, we took advantage of the observation that starvation-induced autophagy limits survival of BCG in a macrophage cell line (Gutierrez et al., 2004). Atg5-deficient macrophages were less effective than control macrophages in killing BCG (Figure 1A), confirming a functional deficiency in autophagy protein-dependent control of an intracellular pathogen in these cells (Gutierrez et al., 2004).

Role of Atg5 In Vivo for Cellular Immunity to Intracellular Bacteria and Parasites

To determine the physiologic importance of Atg5 in vivo, we challenged *ATG5^{flox/flox}-Lyz-Cre* and control mice with *T. gondii*-expressing firefly luciferase and followed infection over time (Figures 1B–1F). *ATG5^{flox/flox}-Lyz-Cre* female mice were more susceptible to *T. gondii* infection (Figure 1B, $p < 0.0001$) and exhibited greater weight loss (Figure 1C, $p = 0.0008$) than control mice. As measured by light detected in whole animals after luciferin injection (Saeij et al., 2005), *ATG5^{flox/flox}-Lyz-Cre* mice were unable to control *T. gondii* replication normally (Figure 1D, $p = 0.0004$, $p = 0.0049$). Quantification of *T. gondii* parasites in spleen and mesenteric lymph nodes revealed increased parasite

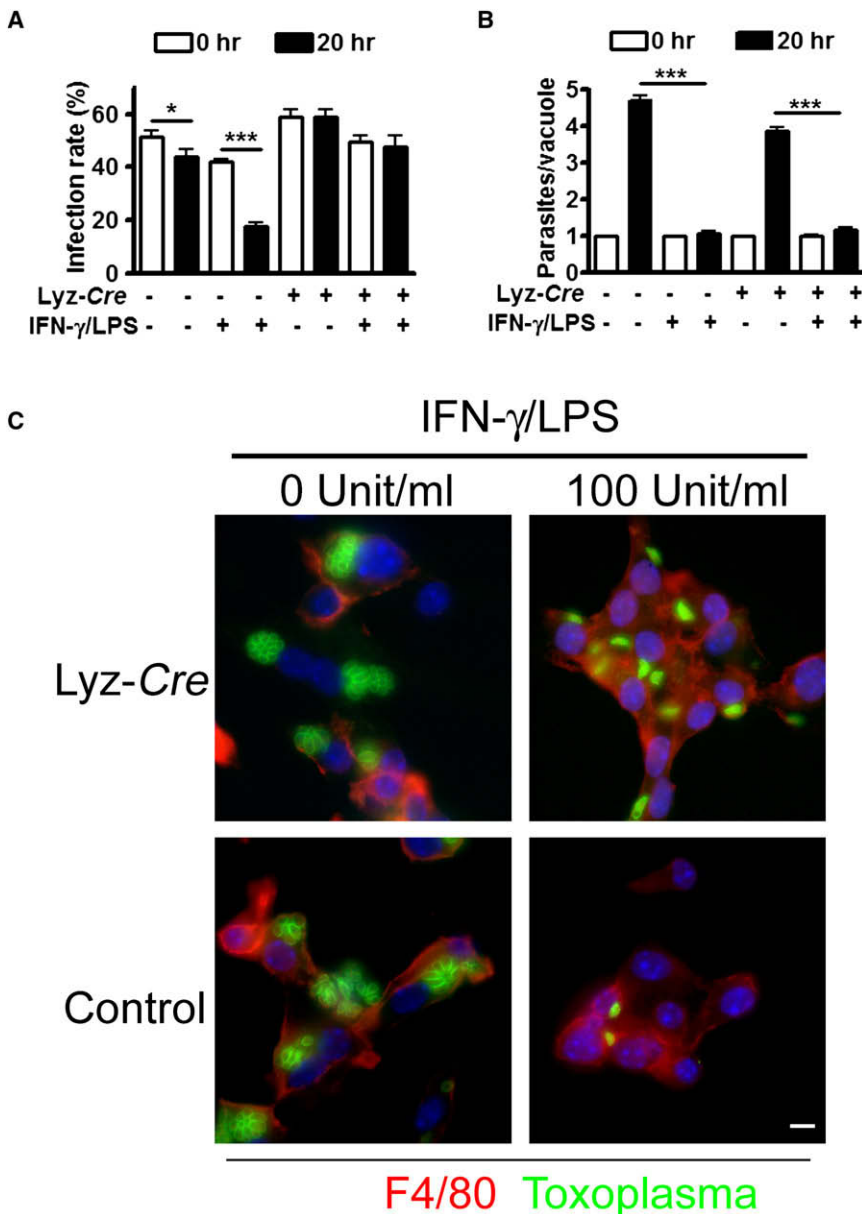


Figure 2. Atg5 Is Required for IFN- γ -Induced Clearance of *Toxoplasma gondii* from Macrophages

(A) The proportion of macrophages containing at least one *T. gondii* parasite; * $p < 0.05$, *** $p < 0.0001$.

(B) The number of *T. gondii* parasites per vacuole in infected cells; *** $p < 0.0001$. For (A) and (B), data were pooled from at least three independent experiments in which at least 210 cells were counted per condition and presented as mean \pm SEM.

(C) Representative immunofluorescence images of *T. gondii*-infected macrophages 20 hr after infection, stained with anti-mouse F4/80 (red), anti-*T. gondii* (green), and DAPI (blue). Scale bar = 10 μ m.

were more susceptible to lethal *L. monocytogenes* infection than control mice (Figure 1H, $p = 0.02$), and *L. monocytogenes* replicated to higher levels in both spleen and liver of *ATG5^{flox/flox}-Lyz-Cre* than in control mice (Figure 1I; $p = 0.0054$, $p < 0.001$). These data show that Atg5 is essential for effective cellular immunity to intracellular pathogens in vivo.

