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Atg5 is Essential for Cellular Immunity *in vivo* and recruitment of a p47 GTPase to the *Toxoplasma gondii* Parasitophorous Vacuole in Macrophages

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SUMMARY

The physiologic importance of autophagy proteins for control of mammalian bacterial and parasitic infection *in vivo* is unknown. We show that expression of the essential autophagy protein Atg5 in granulocytes and macrophages is required for *in vivo* resistance to infection with *L. monocytogenes* and *T. gondii*. In primary macrophages, Atg5 was not required for IFNγ/LPS-mediated transcription, induction of nitric oxide, or inhibition of *T. gondii* replication. However, Atg5 was required for IFNγ/LPS-induced damage to the *T. gondii* parasitophorous vacuole membrane and parasite clearance. While we did not detect autophagosomes enveloping *T. gondii*, Atg5 was required for recruitment of the IFNγ-inducible p47 GTPase IIGP1 (Irga6) to the vacuole membrane. 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 monocyte/macrophages

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by IFNγ. 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 non-activated macrophages by escaping the phagolysosomal system [*L. monocytogenes*, (Edelson and Unanue, 2000)], survival in a specialized parasitophorous vacuole which resists fusion with lysosomes [*T. gondii* (Mordue and Sibley, 1997; Sibley, 2003)], or inhibition of phagosomes acidification [mycobacteria (Gutierrez et al., 2004; Sturgill-Koszycki et al., 1994)]. Activation of macrophages by IFNγ or IFNγ plus bacterial lipopolysaccharide (LPS) overcomes these pathogen survival mechanisms, resulting in blockade of pathogen replication, killing, and clearance of the pathogen from the cell. IFNγ 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 IFNγ (Suzuki et al., 1988). However, the effector mechanisms responsible for IFNγ-induced killing and clearance of intracellular pathogens from activated macrophages are not completely defined. Importantly, a series of IFNγ-inducible p47 GTPases have been implicated in the control of a range of bacterial and parasitic infections including *T. gondii* (Taylor et al., 2007; Taylor et al., 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 to 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. Atg5 may have autophagy-independent functions (Codogno and Meijer, 2006).

Many studies have demonstrated co-localization of LC3 to structures either induced by, or containing bacteria or parasites such as *T. gondii* (Andrade et al., 2006; Martens et al., 2005; Checroun 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 co-localize with various types of structures inside the cell, the physiologic meaning of co-localization *perse* 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 co-localization 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 two hours (Py et al., 2007), decreases the number of viable *Streptococcus pyogenes* by about three-fold at four hours after infection (Nakagawa et al., 2004), and decreases intracellular *Salmonella typhimurium* about two-fold at eight hours after infection (Birmingham et al., 2006).

Furthermore, the induction of autophagy by starvation or treatment with rapamycin decreased mycobacterial viability 40–70% three hours after infection (Gutierrez et al., 2004). One study in transformed fibroblasts suggests a role for Atg5 in IFNγ-mediated control of *T. gondii* (Konen-Waisman and Howard, 2007). Indeed, studies in *Drosophila* clearly indicate that autophagy has a role in 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 parasite 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 herpes simplex virus, 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 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 IFNγ/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; Andrade et al., 2006). Mice lacking CD40 signaling fail to control of chronic *T. gondii* infection, dying more than 50 days after infection (Reichmann et al., 2000). Mice lacking IFNγ succumb within 10 days of infection (Suzuki et al., 1988; Scharton-Kersten et al., 1996) indicate the greater importance of the IFNγ-mediated pathway for control of acute infection. In macrophages or astrocytes activated by the IFNγ, *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 IFNγ-dependent killing and clearance of *T. gondii* is less clear; experiments to date indicate that IFNγ-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 IFNγ-activated cells, overexpression of IIGP1 increases damage to the parasitophorous vacuole membrane, and expression of a dominant negative form of IIGP1 inhibits IFNγ-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 for the activation of autophagy by IFNγ (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 IFNγ 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 monocyte/macrophages and granulocytes (Kuma et al., 2004; Hara et al., 2006; Zhao et al., 2007b) by breeding $ATG5^{flox/flox}$ mice (Hara et al., 2006) to mice expressing the *Cre* recombinase from the endogenous lysozyme M locus to generate $ATG5^{flox/flox}$-Lyz-*Cre* mice (Lyz-Cre throughout this manuscript) (Clausen et al., 1999; Steed et al., 2007). Deletion of the *ATG5* gene in these cells resulted in 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 [ref. (Zhao et al., 2007b) and Supplemental Data Fig. 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 starvion induced autophagy limits survival BCG in a macrophage cell line (Gutierrez et al., 2004) (Fig. 1A). Atg5-deficient macrophages were less effective than control macrophages in killing BCG, 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 $ATG5^{flox/flox}$ mice (hereafter referred to as control mice) with *T. gondii* expressing firefly luciferase and followed infection over time (Fig. 1B to 1F). $ATG5^{flox/flox}$-Lyz-*Cre* female mice were more susceptible to *T. gondii* infection (Fig. 1B, $P<0.0001$) and exhibited greater weight loss (Fig. 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 (Fig. 1D, $P=0.0004$, $P=0.0049$). Quantification of *T. gondii* parasites in spleen and mesenteric lymph nodes revealed the increased parasite numbers in $ATG5^{flox/flox}$-Lyz-*Cre* mice (Fig. 1E, F, Supplemental Fig. S2, 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* (Fig. 1G, $P<0.01$). The majority of both Atg5-deficient and control mice infected with *T. gondii* expressed detectable IFNγ in serum, indicating that Atg5 expression in macrophages and granulocytes is not required for induction of IFNγ *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 IFNγ 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 were more susceptible to lethal *L. monocytogenes* infection than control mice (Fig. 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 (Fig. 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 IFNγ/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 IFNγ and macrophages for resistance to *T. gondii* infection *in vivo* suggested that Atg5 is important for IFNγ-dependent control of *T. gondii* infection in activated primary macrophages. Activation of macrophages by treatment with IFNγ 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 IFNγ/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; Murray et al., 1985b; Ling et al., 2006; Mordue and Sibley, 2003).

