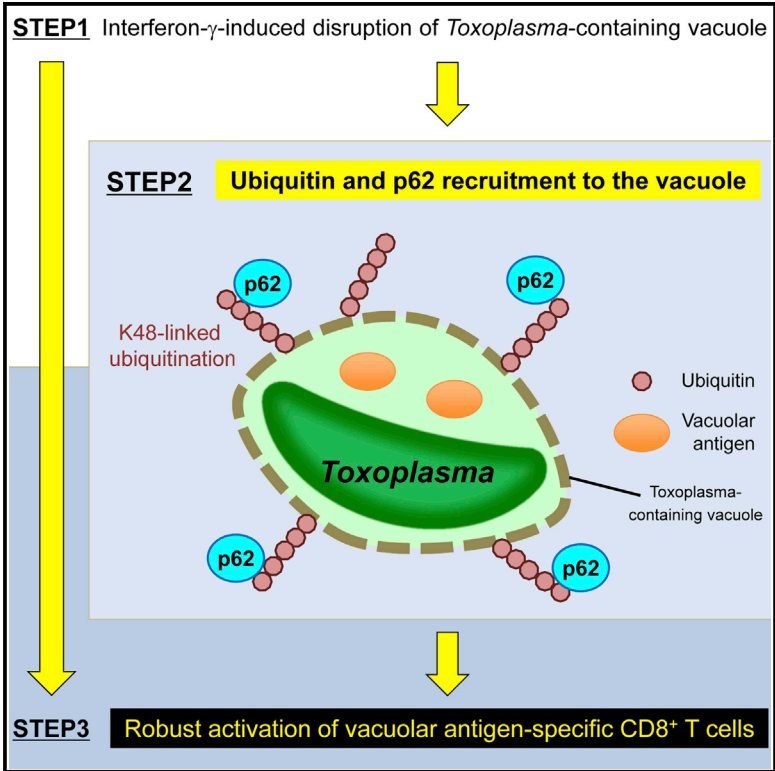# p62 Plays a Specific Role in Interferon-γ-Induced Presentation of a *Toxoplasma* Vacuolar Antigen

Graphical Abstract

STEP1 Interferon-γ-induced disruption of *Toxoplasma*-containing vacuole

STEP2 **Ubiquitin and p62 recruitment to the vacuole**

K48-linked ubiquitination

p62

Toxoplasma

Ubiquitin

Vacuolar antigen

Toxoplasma-containing vacuole

STEP3 Robust activation of vacuolar antigen-specific CD8⁺ T cells

Highlights

- IFN-γ induces ubiquitin and p62 recruitment to *Toxoplasma gondii*-forming vacuoles

- Atg3/5/7/16L1 and Irgm1/m3 are essential for the ubiquitination and p62 recruitment

- p62 is dispensable for IFN-γ-induced clearance of *T. gondii*

- p62 plays a specific role in vacuolar-antigen-specific CD8⁺ T cell activation

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In Brief

Interferon γ (IFN-γ) mediates cell-autonomous host defense against the obligatory intracellular parasite *Toxoplasma gondii*. In this study, Lee et al. demonstrate that IFN-γ stimulates ubiquitination and subsequent accumulation of p62/Sqstm1 on *T. gondii*-forming vacuoles after the damage by IFN-γ-inducible GTPases, specifically leading to activation of the vacuolar-antigen-specific CD8⁺ T cells.

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p62 Plays a Specific Role in Interferon-γ-Induced Presentation of a *Toxoplasma* Vacuolar Antigen

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SUMMARY

Also known as Sqstm1, p62 is a selective autophagy adaptor with a ubiquitin-binding domain. However, the role of p62 in the host defense against *Toxoplasma gondii* infection is unclear. Here, we show that interferon γ (IFN-γ) stimulates ubiquitin and p62 recruitment to *T. gondii* parasitophorous vacuoles (PVs). Some essential autophagy-related proteins, but not all, are required for this recruitment. Regardless of normal IFN-γ-induced *T. gondii* clearance activity and ubiquitination, p62 deficiency in antigen-presenting cells (APCs) and mice diminishes the robust IFN-γ-primed activation of CD8+ T cells that recognize the *T. gondii*-derived antigen secreted into PVs. Because the expression of Atg3 and Irgm1/m3 in APCs is essential for PV disruption, ubiquitin and p62 recruitment, and vacuolar-antigen-specific CD8+ T cell activation, IFN-γ-mediated ubiquitination and the subsequent recruitment of p62 to *T. gondii* are specifically required for the acquired immune response after PV disruption by IFN-γ-inducible GTPases.

## INTRODUCTION

The host immune system produces a proinflammatory cytokine, interferon γ (IFN-γ), against infection of the obligatory intracellular protozoan pathogen *Toxoplasma gondii* (Hunter and Sibley, 2012; Suzuki et al., 1988; Yap and Sher, 1999). IFN-γ stimulates the cell-autonomous innate immune response, causing the disruption of the membranes of parasitophorous vacuole (PV), which *T. gondii* forms inside infected cells, by IFN-γ-inducible GTPases, such as immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs) (MacMicking, 2012). GBPs and effector IRGs (called GKS-IRGs), such as Irga6 and Irgb6, are recruited to the *T. gondii* PV membranes and are thought to cooperate in PV disruption (Hunn et al., 2008; Selleck et al., 2013;

Yamamoto et al., 2012). The regulatory IRGs (called GMS-IRGs), such as Irgm1 and Irgm3 (Irgm1/m3), and some essential autophagy components, such as Atg3, Atg5, Atg7, and Atg16L1, positively control the recruitment of IRGs and GBPs to the *T. gondii* PVs (Choi et al., 2014; Haldar et al., 2013, 2014; Hunn et al., 2008; Ohshima et al., 2014; Zhao et al., 2008).

When a healthy host is infected with *T. gondii*, it develops acquired immunity involving a parasite-specific CD8+ T cell response, which is important for both acute and chronic resistance to *T. gondii* and protective immunity against infection (Dzierszinski et al., 2007; Grover et al., 2014). Endogenous parasite-derived proteins, including GRA6, or a model protein such as ovalbumin (OVA) that is secreted into the PVs has been shown to efficiently elicit strong parasite-specific CD8+ T cells (Dzierszinski et al., 2007; Grover et al., 2014; Yap and Sher, 1999). Professional hematopoietic antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, as well as nonprofessional nonhematopoietic cells, such as fibroblasts and astrocytes, process the vacuolar proteins and present the antigens on major histocompatibility complex class I (MHCI) molecules in a TAP1-dependent manner (Dzierszinski et al., 2007; Goldszmid et al., 2007; Gubbels et al., 2005). Furthermore, the direct interaction between the PV and the host endoplasmic reticulum (ER) provides a route of entry for the MHCI-restricted antigen presented on DCs (Goldszmid et al., 2009). The priming of macrophages by the IFN-γ and tumor necrosis factor alpha (TNF-α) further enhances the activation of antigen-specific CD8+ T cells in an Irgm3-dependent manner, suggesting a potential role for IFN-γ-inducible GTPases in antigen presentation (Dzierszinski et al., 2007). However, the molecular link between IFN-γ-induced cell-autonomous innate immunity and vacuolar-antigen-specific CD8+ T cell-dependent acquired immunity remains unclear.