Atg5 Is Essential for IFN- γ /LPS-Induced Clearance of *T. gondii* from Primary Macrophages

We next defined the cellular mechanisms responsible for the essential role of Atg5 in control of intracellular pathogen infection. The importance of IFN- γ and macrophages for resistance to *T. gondii* infection in vivo suggested that Atg5 is important for IFN- γ -dependent control of *T. gondii* infection in activated primary macrophages. Activation of macrophages by treatment with IFN- γ , plus a second signal such as LPS, optimally restricts *T. gondii* growth and results in

clearance of *T. gondii* from infected cells (Sibley et al., 1991). We therefore determined the role of Atg5 in IFN- γ /LPS-induced inhibition of *T. gondii* growth in, and clearance from, primary macrophages by measuring the proportion of macrophages infected (Ling et al., 2006) as a measure of clearance, and the number of parasites per parasitophorous vacuole as a measure of replication (Murray et al., 1985a, 1985b; Ling et al., 2006; Mordue and Sibley, 2003).

T. gondii efficiently infected and replicated in nonactivated control and Atg5-deficient macrophages (Figure 2). IFN- γ /LPS treatment significantly decreased the proportion of *T. gondii*-infected control macrophages (Figures 2A and 2C, $p < 0.0001$) 20 hr after infection, reflecting the capacity of these cells to clear infection. In contrast, Atg5-deficient macrophages treated with IFN- γ /LPS failed to clear *T. gondii* infection (Figures 2A and 2C, $p > 0.72$). The observation that Atg5 is essential for clearance

numbers in *ATG5^{flox/flox}-Lyz-Cre* mice (Figures 1E, 1F, S2, and S3). Experiments in male mice using the relevant dose of *T. gondii* confirmed the critically important role of Atg5 expression to resistance to *T. gondii* (Figure 1G, $p < 0.01$). The majority of both Atg5-deficient and control mice infected with *T. gondii* expressed detectable IFN- γ in serum, indicating that Atg5 expression in macrophages and granulocytes is not required for induction of IFN- γ in vivo (data not shown). Therefore, Atg5 is essential for resistance to *T. gondii* in vivo. The fact that mice succumb rapidly suggests a failure in the innate immune response, which is highly dependent on IFN- γ activation of macrophages, but not granulocytes (Robben et al., 2005).

To determine whether the role of Atg5 in resistance to *T. gondii* represents a more general role of Atg5 in resistance to intracellular pathogens in vivo, we challenged mice with a second intracellular pathogen, *L. monocytogenes*. *ATG5^{flox/flox}-Lyz-Cre* mice

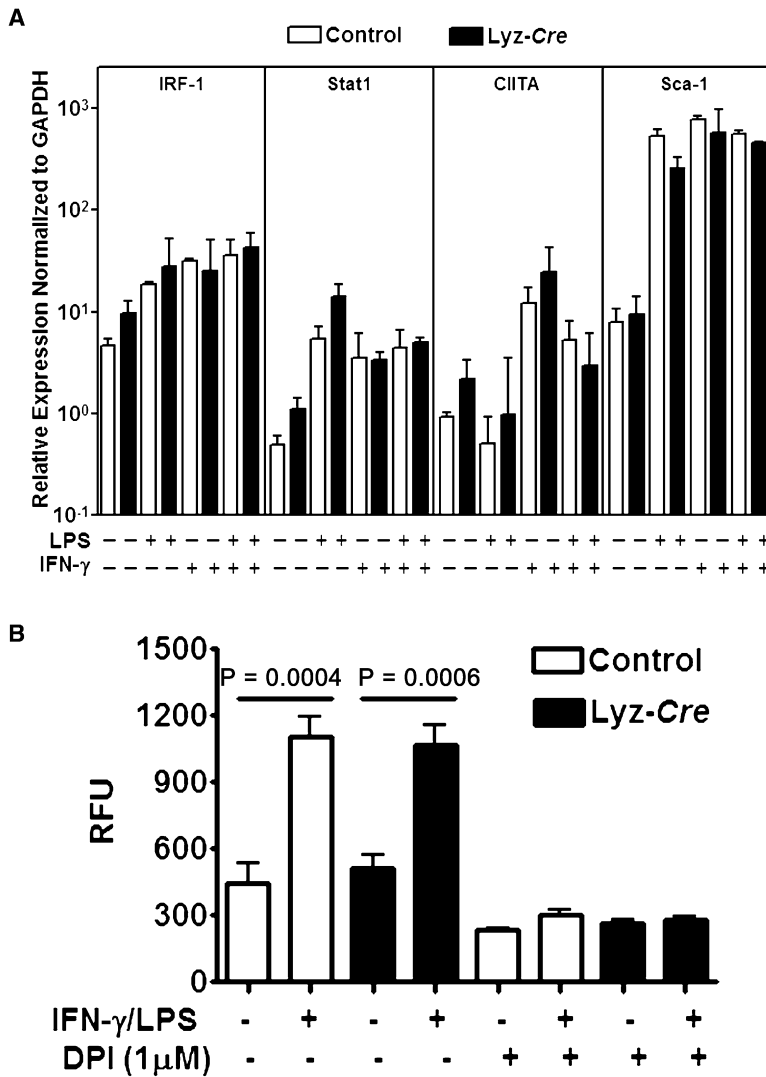


Figure 3. Atg5 Is Not Globally Required for IFN- γ -Induction of Transcription or NO Production

(A) Shown is the induction of expression of the indicated genes as measured by qRT-PCR at 16 hr after treatment of control, or Atg5-deficient macrophages with LPS, IFN- γ , or the combination of IFN- γ /LPS. Data were pooled from two independent experiments and presented as mean \pm SEM. There were no statistically significant differences between control and Atg5-deficient macrophages.