T. gondii efficiently infected and replicated in non-activated control and Atg5-deficient macrophages (Fig. 2). IFNγ/LPS treatment significantly decreased the proportion of *T. gondii* infected control macrophages (Fig. 2A and C; $P<0.0001$) twenty hours after infection, reflecting the capacity of these cells to clear infection. In contrast, Atg5-deficient macrophages treated with IFNγ/LPS failed to clear *T. gondii* infection (Fig. 2A and C, $P>0.72$). The observation that Atg5 is essential clearance 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 (Fig. 1), which are essential for resistance to acute *T. gondii* infection (Robben et al., 2005).

Atg5 is not required for IFNγ or IFNγ/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, IFNγ/LPS treated control cells that had not cleared infection had *ca*. one parasite per vacuole (Fig. 2B and C; $P<0.001$). Activation of Atg5-deficient macrophages with IFNγ/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 (Fig. 2B and C, $P<0.0001$). This shows that IFNγ/LPS efficiently generates *Toxoplasma*-static responses in the absence of Atg5, indicating Atg5 deficiency does not globally inhibit IFNγ/LPS-induced macrophages activation. To evaluate the possible role of Atg5 in IFNγ-induced transcription and macrophage activation, we quantified several IFNγ-induced transcripts using qRT-PCR (Fig. 3A). IRF-1, Stat1, CIITA, and Sca-1 were all induced comparably in Atg5-deficient as compared to control macrophages after stimulation with IFNγ, LPS, or IFNγ/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 IFNγ/LPS was comparable between control and Atg5-deficient macrophages (Fig. 3B). Thus, Atg5 is not required for clearance of *T. gondii* based on a role in IFNγ-dependent transcription or induction of NO. We therefore evaluated the role of Atg5 in proximal events in the killing of *T. gondii* by IFNγ/LPS activated macrophages.

Atg5 is required for disruption of the parasitophorous vacuole membrane in IFNγ activated macrophages

Killing of *T. gondii* by activated macrophages is associated with blebbing and ultimately stripping of the parasitophorous vacuole membrane early after cell entry followed by parasite destruction (Ling et al., 2006). We therefore defined the role of Atg5 in IFNγ/LPS induced damage to the membrane of the parasitophorous vacuole in activated macrophages five hours after infection. Electron microscopy (EM) revealed marked differences in the vacuole occupied by *T. gondii* in IFNγ-treated control macrophages versus Atg5-deficient macrophages (Fig. 4).

The majority of parasites were found within intact vacuoles in untreated cells (10/10 control and 8/8 Atg5-deficient cells). In control cells treated with IFNγ/LPS, the majority of parasites were found in partially or fully disrupted vacuoles (10/12 control cells). In contrast, the majority of parasites in IFNγ/LPS treated Atg5-deficient cells remained in intact vacuoles (7/8 cells). Parasites within Atg5-deficient IFNγ/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 (Fig. 4A, B). There was no consistent difference in the amount of open space within parasitophorous vacuoles between control and Atg5-deficient cells. Inspection revealed that regions of the parasitophorous vacuole membrane that appeared to have more than one bilayer (e.g. Fig. 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 IFNγ/LPS-activated control macrophages showed extensive vesiculation and blebbing with clusters of small vesicles in the vicinity of the vacuole (Fig. 4C, D). Membrane vesiculation and damage to the parasitophorous vacuole was not observed in the absence of IFNγ/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 IFNγ/LPS-activated control macrophages were often found free in the cytosol and showed extensive membrane damage (Fig. 5A). Frequently, double membrane bound compartments and even membrane crescents were observed in the vicinity of such damaged parasites (Fig. 5B–E). Occasionally, vacuoles that showed extensive vesiculation were adjacent to distinctive flattened membrane stacks (Fig. 5F–I). These flattened membrane structures were not observed associated with the normal-appearing parasitophorous vacuoles in Atg5-deficient IFNγ/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 IFNγ/LPS-induced damage to the parasitophorous vacuole 11 membrane and stripping of 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.