Here we show that p62/Sqstm1, a protein with a ubiquitin-binding domain and that is involved in the selective autophagic elimination of intracellular pathogens (Dupont et al., 2009; Mostowy et al., 2011; Zheng et al., 2009), is recruited together with ubiquitin to the *T. gondii* PVs, following IFN-γ stimulation, and plays a specific role in vacuolar-antigen-specific CD8+ T cell activation after PV disruption by IFN-γ-inducible GTPases.
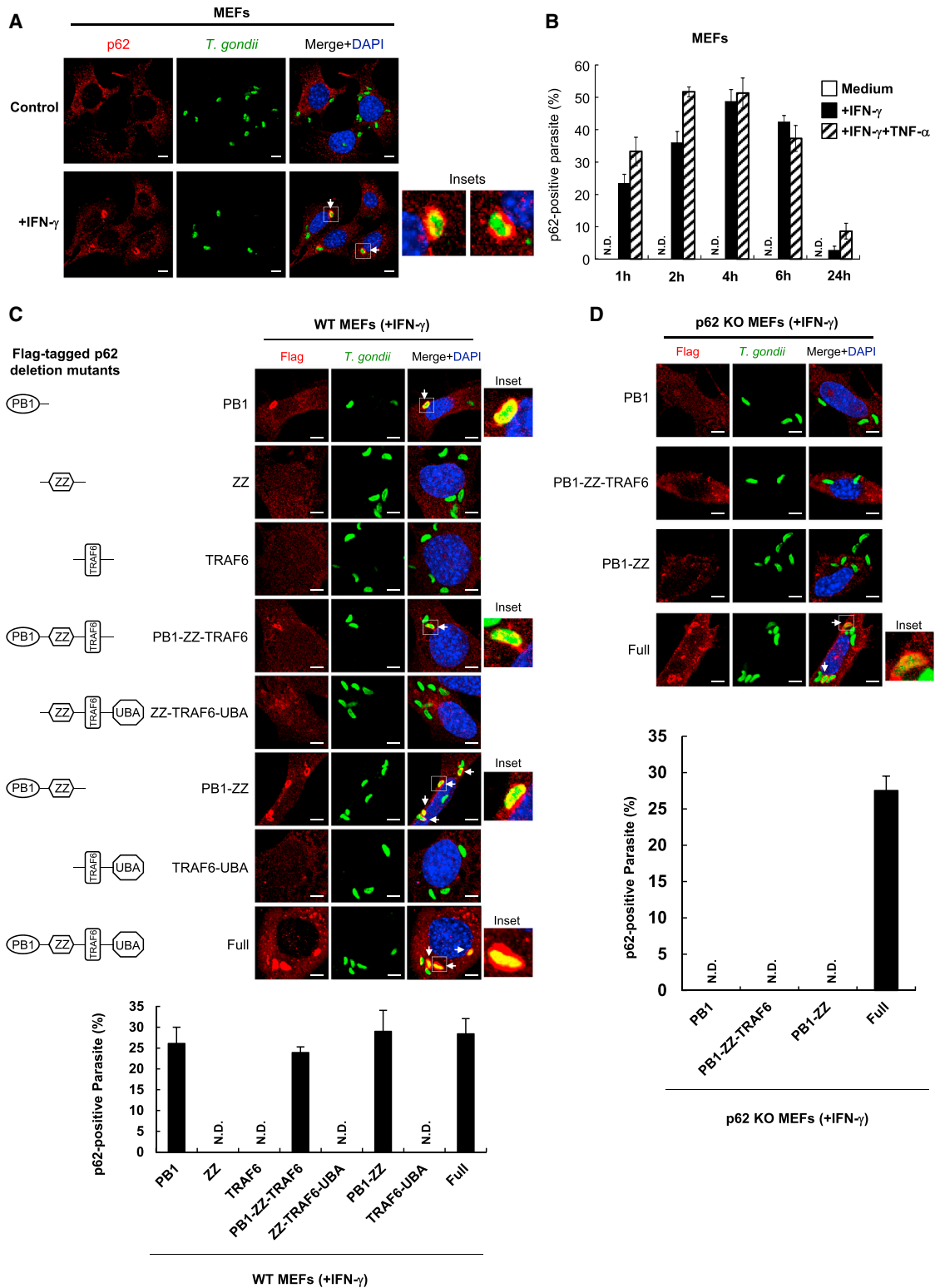
Figure 1. IFN-γ-Dependent p62 Recruitment to *T. gondii* via PB1 and UBA Domains

WT MEFs were treated with IFN-γ (10 ng/ml) or IFN-γ and TNF-α (10 ng/ml) or not for 24 hr, infected with ME49 *T. gondii* for the indicated time point, fixed, and then stained with antibodies against p62 (red) and *T. gondii* (green) and DAPI (blue).

(A) Representative confocal microscope images show p62 localization with *T. gondii* at 4 hr p.i.

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RESULTS

IFN-γ Stimulates p62 Recruitment to *T. gondii* PVs via Its PB1 and UBA Domains

The previous finding that p62 accumulates in aggregates with GBPs in IFN-γ-stimulated cells defective in the machinery for *T. gondii* clearance prompted us to examine the localization of p62 in IFN-γ-stimulated wild-type (WT) mouse embryonic fibroblasts (MEFs) infected with *T. gondii* (Selleck et al., 2013). Surprisingly, p62 colocalized strongly with *T. gondii* (Figure 1A). Moreover, the recruitment of p62 to the parasite was totally dependent on IFN-γ stimulation, because no p62-positive parasites were detected in unstimulated cells (Figure 1B). TNF-α, in addition to IFN-γ, mediates the full anti-*T. gondii* effect (Sibley et al., 1991), but the costimulation of cells with TNF-α and IFN-γ, or with IFN-γ alone, resulted in the transient accumulation of p62 on *T. gondii* (Figure 1B).

p62 encodes an N-terminal PB1 domain, a ZZ-type zinc-finger domain, a TRAF6-binding domain, and a ubiquitin-associated (UBA) domain (Nakamura et al., 2010). To identify the domains of p62 that are important for its recruitment to *T. gondii*, we generated WT and p62-deficient MEFs overexpressing FLAG-tagged WT p62 or p62 deletion mutants using a retroviral expression system. Only the p62 deletion mutants containing the PB1 domain and WT p62 were recruited to the PVs in the WT MEFs (Figure 1C). Because the PB1 domain plays a role in the self-oligomerization of the p62 protein, these results suggest that the PB1 domain of the p62 deletion mutants associated with endogenous p62 and localized on *T. gondii*. In sharp contrast, all the deletion mutants lacking the UBA domain failed to accumulate on *T. gondii* in the p62-deficient cells, even if they contained the PB1 domain (Figures 1D and S1), suggesting that both the PB1 and UBA domains of p62 are necessary and sufficient for its recruitment to *T. gondii* after IFN-γ stimulation.