(B) Shown are the levels of NO produced by macrophages stimulated by IFN- γ /LPS for 18 hr. DPI is an inhibitor of NO production. Data were pooled from two independent experiments and presented as mean \pm SEM.

macrophage activation. To evaluate the possible role of Atg5 in IFN- γ -induced transcription and macrophage activation, we quantified several IFN- γ -induced transcripts using qRT-PCR (Figure 3A). IRF-1, Stat1, CIITA, and Sca-1 were all induced comparably in Atg5-deficient (as compared to control) macrophages after stimulation with IFN- γ , LPS, or IFN- γ /LPS. Inhibition of *T. gondii* replication has been assigned to reactive nitric oxide (NO) generated by iNOS (Adams et al., 1990). The induction of NO by IFN- γ /LPS was comparable between control and Atg5-deficient macrophages (Figure 3B). Thus, Atg5 is not required for clearance of *T. gondii* due to a role in IFN- γ -dependent transcription or induction of NO. We therefore evaluated the role of Atg5 in proximal events in the killing of *T. gondii* by IFN- γ /LPS-activated macrophages.

Atg5 Is Required for Disruption of the Parasitophorous Vacuole Membrane in IFN- γ Activated Macrophages

Killing of *T. gondii* by activated macrophages is associated with blebbing and ultimately stripping of

of *T. gondii* from activated primary macrophages provides a likely explanation for the rapid death of *T. gondii*-infected mice lacking Atg5 expression in macrophages (Figure 1), which are essential for resistance to acute *T. gondii* infection (Robben et al., 2005).

Atg5 Is Not Required for IFN- γ or IFN- γ /LPS Signaling, Inhibition of *T. gondii* Replication within Vacuoles, or Induction of Nitric Oxide in Primary Macrophages

We next defined the role of Atg5 in control of *T. gondii* replication within the parasitophorous vacuole. Untreated control cells had ca. four parasites per vacuole, reflecting replication within the vacuole. In contrast, IFN- γ /LPS-treated control cells that had not cleared infection had ca. one parasite per vacuole (Figures 2B and 2C, $p < 0.001$). Activation of Atg5-deficient macrophages with IFN- γ /LPS also reduced the number of *T. gondii* parasites per vacuole from ca. three to four to ca. one ($p < 0.0001$), indicating that inhibition of replication of *T. gondii* within the parasitophorous vacuole did not require Atg5 (Figures 2B and 2C, $p < 0.0001$). This shows that IFN- γ /LPS efficiently generates *Toxoplasma*-static responses in the absence of Atg5, indicating that Atg5 deficiency does not globally inhibit IFN- γ /LPS-induced

the parasitophorous vacuole membrane early after cell entry, followed by parasite destruction (Ling et al., 2006). We therefore defined the role of Atg5 in IFN- γ /LPS-induced damage to the membrane of the parasitophorous vacuole in activated macrophages 5 hr after infection. Electron microscopy (EM) revealed marked differences in the vacuole occupied by *T. gondii* in IFN- γ -treated control macrophages versus Atg5-deficient macrophages (Figure 4). The majority of parasites were found within intact vacuoles in untreated cells (ten out of ten control and eight out of eight Atg5-deficient cells). In control cells treated with IFN- γ /LPS, the majority of parasites were found in partially or fully disrupted vacuoles (ten out of twelve control cells). In contrast, the majority of parasites in IFN- γ /LPS-treated Atg5-deficient cells remained in intact vacuoles (seven out of eight cells). Parasites within Atg5-deficient IFN- γ /LPS-activated macrophages were found within typical parasitophorous vacuoles bound by a single membrane and often surrounded with host ER. The vacuole membrane remained intact, and the intracellular membranes of the parasite showed no signs of damage (Figures 4A and 4B). There was no consistent difference in the amount of open space within parasitophorous vacuoles between control