Role of Atg5 in recruitment of IIGP1 to the parasitophorous vacuole

The failure of IFNγ/LPS-activated, but Atg5-deficient macrophages, to damage and strip the parasitophorous vacuole membrane (Fig. 4, Fig. 5) suggested that Atg5 might be required for recruitment of IFNγ-inducible p47 GTPases, several of which are required for efficient IFNγ-mediated clearance of T. gondii *in vitro* and/or *in vivo* (Taylor et al., 2007). We therefore determined whether IIGP1 (Irga6) is properly recruited to the parasitophorous vacuole membrane in IFNγ/LPS-activated, but Atg5-deficient cells. We focused on early times after infection concurrent with or preceding damage to the parasitophorous vacuole membrane (Fig. 6). IIGP1 protein expression was induced by IFNγ/LPS treatment normally in Atg5-deficient macrophages (Supplemental Figure S1B). Within one hour, IIGP1 was recruited to the parasitophorous vacuole in control cells activated by treatment with IFNγ/LPS (Fig. 6A). We did not observe efficient recruitment of IGTP to the parasitophorous vacuole in similar

experiments (data not shown). In contrast to control cells, recruitment of IIGP1 was abrogated in Atg5-deficient cells (Fig. 6A, B). 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 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 LAMP-1 positive vesicles, resulting in a significant percentage of vacuoles that are uniformly LAMP-1 positive (Ling et al., 2006). However, we did not observed parasite-containing vacuoles that were uniformly LAMP-1 positive in either control cells or Atg5-deficient cells activated with IFNγ/LPS (data not shown). This difference between our results and the results of Ling et al. (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 fusigenic 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 (Fig. 6C–F). LAMP-1 signal was often associated with material that was sloughed from the parasite-containing vacuole. We speculate that in our experiments LAMP-1 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 LAMP-1 positive vesicles to region of parasites containing vacuoles in Atg5-deficient cells, consistent with a failure of IIGP1 to be recruited (Fig. 6D,F) and the fact that parasite containing vacuole remain intact in the absence of Atg5 (Fig. 4).

One possible explanation for the failure of IIGP1 to be recruited in Atg5 deficient cells is that it was sequestered in intracellular inclusions (Fig. 5B). These intracellular inclusions consisted of clusters of small vesicles that were IIGP1 positive and which occurred in close association with IIGP1-negative but LAMP-1-positive vesicles, despite the fact that they did not precisely colocalize in the same vesicular structure (Fig. 6E insert). The nature of these inclusions is presently unknown, but their existence suggests a failure for 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 new 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 which 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 targeting 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 IFNγ-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; Kim et al., 2001). *T. gondii* (type-II strains): wild type (PTG), expressing luciferase (PRU-LUC), or expressing GFP (GFP-PTG) 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 media prior to enumeration of viable bacteria (Gutierrez et al., 2004). *ATG5*$^{flox/flox}$ (control) mice and *ATG5*$^{flox/flox}$ Lyz-Cre mice were bred and genotyped as described (Zhao et al., 2007b; Hara et al., 2006). IFNγ levels in serum were determined using a Becton-Dickinson CBA mouse inflammation kit. Female mice 8 to 12 weeks of age were used for *in vivo* studies.

In vitro and *in vivo* infections and immunofluorescence microscopy

Macrophages were pre-treated with 100 U/ml recombinant murine IFN-γ (R&D Systems, Minneapolis MN) plus 1ng/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% CO_2 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*, LAMP-1 (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), Alex 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 were inoculated into mice intraperitoneally. *L. monocytogenes* replication was quantified as cfus in spleen and liver three days after infection (Barton et al., 2007). *T. gondii* replication was quantified by light emission after injection of 0.15 mg/kg of firefly Dluciferin (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 μm cell strainer) prior to distribution into 96 well plates (4 wells/tissue sample). To detect luciferase

expression 2 μl of firefly D-luciferin (30mg/ml) was added per well, incubated for 10 min at room temperature, and light emission was measured (Xenogen IVIS 100). To prepare a standard curve (Supplemental Figure S3), serial dilutions of PRU-LUC (10^6 to 10^2 parasites per well) were made in 96 well plates and luciferase assessed as 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 1hr 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, rinsed extensively in dH_20 prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 hr. Following several rinses in dH_20, 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 80kV.

Western blotting and assay of NO production

2×10^5 macrophages were treated with LPS, IFNγ, or IFNγ/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, IFNγ, or IFNγ/LPS for 18 hr, washed, and incubated with 200 μl of reaction buffer or 200 μl inhibition reaction buffer containing 1 μM diphenyleneiodoum chloride (DPI), and incubated at room temperature for 2 hr in dark prior to detection of fluorescence.