K48-Linked Ubiquitination after IFN-γ Stimulation

Because the UBA domain of p62 is required for its recruitment to the parasite, we examined the localization of ubiquitin in infected cells. Notably, IFN-γ stimulated the accumulation of ubiquitin on the *T. gondii* PVs (Figures 2A and 2B). All the p62-positive parasites colocalized with ubiquitin in ~31% of *T. gondii*, and only about 9% of parasites were ubiquitin positive and p62 negative (Figure 2C). Next we tested the requirement for p62 in the IFN-γ-mediated ubiquitination of *T. gondii*. p62 deficiency resulted in even higher *T. gondii* ubiquitination after IFN-γ stimulation (Figures 2D and 2E), suggesting that IFN-γ-induced ubiquitination of *T. gondii* precedes the recruitment of p62 to *T. gondii* through its UBA domain.

The UBA domain of p62 has been shown to associate with both K48-linked and K63-linked polyubiquitin chains (Cavey et al., 2005; Seibenhener et al., 2004; Vadlamudi et al., 1996;

Waters et al., 2009). Therefore, we examined the type of IFN-γ-induced ubiquitination on *T. gondii* with indirect immunofluorescence using chain-specific antibodies. IFN-γ-stimulated ubiquitination also was detected with anti-K48-linked polyubiquitin antibody, but not with anti-K63-linked ubiquitin antibody (Figures 2F and 2G). In contrast, the treatment of MEFs with the protonophore m-chlorophenylhydrazone, which induces mitochondrial uncoupling and Parkin-dependent K63-linked ubiquitination (Geisler et al., 2010), resulted in the formation of p62 aggregates costained with anti-K63-linked polyubiquitin, but not with anti-K48-linked polyubiquitin (Figures S2A and S2B). When we quantified this reaction, we found that more than 90% of the ubiquitinated parasites were costained with the anti-K48-linked polyubiquitin and anti-ubiquitin antibodies (Figure 2H).

UBC13 is an E2-conjugating enzyme essential for the synthesis of K63-linked polyubiquitin chains (Deng et al., 2000). Consistent with the dominant K48-linked ubiquitination on *T. gondii*, IFN-γ-induced ubiquitination was normally observed in Ubc13-deficient MEFs (Figures 2I, S2C, and S2D). p62 has been shown to associate with an E3 ubiquitin ligase, TRAF6 (Deng et al., 2000). Traf6-deficient cells exhibited the normal recruitment of ubiquitin and p62 to the parasite following stimulation with IFN-γ (Figures 2J and S2E). To explore the substrate of the ubiquitination on PVs, we used MEFs lacking GBPs (Gbp1/Gbp2/Gbp3/Gbp5/Gbp7; GBP^{chr3}) or Irga6 and tested for IFN-γ-induced ubiquitination on *T. gondii*. Both GBP^{chr3}- and Irga6-deficient MEFs showed normal accumulation of ubiquitin and p62 on *T. gondii* in response to IFN-γ (Figures 2K and 2L). Taken together, our data suggest that IFN-γ predominantly stimulates K48-linked polyubiquitination on PVs in a Traf6- and Ubc13-independent manner, and that neither Irga6 nor GBP^{chr3} are substrates of this ubiquitination.

Some Atg Proteins and GMS-IRGs Are Critical for the Recruitment of Ubiquitin and p62 to *T. gondii* PVs

Autophagy-related proteins including Atg3, Atg5, Atg7, and Atg16L1, but not Atg14, are essential for *T. gondii* clearance because they regulate the recruitment of IFN-γ-induced effector molecules, such as GKS-IRGs and GBPs, to the PV membrane (Haldar et al., 2014; Ohshima et al., 2014; Zhao et al., 2008). A deficiency in Atg3, Atg5, Atg7, or Atg16L1 in MEFs significantly reduced the recruitment of ubiquitin and p62 to the PV membrane and caused defective *T. gondii* clearance and the accumulation of IRGs and GBPs after IFN-γ stimulation (Figures 3A–3D and S3A–S3H; Ohshima et al., 2014). In sharp contrast, Atg14-deficient cells showed no significant change in the recruitment of ubiquitin or p62 relative to that in WT cells (Figures 3A–3D), even though MEFs lacking these Atg proteins showed greater accumulation of p62 protein than WT cells because their autophagic flux was impaired (Figure S3I). This indicates that the IFN-γ-mediated recruitment of ubiquitin, p62, GKS-IRGs, and

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(B) The graph represents the mean ± SE of p62-positive parasites after infection for the indicated time points in three independent experiments.

(C and D) WT (C) or p62 knockout (KO) (D) MEFs overexpressing FLAG-tagged p62 WT or deletion mutants were treated with 10 ng/ml IFN-γ for 24 hr, infected with ME49 *T. gondii* for 4 hr, fixed, and then stained with antibodies against FLAG (red) and GAP45 (green) and DAPI (blue). Representative confocal microscope images represent p62 localization with *T. gondii* in three independent experiments and are shown at ×1,000 magnification. The graphs represent the mean ± SE of p62-positive parasites in three independent experiments. Scale bars, 5 μm. N.D., not detected.
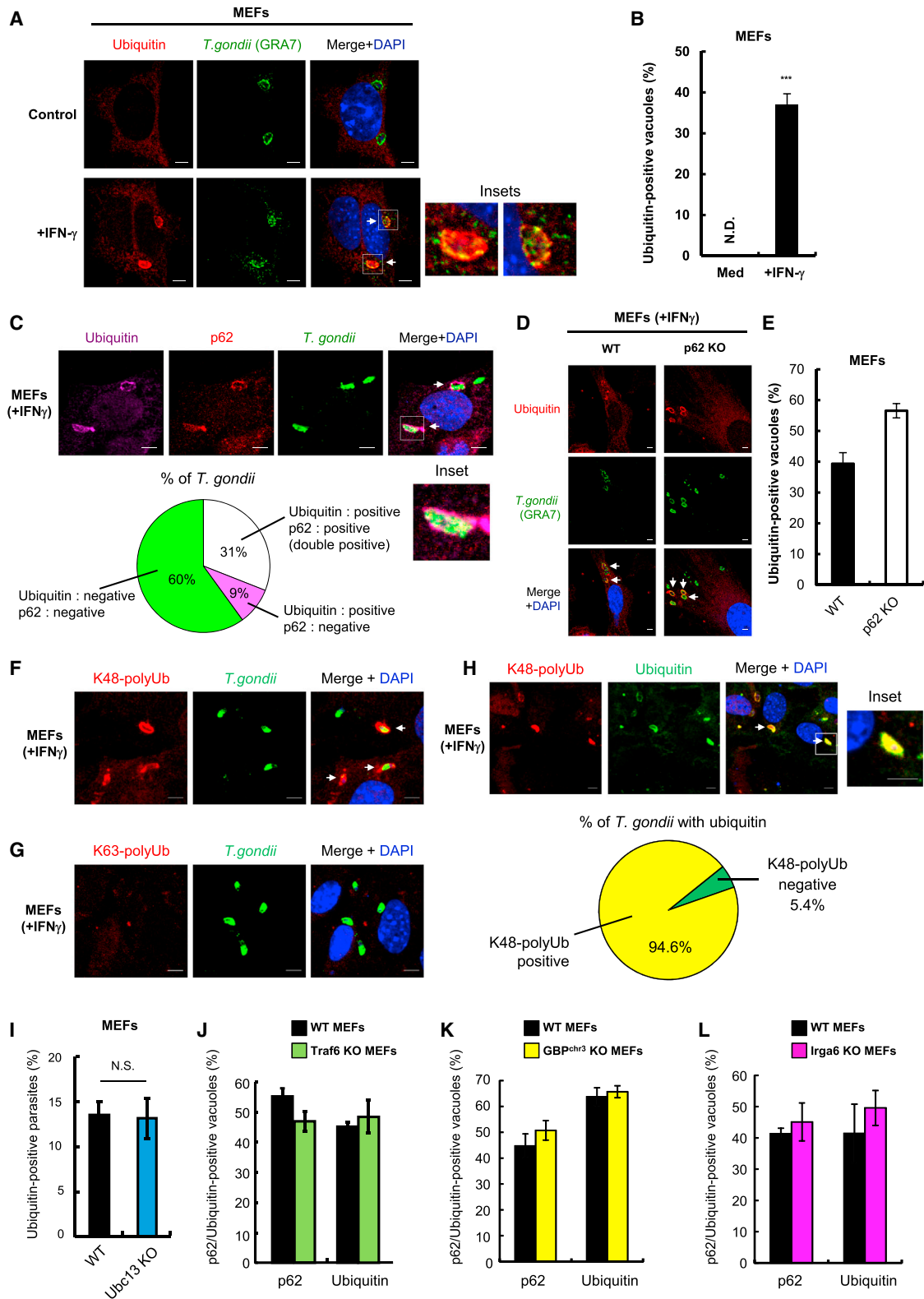
Figure 2. Ubiquitin Colocalization with p62-Positive Parasites upon IFN-γ Stimulation