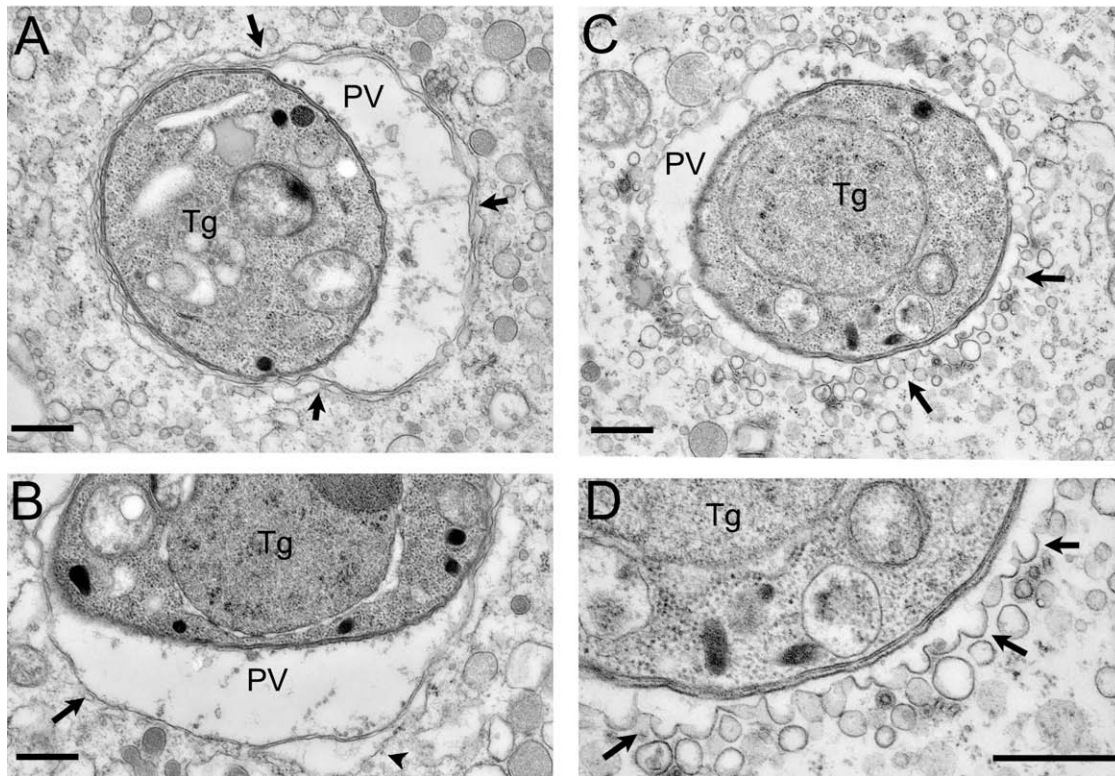
Lyz-Cre (IFN- γ /LPS)Control (IFN- γ /LPS)

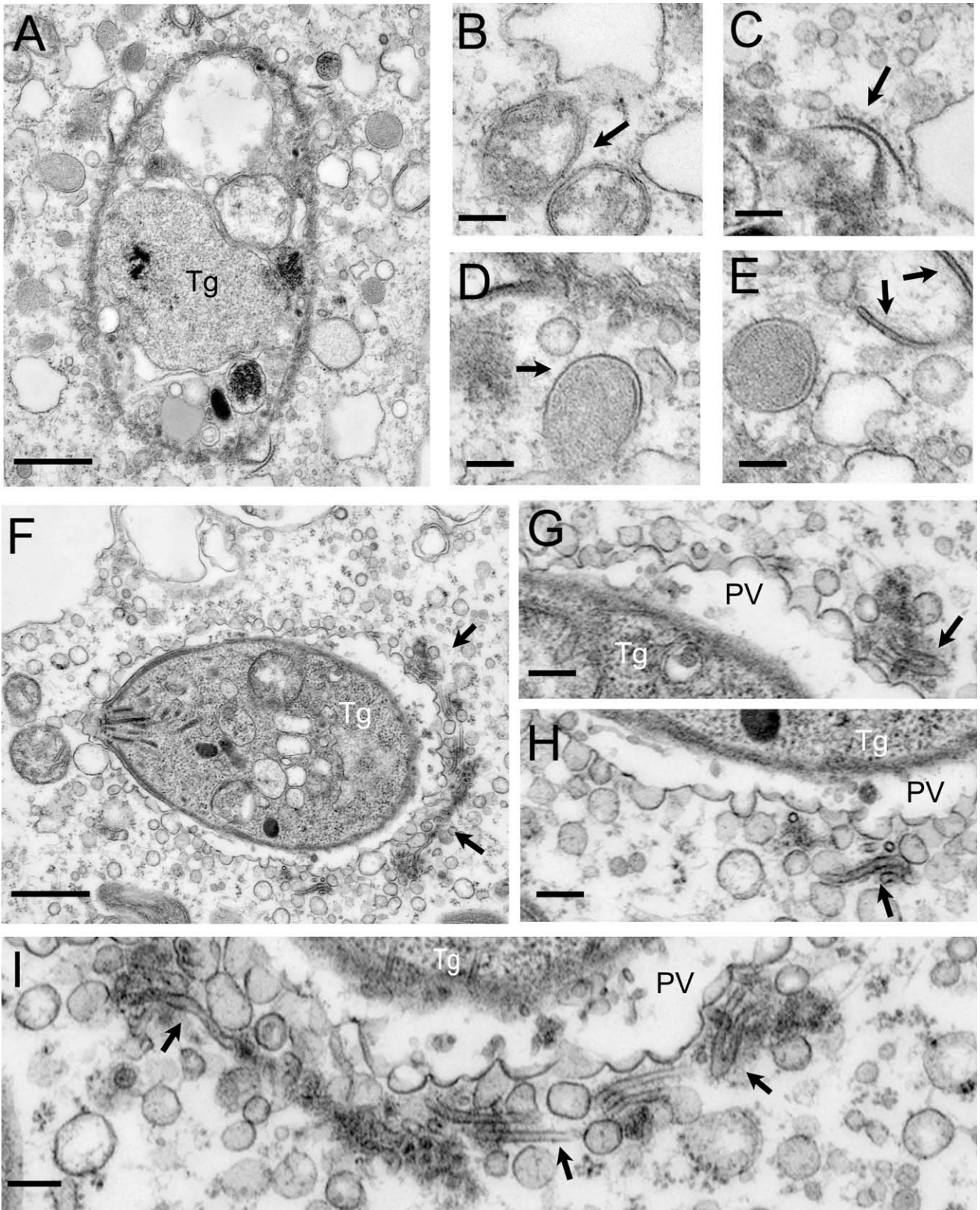
Figure 4. Electron Microscopic Analysis of the Clearance of *T. gondii* by IFN- γ -Activated Macrophages