Measurement of gene expression

Total RNA was isolated from bone-marrow derived macrophages using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA (2ug) was treated with DNase I (Ambion, Austin, TX) before subject to reverse transcriptase cDNA synthesis using $Oligo(dT)_{12–18}$ and Superscript II (Invitrogen, Carlsbad, CA) as per manufacture'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'CACCCCCAGATGTGTATGTGC and 5'CGAGGTTTCCCAGTCCAGAAG, GAPDH 5'TGCCCCCATGTTTGTGATG and 5'TGTGGTCATGAGCCCTTCC, IRF-1 5'ACACTAAGAGCAAAACCAAGAG and 5'TTTCCATATCCAAGTCCTGA, Sca-1 5'CTTGCCCATCAATTACCTGCCC and 5'GGAGGGCAGATGGGTAAGCAAA, Stat-1 5'CTCTTAGCTTTGAAACCCAGTT and 5'TTGTACCACAGGATAGACGC. Transcript levels were normalized to GAPDH within each sample and data was 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 +/− S.E.M.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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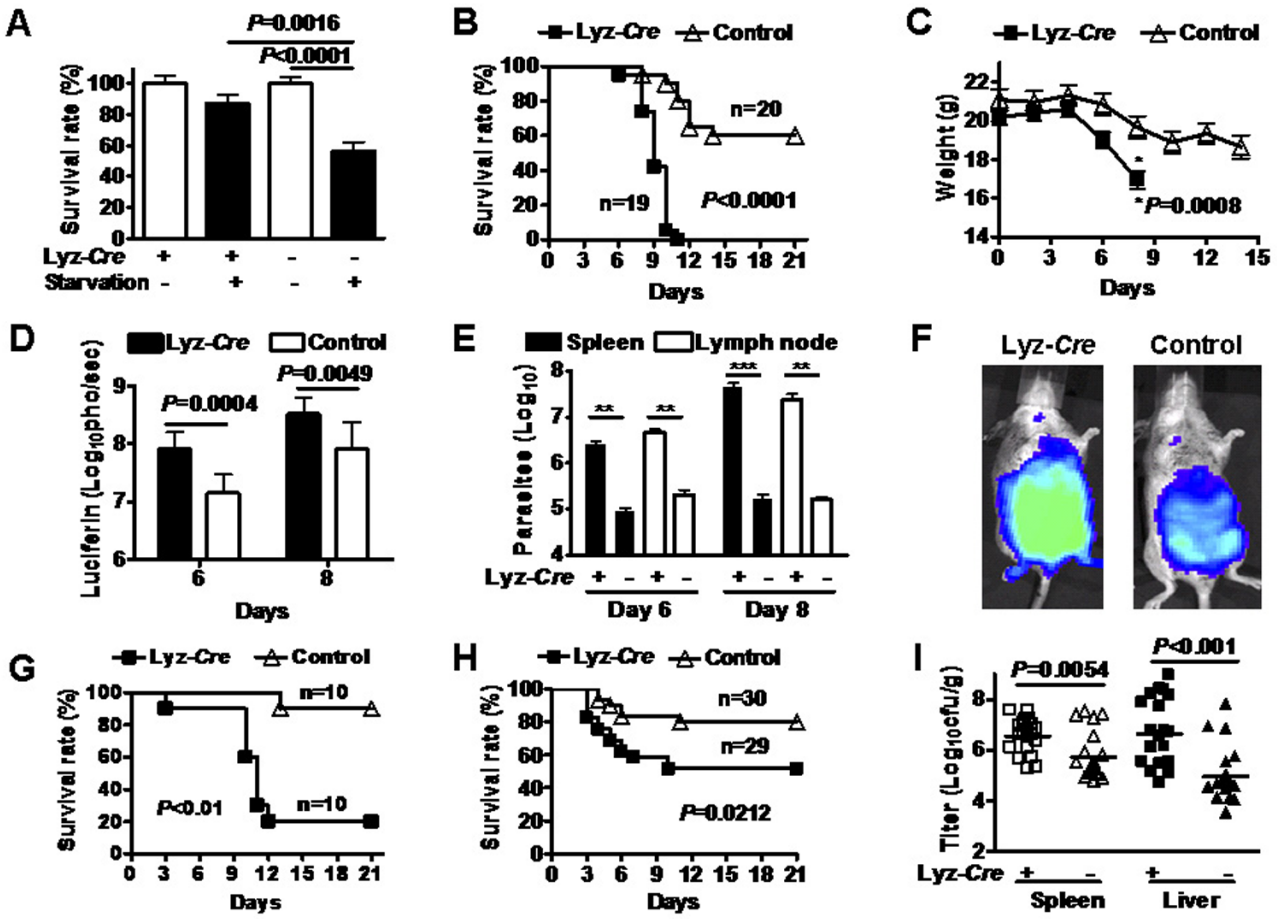

Fig. 1. Atg5 is required for cellular immunity to *Toxoplasma gondii* and *L. monocytogenes in vivo*

(A) Survival of BCG in Atg5 deficient and control macrophages after starvation (Gutierrez et al., 2004). Results are pooled from three independent experiments.

(B) Survival of female mice after infection with 100 *T. gondii* parasites expressing luciferase. These data were pooled from 4 independent experiments.

(C) Weight of mice in Fig. 1B over the course of *T. gondii* infection.

(D) Light emission from mice in Fig. 1B after injection of luciferin.

(E) Quantification of parasites in the indicated tissues using the methods and standard curve in Supplemental Figure S3. These data were pooled from 2 independent experiments. ** $P<0.001$, *** $P<0.0001$.

