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Murine Irgm Paralogs Regulate Nonredundant Functions To Execute Host Defense to *Toxoplasma gondii*

Jacob Dockterman,[a] Brian E. Fee,[b,c] Gregory A. Taylor,[a,b,c,d] Jörn Coers[a,d]

[a]Department of Immunology, Duke University Medical Center, Durham, North Carolina, USA
[b]Geriatric Research, Education, and Clinical Center, VA Health Care Center, Durham, North Carolina, USA
[c]Departments of Medicine, Division of Geriatrics, and Center for the Study of Aging and Human Development, Duke University Medical Center, Durham, North Carolina, USA
[d]Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina, USA

ABSTRACT Gamma interferon (IFN-γ)-induced immunity-related GTPases (IRGs) confer cell-autonomous immunity to the intracellular protozoan pathogen *Toxoplasma gondii*. Effector IRGs are loaded onto the *Toxoplasma*-containing parasitophorous vacuole (PV), where they recruit ubiquitin ligases, ubiquitin-binding proteins, and IFN-γ-inducible guanylate-binding proteins (Gbps), prompting PV lysis and parasite destruction. Host cells lacking the regulatory IRGs Irgm1 and Irgm3 fail to load effector IRGs, ubiquitin, and Gbps onto the PV and are consequently defective for cell-autonomous immunity to *Toxoplasma*. However, the role of the third regulatory IRG, Irgm2, in cell-autonomous immunity to *Toxoplasma* has remained unexplored. Here, we report that Irgm2 unexpectedly plays a limited role in the targeting of effector IRGs, ubiquitin, and Gbps to the *Toxoplasma* PV. Instead, Irgm2 is instrumental in the decoration of PVs with γ-aminobutyric acid receptor-associated protein-like 2 (GabarapL2). Cells lacking Irgm2 are as defective for cell-autonomous host defense to *Toxoplasma* as pan-*Irgm*$^{-/-}$ cells lacking all three Irgm proteins, and *Irgm2*$^{-/-}$ mice succumb to *Toxoplasma* infections as readily as pan-*Irgm*$^{-/-}$ mice. These findings demonstrate that, relative to Irgm1 and Irgm3, Irgm2 plays a distinct but critically important role in host resistance to *Toxoplasma*.

KEYWORDS GABARAP, GBPs, *Toxoplasma*, autophagy, cell-autonomous immunity, guanylate binding proteins, immunity-related GTPases, interferons, intracellular parasites, ubiquitination

Toxoplasma gondii is an intracellular protozoan pathogen that infects its definitive feline host, as well as a variety of intermediate mammalian hosts, including mice and humans (1). Human *Toxoplasma* infections during pregnancy can cause miscarriage, stillbirth, or severe disease in newborns. In immunocompromised adults, *Toxoplasma* infections are associated with encephalitis and other disseminated systemic diseases (2–5). Like many intracellular bacterial pathogens, *Toxoplasma* forms a pathogen-containing or parasitophorous vacuole (PV) that shelters the parasite from cytosolic host defenses, ensures nutrient acquisition, and thus provides the parasite with an intracellular replicative niche (6–9). Many cell-autonomous defenses directed at the parasite therefore require recognition of the PV as foreign, allowing for the xenophagic destruction of PVs, as well as the direct lytic disruption of the PV membrane (PVM) (1, 10–12).

Many cell-autonomous immune programs targeting *Toxoplasma* are activated by the cytokine gamma interferon (IFN-γ), which induces the expression of hundreds of proteins that play various roles in defense against intracellular pathogens (13). Both human and murine cells rely on a host of IFN-γ-inducible GTPases for resistance to *Toxoplasma*. In mice, these GTPases include 11 guanylate-binding proteins (Gbps) (14) and the p47 immunity-

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Address correspondence to Jörn Coers, jorn.coers@duke.edu.

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related GTPases (IRGs), which themselves are divided into approximately 20 effector IRGs (such as Irga6, Irgb6, and Irgb10) and three clade M regulatory IRGs (Irgm1, Irgm2, and Irgm3) (15, 16). Decoration of the PV with effector IRGs (Irgb6 in particular) leads to ubiquitination of the PV in IFN-γ-treated murine cells, recruitment of autophagy-related protein sequestome-1 (p62) and Gbps, vesiculation of the PV, and killing of *Toxoplasma* (17–27). The targeting of effector IRGs to the PVM is regulated by Irgm proteins, which themselves reside on host membranes such as mitochondria, ER, the Golgi apparatus, and lipid droplets (26, 28–32). In the absence of Irgm1 and/or Irgm3, effector IRGs fail to target the PVM and instead form cytoplasmic aggregates (31, 33, 34). Consequently, PV ubiquitination and rupture, as well as IFN-γ-mediated restriction of *Toxoplasma* growth, are absent in Irgm1/Irgm3-deficient cells (24, 35), and mice lacking Irgm1 and/or Irgm3 are deficient for immunity to *Toxoplasma in vivo* (36–38). While organelle-resident Irgm proteins are able to transiently interact with effector IRGs, inhibit their GTP binding, and impede their stable association with organelles such as lipid droplets (31, 33), the mechanism by which Irgm proteins escort effector IRGs to the PVM is not well defined.