(A) Macrophages treated as indicated were infected for 5 hr and then analyzed by EM. All images shown are from IFN- γ /LPS-activated macrophages. In (A), parasites (Tg) within Atg5-deficient cells reside within conventional parasitophorous vacuoles (PV) bounded by host ER (arrow heads). (B) The parasitophorous vacuole membrane is a single unit membrane (arrow), surrounded in places by host ER (arrow head). (C) Parasites within activated control macrophages are found within vacuoles that are undergoing membrane blebbing and vesiculation (arrows). (D) Enlarged view from (C) shows membrane blebs protruding from the parasitophorous vacuole membrane (arrows). Scale bars = 0.5 μ m.

and Atg5-deficient cells. Inspection revealed that regions of the parasitophorous vacuole membrane that appeared to have more than one bilayer (Figure 4A) represented a single parasitophorous vacuole membrane apposed to endoplasmic reticulum. This tight apposition of the endoplasmic reticulum to the parasitophorous vacuole membrane has been well described (Sibley, 2003; Jones et al., 1972; Jones and Hirsch, 1972).

In contrast, the parasitophorous vacuole membrane surrounding parasites within IFN- γ /LPS-activated control macrophages showed extensive vesiculation and blebbing, with clusters of small vesicles in the vicinity of the vacuole (Figures 4C and 4D). Membrane vesiculation and damage to the parasitophorous vacuole was not observed in the absence of IFN- γ /LPS treatment (data not shown). This is similar to previous reports of activated macrophages or astrocytes infected with *T. gondii* (Ling et al., 2006; Martens et al., 2005). Also similar to these previous reports, parasites in IFN- γ /LPS-activated control macrophages were often found free in the cytosol and showed extensive membrane damage (Figure 5A). Frequently, double-membrane-bound compartments and even membrane crescents were observed in the vicinity of such damaged parasites (Figures

5B–5E). Occasionally, vacuoles that showed extensive vesiculation were adjacent to distinctive flattened membrane stacks (Figures 5F–5I). These flattened membrane structures were not observed associated with the normal-appearing parasitophorous vacuoles in Atg5-deficient, IFN- γ /LPS-activated macrophages, suggesting a role for Atg5 in the generation of these structures. Notably, we did not observe envelopment within double membranes of either the parasitophorous vacuole or partially degraded parasites in the cytosol, suggesting that Atg5-dependent damage to the parasitophorous vacuole did not involve envelopment within autophagosomes. This is consistent with results obtained in activated primary astrocytes (Martens et al., 2005). Collectively, these results show that Atg5 is required for IFN- γ /LPS-induced damage to the parasitophorous vacuole membrane and stripping the membrane away from the parasite. While these observations do not rule out a role for classical autophagy in clearance of debris from damaged parasites, such events would appear to be downstream of a critical Atg5-dependent step in damaging the parasitophorous vacuole. Therefore, these data are consistent with a critical role for Atg5 in a process that does not represent classical autophagy.



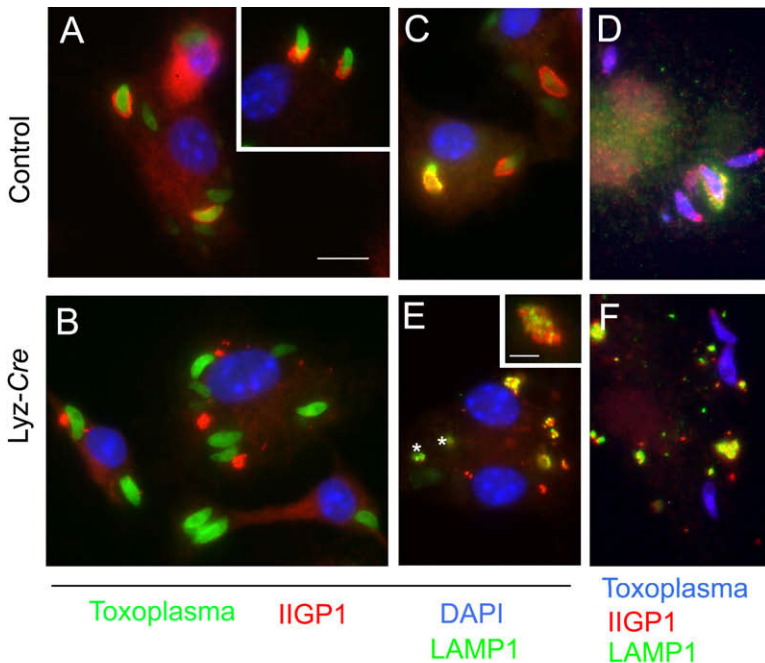


Figure 6. Atg5 Is Required for IFN- γ /LPS-Induced Targeting of IIGP1 to the *T. gondii* Parasitophorous Vacuole