(F) Representative images of mice 8 days after infection with *T. gondii*. The full data set of which these are representatives is provided in Supplemental Figure S2.

(G) Survival of male mice after infection with 200 *T. gondii* parasites. This dose is higher than that used in female mice in 1B.

(H) Survival of mice after inoculation with 2×10^5 CFUs of *L. monocytogenes*.

(I) *L. monocytogenes* colony forming units in spleen or liver three days after infection. These data were pooled from at least three independent experiments (20 control mice and 19 $ATG5^{flox/flox}$-Lyz-*Cre* mice).

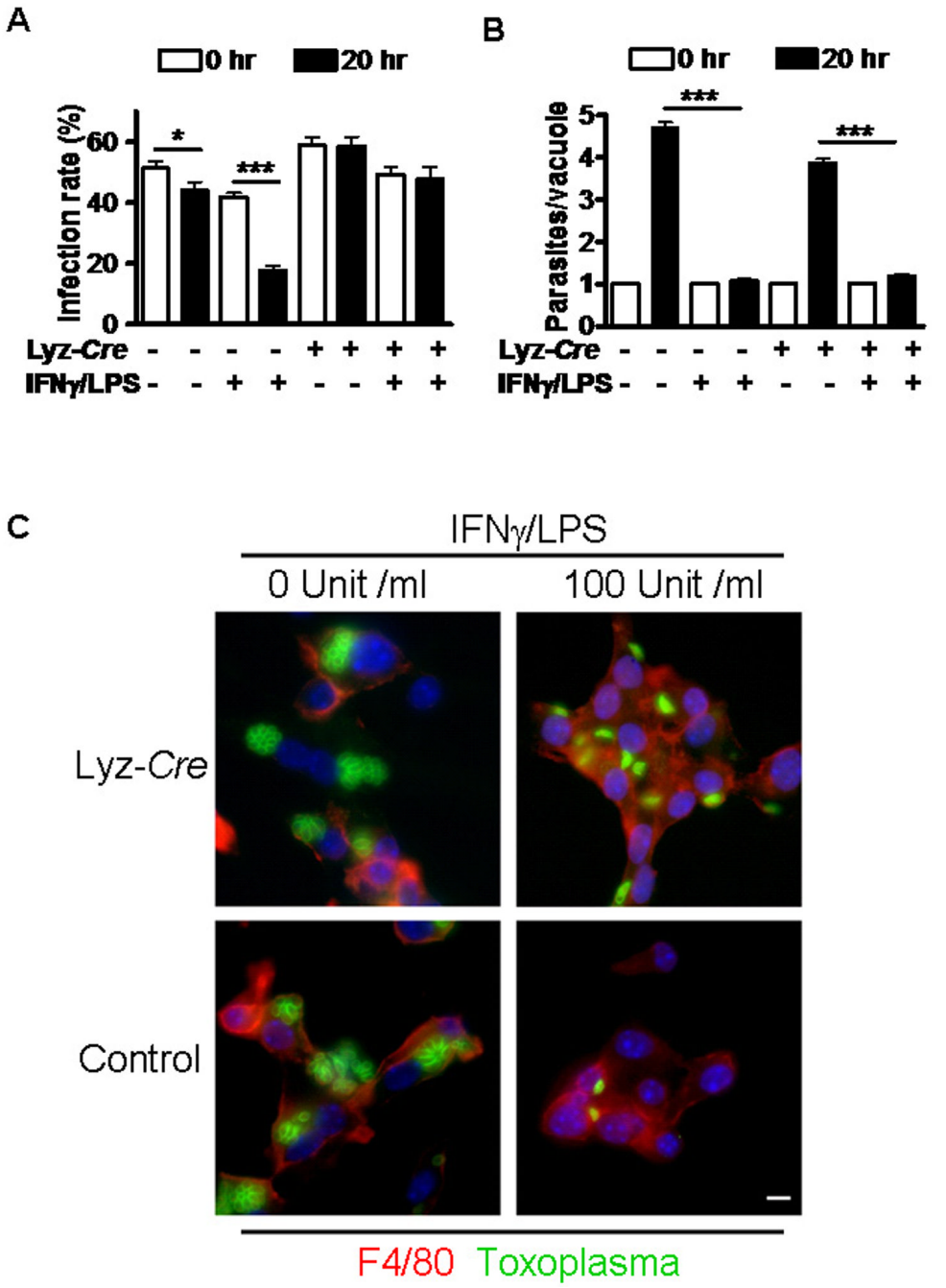

Fig. 2. Atg5 is required for IFNγ-induced clearance of *Toxoplasma gondii* from macrophages

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

(B) The number of *T. gondii* parasite 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.

(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). Bar= 100 μm.