WT, p62 KO, Ubc13 KO, TRAF6 KO, Gbp[chr3] KO, or Irga6 KO MEFs were treated with 10 ng/ml IFN-γ or not for 24 hr, infected with Pru or ME49 *T. gondii* for 4 hr, fixed, and then stained with antibodies against p62, ubiquitin, K48-polyubiqutin, K63-polyubiquitin, and GRA7 or GAP45 and DAPI (blue).

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GBPs may be similarly regulated by the nonautophagic functions of Atg3, Atg5, Atg7, and Atg16L1.

To examine whether the disruption of PVs by these GTPases is required for the recruitment of ubiquitin and p62, we tested whether p62 and ubiquitin are recruited to type I *T. gondii*, which inhibits IFN-γ-inducible GTPase-dependent PV disruption using the polymorphic virulence effectors ROP5 and ROP18 (Hunter and Sibley, 2012). In sharp contrast to cells infected with type II parasites, the accumulation of ubiquitin and p62 was profoundly reduced in cells infected with type I *T. gondii* (Figures 3E and 3F). To examine the phenomenon more directly, we tested the recruitment of p62 and ubiquitin in cells lacking Irgm1 and Irgm3 (Figures S3J and S3K), which are completely devoid of IFN-γ-inducible GTPase-dependent cell-autonomous immunity (Figures S3L and S3M; Haldar et al., 2013). Compared with WT cells, no IFN-γ-induced recruitment of p62 and ubiquitin to PVs was observed at all in Irgm1/m3 doubly deficient MEFs (Figures 3G and 3H), indicating that the disruption of PVs by IFN-γ-inducible GTPases, which is critically controlled by GMS-IRGs, may be a prerequisite for the IFN-γ-induced localization of ubiquitin and p62 to the PVs.

p62 Is not Essential for the Cell-Autonomous Clearance of *T. gondii*

We then examined the biological significance of p62 recruitment to the *T. gondii* PVs after IFN-γ stimulation. We first tested whether IFN-γ stimulates the recruitment of ubiquitin and p62 to PVs in macrophages and DCs. We found that both ubiquitin and p62 accumulated on the PVs in IFN-γ-activated macrophages and DCs (Figures S4A–S4C). The recruitment of ubiquitin and p62 was completely abrogated in Irgm1/m3 doubly deficient DCs (Figures S4B and S4C), indicating that IFN-γ-mediated p62 recruitment and ubiquitination occur in immune cells as well as in MEFs. We then tested whether p62 also is involved in pathogen clearance by comparing the survival rates, infection rates, and replication abilities of the parasite in bone marrow-derived macrophages (BMMs) from WT and p62-deficient mice. The survival rate, infection rate, and replication ability of the parasite in p62-deficient macrophages were similar to those in the WT cells after IFN-γ stimulation (Figures 4A–4C). The recruitment of GBPs and GKS-IRGs to the parasite, which are important for PV disruption, was also normal in the p62-deficient macrophages (Figures 4D and S4D). Furthermore, the IFN-γ-induced reduction in *T. gondii* was similar in the WT and p62-deficient MEFs (Fig-

ure S4E), indicating that p62 is not required for the cell-autonomous immune response.

Atg3 and Irgm1/m3 Are Required for the Priming of APCs by IFN-γ to Activate Vacuolar-Antigen-Specific CD8+ T Cells during *T. gondii* Infection

We next compared the ability of unstimulated or IFN-γ-stimulated APCs to activate CD8+ T cells during *T. gondii* infection. IFN-γ-primed DCs infected with irradiated *T. gondii* engineered to secrete OVA (*T. gondii* p30-OVA) into PVs elicited a 5.7-fold higher proportion of IFN-γ-producing OT-I T cells than did the corresponding unprimed DCs (Figures 4E and S4F), and they robustly enhanced the IFN-γ production by OT-I T cells compared with that induced by unprimed DCs (Figure 4F). The effect of IFN-γ priming also was observed on nonprofessional APCs, such as MEFs (Figures 4G and 4H), as previously reported (Dzierszinski et al., 2007). In contrast, when we used *T. gondii* that expressed OVA only within the parasite cytoplasm, no increase in the proportion of IFN-γ-producing OT-I T cells was observed after IFN-γ stimulation (Figure 4E).