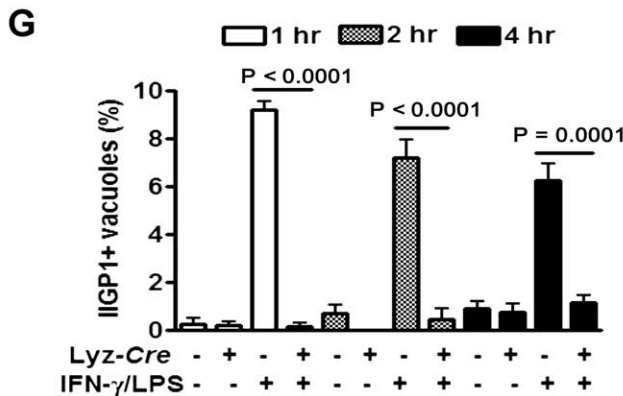
(A and B) Macrophages were stimulated with IFN- γ /LPS and infected with *T. gondii* as in Figure 2, and then stained for the indicated markers 2 hr after infection. Scale bar = 10 μ m for (A)–(F). In (A) and (B), localization of IIGP1 (red) with GFP-expressing *T. gondii* (green) after infection of IFN- γ /LPS-activated control or Atg5-deficient macrophages is shown. DAPI (blue) staining of nuclei. In control cells, IIGP1 decorates the parasitophorous vacuole in a cap, while in Atg5-deficient cells, IIGP1 (red) was shunted to large intracellular inclusions and was not recruited to the parasite-containing vacuole.

(C) Intracellular fate of GFP-expressing parasites in IFN- γ /LPS-activated control cells. LAMP1 (green) was recruited selectively to IIGP1 (red) positive vacuoles and often formed a partial cap, associated with material sloughing from the vacuole surface. Nuclei were visualized with DAPI (blue) staining. (D) Similar to (C), except wild-type parasites were detected with rabbit anti-*Toxoplasma* and secondary antibodies conjugated to Alexa355 (blue).

(E) Parasites within Atg5-deficient cells did not recruit IIGP1 or become LAMP1-positive. Asterisks indicate GFP-expressing parasites. However, IIGP1-positive inclusions (red in Figure 6B) were strongly associated with LAMP1. Insert shows enlarged view of intracellular inclusion. IIGP1 (red) and LAMP1 (green) were closely associated, but not strictly colocalized (scale bar = 1 μ m).

(F) Similar to (E), except wild-type parasites were detected with rabbit anti-*Toxoplasma* and secondary antibodies conjugated to Alexa355 (blue).

(G) Quantitation of IIGP1 colocalization with *T. gondii* parasites in IFN- γ /LPS-activated macrophages 1, 2, and 5 hr postinfection. Data were collected from two independent experiments counting at least 600 vacuoles and presented as mean \pm SEM.



Role of Atg5 in Recruitment of IIGP1 to the Parasitophorous Vacuole

The failure of IFN- γ /LPS-activated, but Atg5-deficient, macrophages to damage and strip the parasitophorous vacuole membrane (Figures 4 and 5) suggested that Atg5 might be required for recruitment of IFN- γ -inducible p47 GTPases, several of which are required for efficient IFN- γ -mediated clearance of *T. gondii* in vitro and/or in vivo (Taylor et al., 2007). We therefore determined whether IIGP1 (Irge6) is properly recruited to the parasitophorous vacuole membrane in IFN- γ /LPS-activated, Atg5-deficient cells. We focused on early times after infection,

concurrent with or preceding damage to the parasitophorous vacuole membrane (Figure 6). IIGP1 protein expression was induced by IFN- γ /LPS treatment normally in Atg5-deficient macrophages (Figure S1B). Within 1 hr, IIGP1 was recruited to the parasitophorous vacuole in control cells activated by treatment with IFN- γ /LPS (Figure 6A). We did not observe efficient recruitment of IIGP1 to the parasitophorous vacuole in similar experiments (data not shown). In contrast to control cells, recruitment of IIGP1 was abrogated in Atg5-deficient cells (Figures 6A–6B). It is interesting that, at each time point evaluated, less than 10% of vacuoles were IIGP1-positive in control cells. This is consistent with rapid transit of the parasitophorous vacuole through a process involving IIGP1 recruitment. Together, these data identify a mechanism, recruitment of a key GTPase, by which

Figure 5. Electron Microscopic Analysis of Damage to the Parasitophorous Vacuole in IFN- γ /LPS-Activated Control Macrophages

(A) Clearance of *T. gondii* infection by control macrophages treated with IFN- γ /LPS is associated with a process of vacuolar membrane damage including vesiculation and blebbing 5 hr after infection. Findings here were specific for control macrophages and were not observed in Atg5-deficient macrophages. (A) shows a heavily damaged parasite within the cytosol following dissolution of the parasitophorous vacuole membrane. The parasite plasma membrane shows evidence of damage. Scale bar = 0.5 μ m.

(B–E) Examples of double-membrane-bound compartments forming in the vicinity of the degraded parasite. Scale bars = 0.1 μ m.

(F) Parasite residing within a parasitophorous vacuole that is undergoing extensive membrane blebbing and vesiculation. A prominent cluster of membrane vesicles and flattened cisternae are found at the posterior end (arrows). Scale bar = 0.5 μ m.

(G–I) Enlarged views of the membrane vesicles showing flattened cisternae (arrows). Scale bars = 0.1 μ m.

Atg5 plays an essential role in control of *T. gondii* in activated primary macrophages.

Role of Atg5 in Recruitment of Lysosomes

Previous studies have indicated that in-vivo-activated macrophages infected in vivo with GFP-expressing parasites contain parasitophorous vacuoles that undergo fusion with LAMP1-positive vesicles, resulting in a significant percentage of vacuoles that are uniformly LAMP1-positive (Ling et al., 2006). However, we did not observe parasite-containing vacuoles that were uniformly LAMP1-positive in either control cells or Atg5-deficient cells activated with IFN- γ /LPS (data not shown). This difference between our results and the results of Ling et al. (2006) may be the result of analysis of different types of macrophages, or the possibility that opsonization that occurs in vivo can direct the parasite to a fusogenic vacuole (Mordue and Sibley, 1997; Joiner et al., 1990).