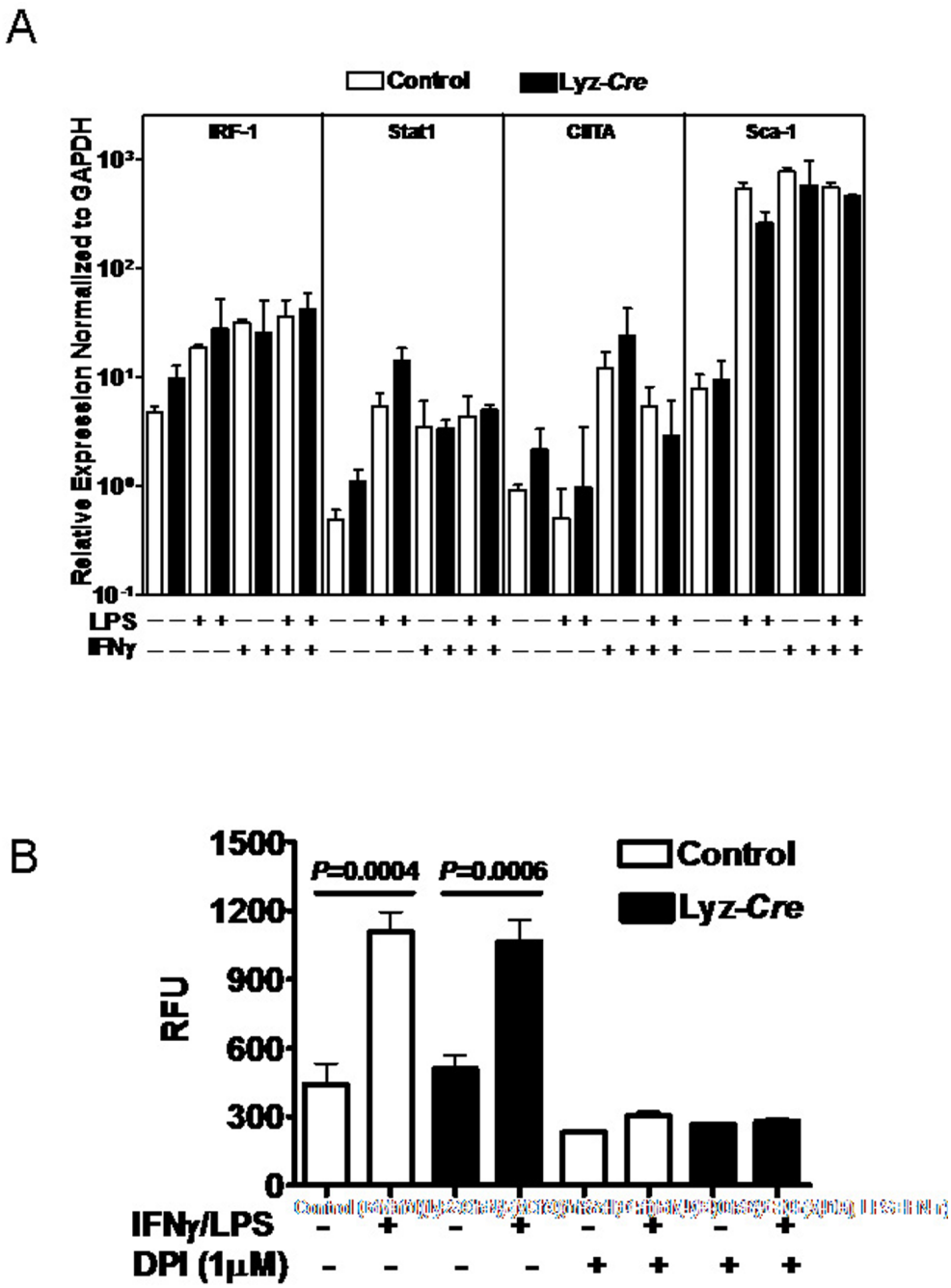

Fig. 3. Atg5 is not globally required for IFNγ-induction of transcription

(A) Shown is the induction of expression of the indicated genes as measured by qRT-PCR at 16 hours after treatment of control or Atg5-deficient macrophages with LPS, IFNγ, or the combination of IFNγ/LPS. Data was pooled from two independent experiments. There were no statistically significant differences between control and Atg5-deficient macrophages. (B) Shown are the levels of NO produced by macrophages stimulated by IFNγ/LPS for 18 hours. DPI is an inhibitor of NO production. Data were pooled from two independent experiments.