Next we tested whether the disruption of PVs by IFN-γ-inducible GTPases in APCs plays a role in the activation of vacuolar-antigen-specific CD8+ T cells. MEFs lacking Atg3 or Irgm1/m3 displayed severely impaired IFN-γ priming effect on the activation of CD8+ T cells (Figures 4G and 4H). However, the treatment of WT and Irgm1/m3 doubly deficient cells with the OVA$_{257-264}$ peptide specific for OT-I resulted in similar IFN-γ production (Figure 4H), indicating that the IFN-γ priming of both professional and nonprofessional APCs to robustly activate CD8+ T cells specifically recognizing the vacuolar antigen derived from *T. gondii* requires the IFN-γ-GTPase-mediated disruption of PVs.

p62 in APCs Plays a Role in the Activation of Vacuolar-Antigen-Specific CD8+ T Cells

When we examined the localization of OVA and p62 or ubiquitin, the OVA signal overlapped the p62 and ubiquitin signals in MEFs infected with *T. gondii* p30-OVA (Figures 4I and 4J). Therefore, we asked whether p62 in APCs is involved in the IFN-γ priming effect on the activation of CD8+ T cells. The levels of MHCI expression were similar in the WT and p62-deficient MEFs and DCs (Figure S4G). IFN-γ-stimulated p62-deficient DCs induced significantly reduced IFN-γ production (by ~50%) from OT-I T cells compared with that of WT cells, whereas unstimulated p62-deficient DCs elicited slightly higher (albeit not significantly) IFN-γ production by OT-I T cells than did WT cells (Figure 4K). In

(A) Confocal microscope images show ubiquitin (red) localization with vacuoles (green).

(B) The graph represents the mean ± SE of ubiquitin-positive vacuoles.

(C) Confocal microscope images (top) show the colocalization of p62 (red), ubiquitin (violet), and *T. gondii* (green), and the pie chart (bottom) represents the mean of p62 and ubiquitin double- or ubiquitin single-positive vacuoles.

(D and E) Confocal microscope images (D) show ubiquitin (red) localization to the vacuoles (green) and the graph (E) represents the mean ± SE of ubiquitin-positive vacuoles in WT and p62 KO MEFs.

(F–H) Confocal microscope images show the localization of K48-polyubiquitin (red) to *T. gondii* (GAP45, green) or to ubiquitin (green) (F and H, respectively) and the localization of K63-polyubiquitin (red) with *T. gondii* (green) (G). The pie chart (H, bottom) represents the mean of K48-polyubiquitin-positive or -negative *T. gondii*.

(I) The graph represents the mean ± SE of ubiquitin-positive parasites in WT and Ubc13 KO MEFs.

(J) The graph represents the mean ± SE of ubiquitin- or p62-positive parasites in WT and Traf6 KO MEFs.

(K and L) The graphs represent the mean ± SE of p62-positive or ubiquitin-positive parasites in WT and Gbp^{chr3} KO (K) or WT and Irga6 KO (L) MEFs. Data are representative of three independent experiments. Scale bars, 5 μm. N.D., not detected; N.S., not significant; ***p < 0.001 versus Medium (Med).
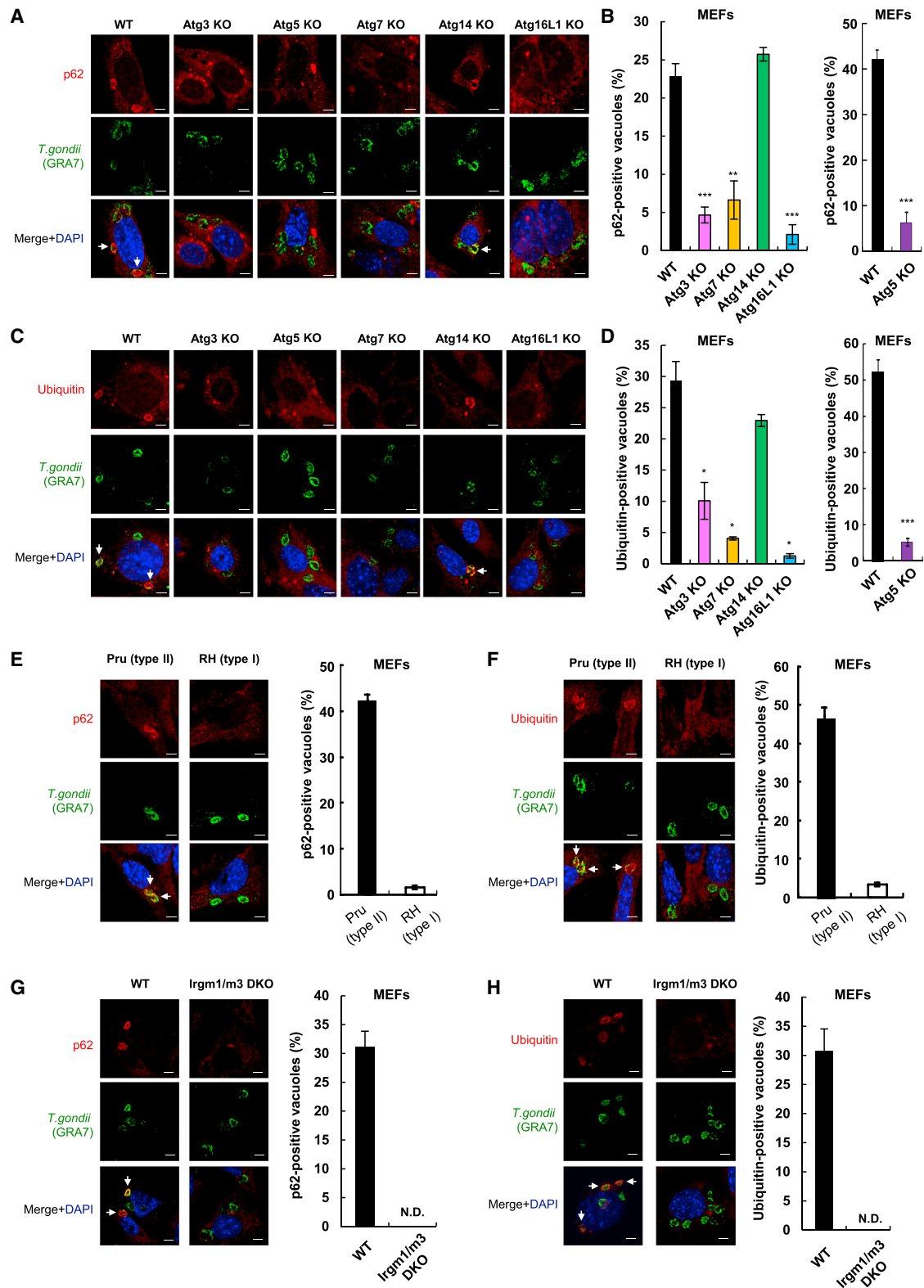
Figure 3. Contribution of Atg Proteins and Irgm1/m3 to Recruitment of p62 and Ubiquitin to the PV

WT, Atg3, Atg5, Atg7, Atg14, or Atg16L1 single KO or Irgm1/m3 double KO (DKO) MEFs were treated with 10 ng/ml IFN-γ for 24 hr, infected with Pru *T. gondii* for 4 hr, fixed, and then stained with anti-p62 or anti-ubiquitin (red) and anti-GRA7 (green) antibodies and DAPI (blue).

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contrast, both IFN-γ-primed WT and p62-deficient DCs infected with the parental *T. gondii* failed to activate OT-I T cells. In addition, the IFN-γ production by OT-I T cells did not change after co-culture with OVA peptide or soluble OVA protein-treated WT or p62-deficient DCs (Figures 4K and S4H). We also found that a deficiency of p62 in MEFs led to significantly lower levels of IFN-γ production from OT-I T cells after IFN-γ priming and *T. gondii* p30-OVA infection (Figure 4L), suggesting that the role of p62 in IFN-γ priming is not limited to a specific cell type.