Instead of direct fusion with the vacuole, prominent clusters of lysosomes were observed to colocalize with IIGP1-positive regions of vacuoles containing *T. gondii* in control, but not Atg5-deficient cells (Figures 6C–6F). LAMP1 signal was often associated with material that was sloughed from the parasite-containing vacuole. We speculate that, in our experiments, LAMP1-positive vesicles do not fuse with the vacuole, resulting in uniform staining, but rather fuse with the remnants of the membrane that is stripped off by IIGP1 and possibly captured nearby by autophagosomes, resulting in shunting of this material to lysosomes. We failed to observe efficient recruitment of LAMP1-positive vesicles to the region of parasite-containing vacuoles in Atg5-deficient cells, consistent with a failure of IIGP1 to be recruited (Figures 6D–6F) and the fact that parasite-containing vacuoles remain intact in the absence of Atg5 (Figure 4).

One possible explanation for the failure of IIGP1 to be recruited in Atg5-deficient cells is that it was sequestered in intracellular inclusions (Figures 6B and 6E). These intracellular inclusions consisted of clusters of small vesicles that were IIGP1-positive, and which occurred in close association with IIGP1-negative but LAMP1-positive vesicles. IIGP1 and LAMP1 did not precisely colocalize in the same vesicular structure (Figure 6E, insert). The nature of these inclusions is presently unknown, but their existence suggests a failure of IIGP1 to correctly traffic in the absence of Atg5, thus disrupting its cellular function in control of intracellular pathogens.

Coda: The Role of Atg5 in Cellular Immunity

Together, these studies establish Atg5 as a crucial in vivo mediator of cellular immunity to intracellular pathogens and provide mechanistic insight into the roles of Atg5 in the cell. The impressive increase in susceptibility of mice lacking Atg5 in phagocytic cells argues for serious consideration of drugs that activate relevant Atg5-dependent processes as anti-infectives. With regard to mechanism, it is important to note that the parasitophorous vacuole is not a phagosome, but rather a pathogen-driven derivative of the plasmalemma that is autonomous in formation and fate within the cell (Dobrowolski and Sibley, 1996; Joiner et al., 1990; Suss-Toby et al., 1996). However, our results are strikingly similar to recent findings for newly formed phagosomes containing latex beads coated with Toll-receptor ligand (Sanjuan et al., 2007). In both cases, Atg5 is important for target-

ing critically important proteins to recently formed membrane structures.

In our case, Atg5 is important for recruitment of a critical p47 GTPase to the parasitophorous vacuole. The molecular mechanism responsible for this role of Atg5 in recruitment of a GTPase is currently unknown. However, it is interesting that classical autophagosomes were not observed as involved in either fusion of lysosomes to phagosomes (Sanjuan et al., 2007) or in Atg5-dependent damage to the parasitophorous vacuole membrane in the present study. This indicates that Atg5 can play a previously undescribed role in intracellular membrane dynamics that is independent of classical autophagosome formation.

Our data show that the critical role of Atg5 in killing of *T. gondii* in cultured primary macrophages reflects, at least in part, a role for Atg5 in damage to the parasitophorous vacuole, a process that is in addition to the role of Atg5 in classical autophagy. It will therefore be important to determine, in future studies, whether the results we have obtained here for Atg5 in damage to the parasitophorous vacuole and GTPase recruitment also apply to other components of the ubiquitin-like conjugations systems involved in classical autophagy. Since IFN- γ -induced small GTPases are implicated in resistance to a variety of pathogens (Taylor et al., 2007), it is possible that the role of Atg5 in targeting of GTPases may be of general importance.

EXPERIMENTAL PROCEDURES

Cells, Pathogens, and Mice

Peritoneal exudate cells were obtained by lavage, plated at 5×10^5 cells/ml well (Edelson and Unanue, 2001) for 4 hr at 37°C, and washed vigorously to purify adherent macrophages prior to incubation with complete or starvation medium for 2 hr. Cell lysates were then analyzed by western blot for expression of Atg5, LC3-I/LC3-II, and actin (Edelson and Unanue, 2001; Zhao et al., 2007b). Primary macrophages were prepared from bone marrow of male mice as described (Zhao et al., 2007b). *T. gondii* (type-II strains): wild-type (PTG), expressing luciferase (PRU-LUC), or expressing GFP (GFP-PTG) (Kim et al., 2001) were maintained in HFF cells (Fux et al., 2007). *L. monocytogenes* strain EGD was prepared and quantified as described (Edelson and Unanue, 2001). Macrophages were infected with *M. tuberculosis* var. *bovis* (BCG, MOI = 1) for 1 hr and chased for 4 hr in either complete DMEM or starvation medium prior to enumeration of viable bacteria (Gutierrez et al., 2004). *ATG5^{fllox/fllox}* (control) mice and *ATG5^{fllox/fllox}-Lyz-Cre* mice were bred and genotyped as described (Zhao et al., 2007b; Hara et al., 2006). IFN- γ levels in serum were determined using a Becton Dickinson CBA Mouse Inflammation Kit. Mice 8–12 weeks of age were used for in vivo studies.