Critical insights into the role of Irgm proteins in controlling the subcellular localization of effector IRGs came from studies revealing that some components of the degradative autophagy pathway regulate the targeting of effector IRGs to the intracellular pathogens *Chlamydia* and *Toxoplasma* (39–44). In canonical autophagy, mammalian Atg8 homologs (comprising, in mice, two microtubule-associated protein light chain 3 family members—LC3a and LC3b—and three γ-aminobutyric acid receptor associated protein (GABARAP) family members (Gabarap, GabarapL1, and GabarapL2) are conjugated to the lipid phosphotidylethanolamine by an enzymatic cascade consisting of E1-like Atg3, E2-like Atg7, and the E3-like enzymatic complex Atg5/Atg12/Atg16L1 (45). In addition to their well-established role in the formation and maturation of autophagosomes, Atg3, Atg5, Atg7, and Atg16L1 were also found to facilitate the recruitment of effector IRGs to the *Toxoplasma* PVM, as well as IFN-γ-mediated restriction of *Toxoplasma* growth (39, 41, 43). In contrast to the Atg8 lipidation machinery, factors such as Atg14L and ULK1 known to regulate the initiation of canonical autophagy are dispensable for effector IRG targeting and associated host defense to *Toxoplasma* (39). Indeed, ectopic expression of the E3-like Atg5/12/16L1 complex on the host cell plasma membrane or mitochondria is sufficient to trigger effector IRG recruitment to those sites under certain conditions (46), underpinning the importance of Atg8 lipidation at the effector IRG target membrane. Furthermore, LC3 and GABARAP colocalize with *Toxoplasma* PVMs in both mouse and human cells, and deletion of GabarapL2 (also known as Gate16) results in effector IRG and Gbp mislocalization, as well as a defect in resistance to *Toxoplasma* (27, 39, 47–50). These findings suggest a model in which conjugated LC3 and/or GABARAP family members are incorporated into the PVM, serving as a flag marking PVs for the recruitment of effector IRGs and possibly other host defense mediators. However, this mechanism has not yet been fully demonstrated, and it remains unclear whether or not Irgm proteins play any role in Atg8 conjugation or the incorporation of lipidated Atg8 molecules into *Toxoplasma* PVMs. Nonetheless, recent observations that Irgm2 physically interacts with GabarapL2 to temper noncanonical inflammasome activation provide compelling evidence for the existence of direct functional relationships between Irgm and Atg8 proteins (51, 52).

The role for Irgm2 in host defense to *Toxoplasma* is not well defined. In order to address this gap in knowledge, we employed the IRG-susceptible type II Prugniaud A7 *Toxoplasma* strain and examined the role of Irgm2 in the targeting of its PV by host effector proteins in IFN-γ-treated primary mouse embryonic fibroblasts. We also tested cells and mice lacking Irgm2 for the ability to restrict growth of the pathogen. We compared these responses to those from cells and mice lacking all three *Irgm* paralogs (pan-*Irgm*$^{-/-}$ mice) that had heretofore not been examined in this context but represent a new model of complete Irgm deficiency. Our findings demonstrate that Irgm2 plays a unique, nonredundant role that is crucial for resistance to *Toxoplasma* infection both in cell culture and *in vivo*.