To examine the role of p62 in the activation of vacuolar-antigen-specific CD8$^+$ T cells in vivo, we intraperitoneally infected WT mice with γ-irradiated *T. gondii* p30-OVA or the parental parasite and monitored the levels of OVA-specific CD8$^+$ T cell activation every 7 days postinfection (p.i.). In the spleens of mice infected with *T. gondii* p30-OVA, but not in those of mice infected with the parental parasite, the number of OVA-tetramer$^+$ CD8$^+$ T cells peaked at 14 days p.i., but was not detected at 21 or 28 days p.i. with flow cytometry (Figures S4I and S4J; data not shown). This suggests that OVA-tetramer$^+$ CD8$^+$ T cells are temporarily induced during infection by nonproliferating *T. gondii* that secrete the vacuolar antigen. WT and p62-deficient mice were then infected intraperitoneally with γ-irradiated *T. gondii* p30-OVA, and the numbers of OVA-tetramer$^+$ CD8$^+$ T cells were counted at 7 or 14 days p.i. The numbers of OVA-tetramer$^+$ CD8$^+$ T cells in the peritoneal cavities of the p62-deficient mice at 14 days p.i. were significantly lower than those in the WT mice (Figure 4M), whereas the numbers in the spleens of the WT and p62-deficient mice were similar at 14 days p.i. and were similar in their peritoneal cavities at 7 days p.i. (Figures 4M and S4K).

Similar levels of interleukin-12 (IL-12) production, which plays a critical role in CD8$^+$ T cell expansion and IFN-γ production (Haring et al., 2006), also were observed in *T. gondii*-infected WT and p62-deficient DCs and mice (Figures 4N and 4O). Anti-CD3/anti-CD28 normally stimulate p62-deficient CD8$^+$ T cells to produce IFN-γ (Figure 4P). When CD45.1$^+$ WT OT-I T cells were adoptively transferred to WT and p62-deficient mice, which were then infected with *T. gondii* p30-OVA, the number of transferred WT OT-I T cells was significantly lower in the peritoneal cavities (but not in the spleens) of the p62-deficient mice than in the WT mice at 14 days p.i. (Figures 4Q and S4L). This suggests that the reduced in vivo OVA-specific CD8$^+$ T cell response in the p62-deficient mice may be caused not by a cell-intrinsic defect in the CD8$^+$ T cells or impaired IL-12 production, but by a failure of antigen presentation. Collectively, these results suggest that p62 plays a role in the *T. gondii*-antigen-presenting pathways to activate vacuolar-antigen-specific CD8$^+$ T cells in vitro and in vivo.

DISCUSSION

Here we have demonstrated that the colocalization of ubiquitin and p62 on *T. gondii* PVs is totally dependent on IFN-γ stimulation and that the ubiquitination of the PV membrane may precede the recruitment of p62 via its UBA domain, because p62 deletion mutants lacking the UBA domain showed no recruitment to the PV membranes, whereas ubiquitin was normally recruited to the PVs in p62-deficient cells. Similarly, a previous study showed that both the PB1 and UBA domains of p62 are required for its proper localization to ubiquitinated protein aggregates by self-oligomerization of p62 and its binding to ubiquitin, respectively (Bjørkøy et al., 2005). p62 preferentially colocalizes with K63-linked polyubiquitin chains and is involved in autophagic degradation in the context of autophagy (Matsumoto et al., 2011; Seibenhener et al., 2004; Wooten et al., 2008). In contrast, a biochemical study demonstrated that the p62 UBA domain associates with K48-linked polyubiquitin more than with K63-linked polyubiquitin (Waters et al., 2009). Therefore, the p62 UBA domain interacts with both K48- and K63-linked polyubiquitin chains. In this context, most ubiquitin-positive *T. gondii* PV membranes were stained with an anti-K48-linked polyubiquitin antibody in our study. Furthermore, a factor essential for autophagy, Atg14, was not essential for the IFN-γ-mediated recruitment of ubiquitin and p62, suggesting that the reaction involving K48-linked ubiquitination may be unrelated to autophagy.

We also found that some Atg proteins (Atg3, Atg5, Atg7, and Atg16L1) and GMS-IRGs (Irgm1 and Irgm3) critically control the recruitment of both ubiquitin and p62 to PVs. Recent studies demonstrated the IFN-γ-dependent cell-autonomous roles of the Atg family (Atg3, Atg5, Atg7, and Atg16L1) during *T. gondii* infection, which contribute to parasite clearance by regulating the recruitment of IFN-γ-inducible GTPases, such as Gbp1, Gbp2, Irga6, and Irgb6, to disrupt the PVs (Choi et al., 2014; Ohshima et al., 2014; Selleck et al., 2013; Zhao et al., 2008). However, in this study, p62 deficiency had no effect on the clearance of *T. gondii* or on the suppression of its intracellular replication. Furthermore, consistent with the similar accumulation of IRGs and GBPs on PVs in WT and p62-deficient cells, we observed that p62-deficient mice were not susceptible to avirulent *T. gondii* infection (data not shown). Therefore, although p62 is known to mediate the killing activity of the antibacterial immune responses (Cemma et al., 2011; Dupont et al., 2009; Zheng et al., 2009), this protein is not essential for the anti-*T. gondii* cell-autonomous innate immunity dependent on IFN-inducible GTPases. However, the recruitment of p62 and some IFN-γ-inducible GTPases and effectors to intracellular pathogens has been demonstrated in several studies (Kim et al., 2011; MacMicking, 2012).

(A–D) Representative confocal microscope images of the recruitment of p62 (A) or ubiquitin (C) to the PV in WT, Atg3-, Atg5-, Atg7-, Atg14-, or Atg16L1-KO MEFs. The graphs represent the mean ± SE of p62-positive (B) or ubiquitin-positive (D) vacuoles in three independent experiments.

(E and F) Representative confocal microscope images (left) of the recruitment of p62 (E) or ubiquitin (F) to the vacuoles of Pru (type II) or RH (type I) *T. gondii*. The graphs (right) represent the mean ± SE of p62- or ubiquitin-positive vacuoles in MEFs.