In Vitro and In Vivo Infections and Immunofluorescence Microscopy

Macrophages were pretreated with 100 U/ml recombinant murine IFN- γ (R&D Systems; Minneapolis, MN) plus 1 ng/ml LPS (*Salmonella*, Sigma; St. Louis, MO) for 18 hr, infected with *T. gondii* tachyzoites (PRU-LUC strain, MOI = 5), incubated at 37°C in 5% CO₂ for 3 hr, washed, and then either fixed immediately or incubated for 20 hr prior to fixation. *T. gondii* infection was assessed by indirect immunofluorescence using antibodies to F4/80 (macrophages), SAG1 (mouse monoclonal antibody DG52) for *T. gondii*, LAMP1 (rat monoclonal antibody 1D4B), or staining with DAPI (Fux et al., 2007; Nagamune et al., 2008; Ling et al., 2006). IIGP1 was detected using monoclonal antibody 5D9 at a 1:500 dilution (Zerrahn et al., 2002). Alexa Fluor350 conjugated goat anti-rabbit IgG (Invitrogen), Alexa Fluor488 conjugated goat anti-rat IgG, and Alexa Fluor594 conjugated goat anti-mouse IgG antibodies (Bioscience) were used at a 1:2000 dilution. *L. monocytogenes* (2×10^5 CFUs) or *T. gondii* strain PRU-LUC was inoculated into mice intraperitoneally. *L. monocytogenes* replication was quantified as CFUs in spleen and liver 3 days after infection (Barton et al., 2007). *T. gondii* replication was quantified by light emission after injection

of 0.15 mg/kg of firefly D-luciferin (Biosynth AG, Switzerland) by a Xenogen IVIS 100 (Saeij et al., 2005; Nagamune et al., 2008). To measure the *T. gondii* titers in spleen and mesenteric nodes, organs were harvested 6 or 8 days after infection, homogenized with 1 ml PBS, and strained (100 μ m cell strainer) prior to distribution into 96-well plates (4 wells/tissue sample). To detect luciferase expression, 2 μ l of firefly D-luciferin (30 mg/ml) was added per well and incubated for 10 min at room temperature, and light emission was measured (Xenogen IVIS 100). To prepare a standard curve (Figure S3), serial dilutions of PRU-LUC (10^6 – 10^2 parasites per well) were made in 96-well plates and luciferase assessed for spleen and lymph node samples.

Transmission Electron Microscopy

For ultrastructural analysis, *T. gondii*-infected macrophages were fixed in 1% glutaraldehyde (Polysciences, Inc.; Warrington, PA)/1% osmium tetroxide (Polysciences, Inc.) in 50 mM phosphate buffer, pH 7.2, for 1 hr at 4°C. This low osmolarity fixation was used to remove dense, soluble cytoplasmic components, allowing for unobscured membrane analysis. Cells were washed in phosphate buffer and rinsed extensively in dH₂O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella, Inc.; Redding, CA) for 1 hr. Following several rinses in dH₂O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella, Inc.). Sections of 70–80 nm were cut, stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA; Peabody, MA) at an accelerating voltage of 80 kV.

Western Blotting and Assay of NO Production

2×10^5 macrophages were treated with LPS, IFN- γ , or IFN- γ /LPS in a 96-well plate for 18 hr. Cells were washed with PBS twice prior to western analysis (Zhao et al., 2007b). NO production was detected using the NO synthase detection system (Sigma). 5×10^4 macrophages were treated with LPS, IFN- γ , or IFN- γ /LPS for 18 hr, washed, incubated with 200 μ l of reaction buffer or 200 μ l inhibition reaction buffer containing 1 μ M diphenyleneiodium chloride (DPI), and incubated at room temperature for 2 hr in the dark prior to detection of fluorescence.

Measurement of Gene Expression

Total RNA isolated from bone-marrow-derived macrophages using TRIzol reagent (Invitrogen; Carlsbad, CA). RNA (2 μ g) was treated with DNase I (Ambion; Austin, TX) before being subjected to reverse transcriptase cDNA synthesis using Oligo(dT)₁₂₋₁₈ and Superscript II (Invitrogen; Carlsbad, CA), as per the manufacturer's protocol. Quantitative RT-PCR was performed with SYBR-Green (Invitrogen; Carlsbad, CA) and the thermal cycler iCycler (Biorad; Hercules, CA). Primer sequences are as follows: *CIITA* 5'CACCCC CAGATGTGTATGTGC and 5'CGAGGTTTCCCAGTCCAGAAG, *GAPDH* 5'TG CCCCATGTTTGTGATG and 5'TGTGGTCATGAGCCCTTCC, *IRF-1* 5'ACAC TAAGAGCAAACCAAGAG and 5'TTCCATATCCAAGTCTGA, *Sca-1* 5'CTT GCCCATCAATTACCTGCC and 5'GGAGGGCAGATGGGTAAGCAA, and *Stat-1* 5'CTCTAGCTTTGAAACCCAGTT and 5'TTGTACCACAGGATAGACGC. Transcript levels were normalized to GAPDH within each sample, and data were calculated using the delta-delta Ct method (Livak and Schmittgen, 2001). Two independent experiments were performed with each qRT-PCR reaction repeated in triplicate.

Statistics

All data were analyzed with Prism software (Graphpad; San Diego, CA), using two-tailed unpaired Student's *t* tests. Unless otherwise indicated, all experiments were performed at least three times and the data pooled for presentation \pm SEM.

SUPPLEMENTAL DATA

Supplemental Data include three figures and can be found online at [http://www.cell.com/cellhostandmicrobe/supplemental/S1931-3128\(08\)00329-6](http://www.cell.com/cellhostandmicrobe/supplemental/S1931-3128(08)00329-6).

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