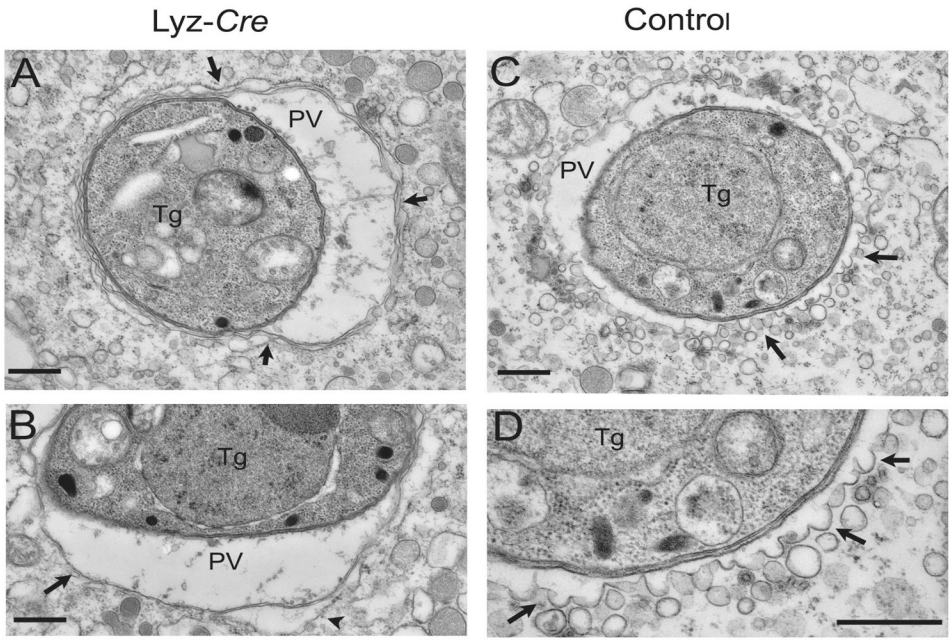

Fig. 4. Electron microscopic analysis of the clearance of *T. gondii* by IFNγ-activated macrophages

Macrophages treated as indicated were infected for 5 hours and then analyzed by EM. All images shown are from IFNγ/LPS activated macrophages.

(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 micrometers.

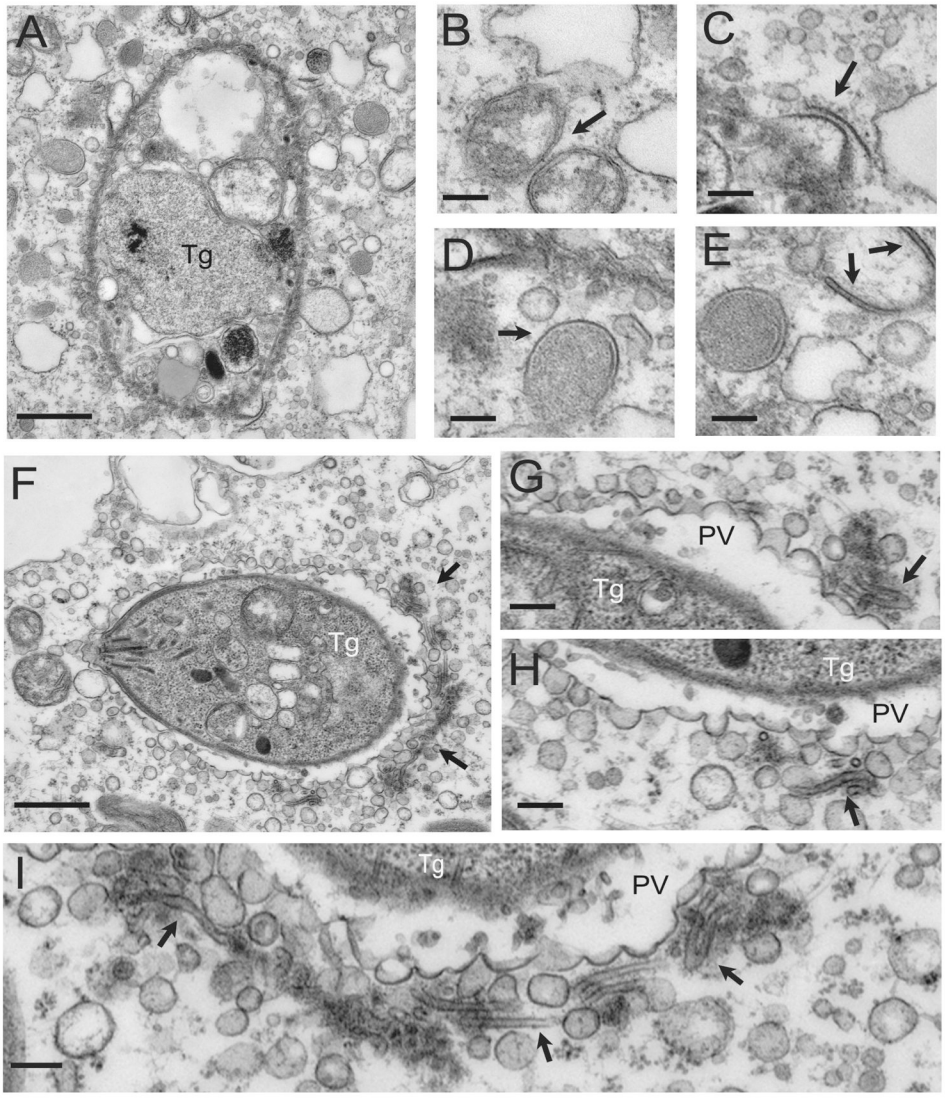

Fig. 5. Electron microscopic analysis of damage to the parasitophorous vacuole in IFNγ/LPS activated control macrophages

Clearance of *T. gondii* infection by control macrophages treated with IFNγ/LPS is associated with a process of vacuolar membrane damage including vesiculation and blebbing 5 hours after infection. Findings here were specific for control macrophages and were not observed in Atg5-deficient macrophages.

(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 micrometers.

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

(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 micrometers.