RESULTS

Effector IRGs mislocalize in Irgm-deficient cells. Effector IRGs such as Irga6, Irgb6, and Irgb10 localize to intracellular pathogens such as *Toxoplasma* and promote lysis of the PV and subsequent destruction of the pathogen (53). Because effector IRGs mislocalize and fail to target *Toxoplasma* in mouse embryonic fibroblasts (MEFs) deficient for Irgm1 and/or Irgm3 (31, 33, 38), we sought to assess the role of Irgm2 in effector IRG targeting to *Toxoplasma*. We primed wild-type, *Irgm2*$^{-/-}$, and pan-*Irgm*$^{-/-}$ (*Irgm1*$^{-/-}$ *Irgm2*$^{-/-}$ *Irgm3*$^{-/-}$) MEFs overnight with IFN-γ to induce IRG expression, infected them with *Toxoplasma*, and fixed cells 1 h postinfection (hpi) for immunofluorescent staining of Irga6, Irgb6, and Irgb10. We confirmed the previous findings that *Toxoplasma* PVs are robustly targeted by all three effector IRGs in wild-type MEFs (Fig. 1A to C). Similarly to *Irgm1*$^{-/-}$ *Irgm3*$^{-/-}$ MEFs (31), effector IRGs were notably absent from *Toxoplasma* PVs in pan-*Irgm*$^{-/-}$ MEFs, with Irga6 and Irgb6 forming large cytoplasmic aggregates (Fig. 1A to C). Although effector IRGs also formed cytoplasmic aggregates in *Irgm2*$^{-/-}$ MEFs, *Toxoplasma* PVs were nonetheless decorated with these effector IRGs in Irgm2-deficient cells, with Irgb6 targeting reduced from 60% to 34% and Irgb10 targeting reduced from 42% to 38% compared to that in the wild type (Fig. 1A to C). Therefore, the delivery of effector IRGs to *Toxoplasma* PVs is decreased but remains largely intact in *Irgm2*$^{-/-}$ MEFs, and it is fully disrupted in pan-*Irgm*$^{-/-}$ MEFs.

Irgm-deficient cells lack ubiquitin-dependent recruitment of Gbp2 to *Toxoplasma* PVs. We and others have previously shown that the recruitment of effector IRGs to *Toxoplasma* and *Chlamydia* PVMs promotes the recruitment of additional host factors, such as the ubiquitin ligases TRIM21, TRAF3, and TRAF6; ubiquitin itself; and cytosolic complexes containing Gbp2 and p62 (24, 25, 27, 35, 54, 55). Gbp2 and p62 both play roles in IFN-γ-mediated restriction of *Toxoplasma* growth (24, 56–58), and p62 also plays a role in the presentation of *Toxoplasma* antigens (35). We sought to determine if the presence or absence of effector IRGs on the *Toxoplasma* PVM in *Irgm2*$^{-/-}$ and pan-*Irgm*$^{-/-}$ MEFs corresponds with recruitment of ubiquitin, Gbp2, or p62. Indeed, pan-*Irgm*$^{-/-}$ MEFs demonstrated a complete lack of PV ubiquitination as well as a complete lack of targeting of Gbp2 and p62 to *Toxoplasma* PVs (Fig. 2A to C). Conversely, *Irgm2*$^{-/-}$ MEFs retained wild-type levels of p62 targeting to PVs (Fig. 2C), although ubiquitination was reduced from 47% to 23% and Gbp2 targeting was reduced from 39% to 24% (Fig. 2A and B). These findings illustrate a significant but incomplete perturbation of the assembly of immune effectors on the *Toxoplasma* PVM in the absence of Irgm2, and a complete failure to target the PV with host effectors in the absence of all Irgm proteins. Additionally, these findings are consistent with previous observations that effector IRG recruitment to the PVM promotes ubiquitination and subsequent Gbp2/p62 recruitment (24, 25, 35).

The targeting of Atg8 family members to the *Toxoplasma* PVM predicts recruitment of host factors and host resistance. Recent studies have demonstrated that the LC3 and GABARAP subfamilies of Atg8 homologs localize to *Toxoplasma* PVs and play roles in recruitment of IRGs and Gbps to PVs as well as IFN-γ-mediated restriction of *Toxoplasma* growth (46, 47). Notably, GabarapL2-deficient cells demonstrated Gbp mislocalization reminiscent of observations made in Irgm-deficient cells (47), and GabarapL2 has recently been shown to cooperate with Irgm2 to regulate inflammasome activation in infection with intracellular bacteria (51, 52). We therefore sought to determine if the absence of Irgm proteins affected the targeting of Atg8 homologs to the *Toxoplasma* PVM. Using a primary antibody reactive to both LC3a and LC3b, we found that pan-*Irgm*$^{-/-}$ MEFs were severely defective for LC3 targeting to *Toxoplasma* PVs (Fig. 3A and B). While *Irgm2*$^{-/-}$ MEFs retained LC3 targeting, we observed large cytoplasmic LC3 aggregates not found in wild-type cells. Irgm1, Irgm3, or a combination of any of the three Irgm proteins is therefore necessary for LC3 targeting, while Irgm2 alone plays a negligible role in this process.