(G and H) Representative confocal microscope images (left) of the localization of p62 (G) or ubiquitin (H) to the vacuoles in WT and Irgm1/m3 DKO MEFs. The graphs (right) represent the mean ± SE of p62- or ubiquitin-positive vacuoles from three independent experiments. Scale bars, 5 μm. N.D., not detected; *p < 0.05, **p < 0.01, ***p < 0.001 versus WT cells.
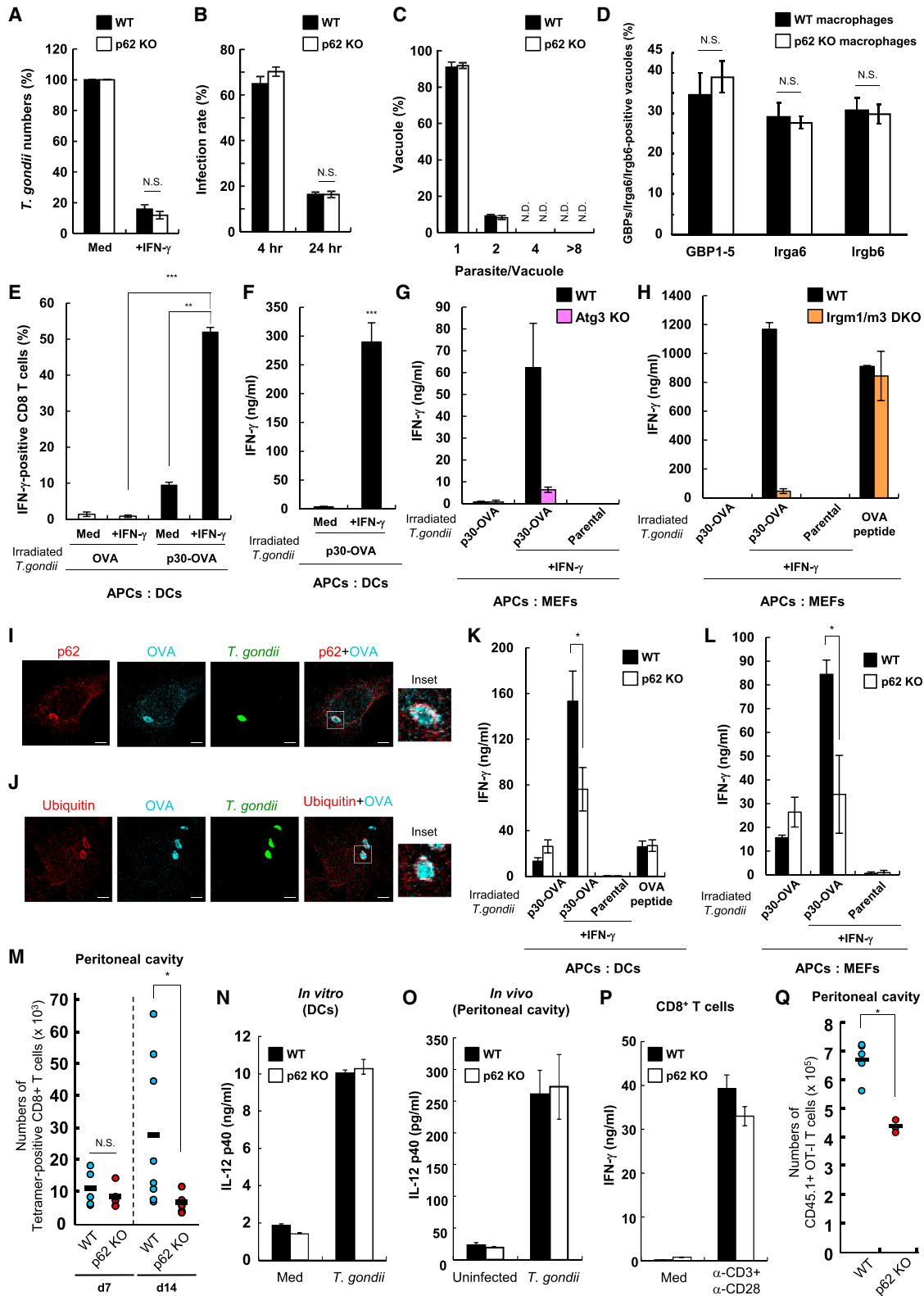
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The GMS-IRGs, which play an important role in the disruption of PVs and parasite clearance by crucially regulating the recruitment of IRGs and GBPs (Haldar et al., 2013), are critical factors in controlling the accumulation of both ubiquitin and p62 on the PV membrane. In contrast, we have demonstrated that Irga6 and GBP[chr3] are not essential for IFN-γ-mediated ubiquitination or p62 recruitment. The IRG- and GBP-dependent clearance of the parasite in either Irga6- or GBP[chr3]-deficient cells was partial, whereas severe or complete defects in clearance were observed in cells lacking the individual Atg proteins or Irgm1/m3, respectively (Liesenfeld et al., 2011; Yamamoto et al., 2012). The ubiquitination of unknown host or parasite-derived substrates on the PV membranes and their subsequent decoration with p62 through its UBA domain might occur if the PV membranes are only partially disrupted (or damaged) by GBPs or IRGs, which is insufficient for complete parasite clearance. However, ubiquitination might be profoundly reduced by the severe blockage of IRG/GBP recruitment resulting from Atg deficiency or prevented by global IRG/GBP dysfunction during multiple GMS-IRG deficiencies, which lead to severe defects in parasite clearance.

In this study, we identified a previously unidentified function of p62 in the IFN-γ-stimulated enhancement of CD8^{+} T cell activation by APCs during T. gondii infection in experiments in vitro and in vivo. CD8^{+} T cell activation mediated by infected APCs is critical for the host's resistance to parasitic infection by producing IFN-γ and cytotoxic killing activity (John et al., 2009; Suzuki and Remington, 1990). Our in vitro results showed that p62 is required for the full activation of CD8^{+} T cells. Also, Atg3-deficient or Irgm1/3 doubly deficient MEFs showed severely impaired IFN-γ production by CD8^{+} T cells compared with that induced by p62 deficiency. A previous study showed that Irgm3 is required for priming macrophages, but not DCs, suggesting a partial or specific role for Irgm3 in the activation of CD8^{+} T cells. In contrast, our study demonstrates the essential role of Irgm1 and Irgm3 in priming CD8^{+} T cells. This discrepancy might be attributable to the different genetic backgrounds of the mice used in each study; mice lacking only Irgm3 were used in

the previous study (Dzierszinski et al., 2007), whereas mice lacking both Irgm1 and Irgm3 were used in the present study. Although Irgm3-deficient cells showed the partial recruitment of IFN-γ-inducible GTPases, Irgm1/m3 doubly deficient cells were completely devoid of this recruitment (Haldar et al., 2013). Therefore, a partial defect in Irgm3 in DCs might be insufficient to abolish their CD8^{+} T cell-priming activity.

According to previous studies, two pathways are needed to load an intracellular-pathogen-derived antigen onto MHCI to activate CD8^{+} T cells: one is the formation of PV membranes by the active invasion of host cells by T. gondii (Dupont et al., 2014), and the other is the proteasome-dependent processing of intracellular antigens when the host ER and PVs are connected in unstimulated DCs (Goldszmid et al., 2009) or by the autophagy machinery-associated disruption of bacteria-containing vacuoles (Fiegl et al., 2013). Therefore, although further studies are required to clarify how p62, Atg3, and Irgm1/m3 in IFN-γ-stimulated APCs regulate the increased activation of vacuolar-antigen-specific CD8^{+} T cells and the molecular mechanism by which p62 participates in processing vacuolar proteins in APCs, our data suggest that p62, Atg3, and Irgm1/m3 are associated with the MHCI antigen-presenting pathways directed against parasite infection through the IFN-γ-dependent disruption of T. gondii PVs.