(G–H) Enlarged views of the membrane vesicles showing flattened cisternae (arrows). Scale bars = 0.1 micrometer.

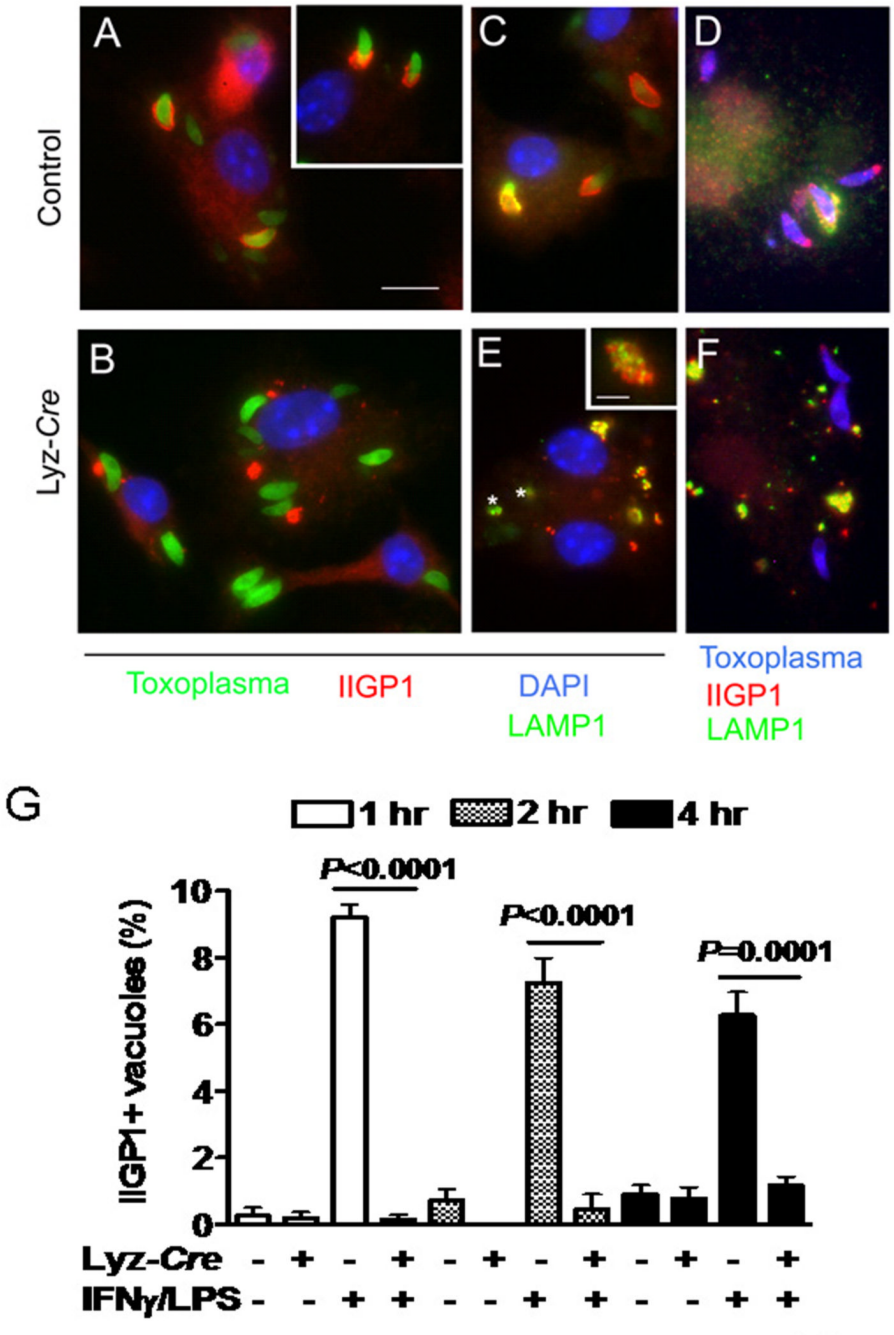

Fig. 6. Atg5 is required for IFNγ/LPS-induced targeting of IIGP1 to the *T. gondii* parasitophorous vacuole

Macrophages were stimulated with IFNγ/LPS and infected with *T. gondii* as in Fig. 2, and then stained for the indicated markers 2 hour after infection. Scale bar = 10 microns for A–F. (A, B) Localization of IIGP1 (red) with GFP-expressing *T. gondii* (green) after infection of IFNγ/LPS activated control or Atg5-deficient macrophages. 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 IFNγ/LPS activated control cells. LAMP-1 (green) was recruited selectively to IIGP1 (red) positive vacuoles and often formed a partial cap, associated with material sloughing from the vacuole surface. DAPI (blue) stains nuclei. (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. * GFP expressing parasites. However, IIGP1 positive clusters (red in 1B) were strongly associated with LAMP1. Insert shows enlarged view of intracellular cluster. IIGP1 (red) and LAMP1 (green) were closely associated but not strictly colocalized. (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 co-localization with *T. gondii* parasites in IFNγ/LPS activated macrophages 1, 2, and 5 hours post-infection. Data were collected from two independent experiments counting at least 600 vacuoles.