Our results support the modest role of p62 in the activation of vacuolar-antigen-specific CD8^{+} T cells during T. gondii infection in vivo. The numbers of peritoneal OVA-tetramer^{+} CD8^{+} T cells or transferred OT-I T cells were reduced in the p62-deficient mice infected with T. gondii p30-OVA. This is consistent with our in vitro co-culture assay using OT-I T cells and APCs, such as MEFs or DCs. However, there was no difference in the numbers of splenic OVA-tetramer^{+} CD8^{+} T cells in WT and p62-deficient mice. In addition to CD8^{+} T cells and DCs or fibroblasts, the spleens include various immune cells that can generate a variety of cytokines, such as IL-2, IL-7, and IL-15. This might lead to clonal expansion of CD8^{+} T cells in an antigen-independent fashion (Ramanathan et al., 2009), probably masking the partial

Figure 4. Roles of p62 in APCs during *T. gondii* Infection

For (A)–(D), WT and p62 KO BMMs were treated with 10 ng/ml IFN-γ or not for 24 hr and infected with ME49 *T. gondii*-expressing luciferase (for clearance assay) or with Pru *T. gondii* (for immunofluorescence assay [IFA]) for 4 and 24 hr. For IFA, the cells were fixed and stained with antibodies against CD11b, GRA7 or GAP45, and DAPI. Then the numbers of non-infected or BMMs infected with parasites were counted. For (E)–(H), (K), and (L), WT and p62 KO BMDCs and WT, Atg3 KO, p62 KO, or Irgm1/m3 DKO MEFs were treated with 10 ng/ml IFN-γ or not for 24 hr, infected with γ-irradiated Pru *T. gondii*-expressing p30-OVA or OVA luciferase or parental strain for 7 hr, or treated with 0.1 nM OVA$_{257-264}$ peptide for 3 hr, and then co-cultured with OT-I T cells for 24 and 72 hr.
(A) Luciferase analysis for the parasite survival rate at 24 hr p.i. is shown.
(B) Quantification analysis for the infection rate of parasites at the indicated time points is shown.
(C and D) Quantification analyses for the parasite numbers per vacuole (C) and GBP1-5-, Irga6-, or Irgb6-positive vacuoles (D) are shown.
(E) IFN-γ-producing OT-I T cells by intracellular staining experiments after co-culture with WT BMDCs for 24 hr are quantified.
(F–L) IFN-γ production by OT-I T cells co-cultured with WT BMDCs (F), WT and Atg3 KO MEFs (G), WT and Irgm1/m3 DKO MEFs (H), WT and p62 KO BMDCs (K), or WT and p62 KO MEFs (L) for 72 hr. Representative confocal microscope images show the colocalization of p62 (I) or ubiquitin (J) with the OVA from Pru *T. gondii* p30-OVA in MEFs.
(M) The numbers of tetramer^{+} CD8^{+} T cells in peritoneal cavities of WT and p62 KO mice at 7 and 14 days after intraperitoneal infection with γ-irradiated Pru *T. gondii* p30-OVA are quantified.
(N and O) IL-12 production by BMDCs in vitro (N) and in the peritoneal cavities in vivo (O) from WT and p62 KO mice after infection with γ-irradiated Pru *T. gondii* p30-OVA for 24 hr or 14 days, respectively, is shown.
(P) IFN-γ production by purified CD8^{+} T cells from WT and p62 KO mice splenocytes, following in vitro stimulation with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) for 72 hr, is shown.
(Q) The numbers of adoptively transferred CD45.1[+] OT-I T cells in the peritoneal cavities of WT and p62 KO mice at 14 days after intraperitoneal infection with γ-irradiated Pru *T. gondii* p30-OVA. All results are the mean ± SE and are representative of two or three independent experiments. Scale bars, 5 μm. N.D., not detected; N.S., not significant; *p < 0.05, **p < 0.01, ***p < 0.001.

phenotype of the p62-deficient APCs observed in the peritoneum and eventually generating similar numbers of OVA-specific CD8^{+} T cells in the spleens of the WT and p62-deficient mice.

In conclusion, our results indicate that, during *T. gondii* infection, the IFN-γ-dependent recruitment of ubiquitin and p62 to the PV membranes in APCs is regulated by Atg proteins, such as Atg3, Atg5, Atg7, Atg16L1, and Irgm1/m3. Atg3 and Irgm1/m3 also have critical functions in the activation of vacuolar-antigen-specific CD8^{+} T cells, probably by mediating the disruption of PVs. This suggests that targeting ubiquitin and p62 to the PVs in infected APCs is involved in inducing an adaptive immune response, which provides the host with protective immunity against *T. gondii* infection in an IFN-γ-dependent fashion.

EXPERIMENTAL PROCEDURES

The detailed materials and methods for cells, mice, reagents, parasites, cell sorting, luciferase assay, western blot, ELISA, fluorescence-activated cell sorting (FACS) analysis, co-culture experiments, and the in vivo transfer experiments are described in the Supplemental Experimental Procedures.

Animals
Animal experiments were approved by the Animal Research Committee of the Research Institute for Microbial Diseases at Osaka University (Supplemental Experimental Procedures).

Generation of MEFs Derived from p62-, Atg3-, Atg5-, or Irgm1/m3-Deficient Mice by Cas9/CRISPR Genome Editing
The synthesized guided RNA (gRNA) and Cas9 mRNA were purified using MEGAclear kit (Life Technologies) and eluted in RNase-free water (Nacalai Tesque). To obtain *p62*-, *Atg3*-, and *Irgm1/m3*-deficient MEFs, C57BL/6 female mice (6 weeks old) were superovulated and mated to C57BL/6 stud males. Fertilized one-cell stage embryos were collected from oviducts and injected into the pronuclei or the cytoplasm with the Cas9 mRNA (100 ng/μl) and the gRNA (50 ng/μl). We transferred the injected live embryos into oviducts of pseudopregnant ICR females at 0.5 dpc to generate primary MEFs or live mice (Supplemental Experimental Procedures).

Immunofluorescence Assays
MEFs, bone marrow-derived DCs (BMDCs), or BMMs were cultured on glass coverslips, infected with *T. gondii* (MOI of 5 or 1) for the indicated time, and fixed in PBS containing 3.7% paraformaldehyde for 10 min at room temperature. Cells were permeabilized with PBS containing 0.1% Triton X-100 or 0.002% Digitonin (to stain GRA7) for 5 min and then blocked with 8% fetal bovine serum (FBS) in PBS for 1 hr at room temperature. Subsequently, cells were incubated with the indicated primary antibodies for 1 hr at 37°C, followed by incubation with Alexa 488-, Alexa 594-, or Alexa 647-conjugated secondary antibodies (Molecular Probes) and DAPI for 1 hr at 37°C in the dark. Finally, coverslips were mounted onto glass slides with PermaFluor (Thermo Scientific) and analyzed using confocal laser microscopy (FV1200 IX-83, Olympus). Images are shown at ×1,000 magnification (scale bars, 5 μm).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.09.005.

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