We also examined the recruitment of GABARAP subfamily members to *Toxoplasma* PVs using a primary antibody reactive with Gabarap, GabarapL1, and GabarapL2. Small GABARAP puncta associated with a large proportion of PVs in wild-type, *Irgm2*$^{-/-}$, and

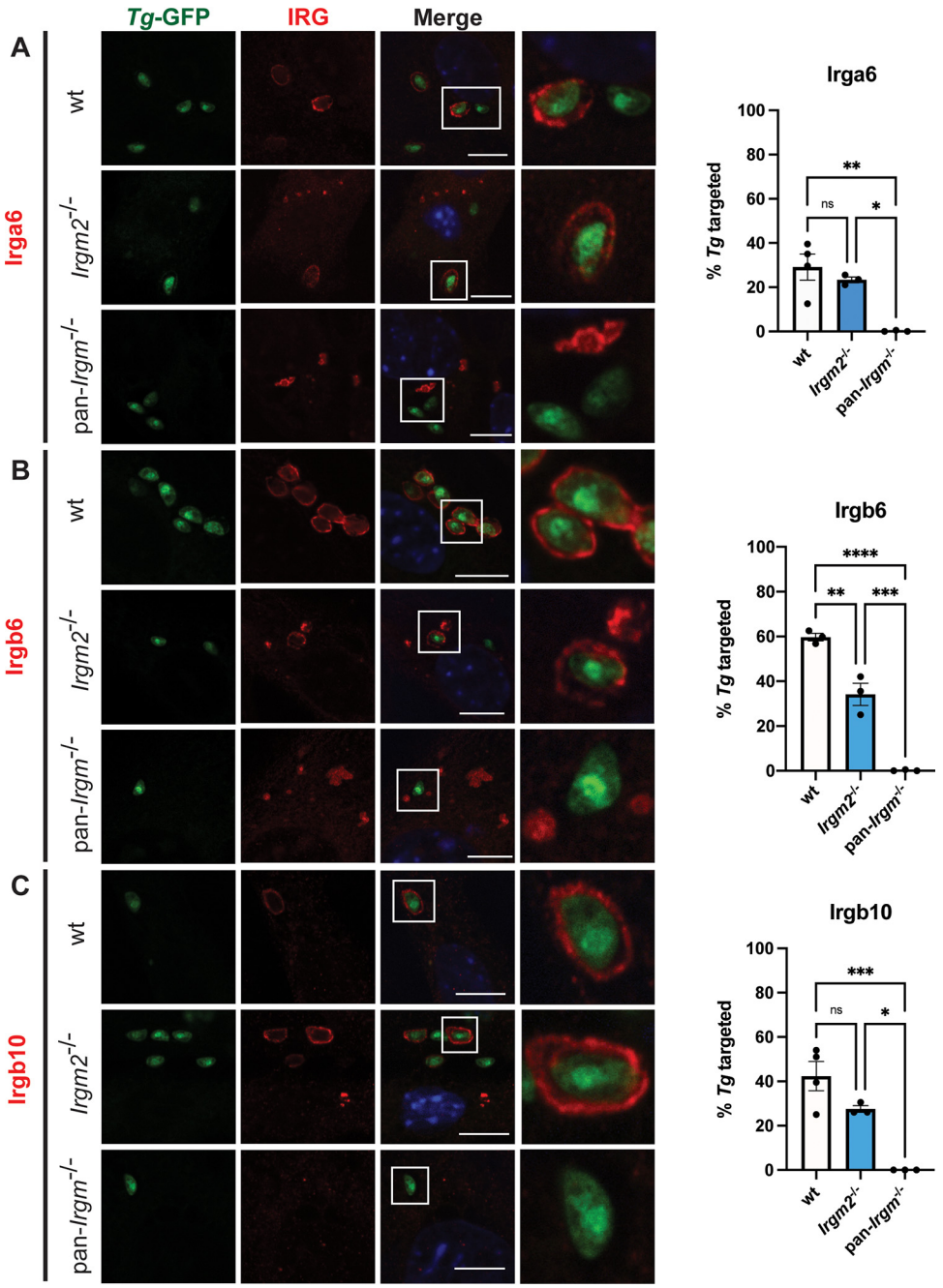

FIG 1 Lack of all Irgm paralogs, but not deletion of Irgm2 alone, eliminates effector immunity-related GTPase (IRG) recruitment to *Toxoplasma* parasitophorous vacuoles (PVs) in mouse embryonic fibroblasts (MEFs). Wild-type, *Irgm2*$^{-/-}$, and pan-*Irgm*$^{-/-}$ MEFs were primed with gamma interferon (IFN-γ) overnight and infected with green fluorescent protein-*Toxoplasma gondi* (*Tg*-GFP) for 1 h prior to fixation and immunofluorescent staining for Irga6 (A), Irgb6 (B), or Irgb10 (C) (red). The rightmost column of images shows magnified views of the areas in white boxes. Bars, 10 μm. Images are representative of at least three independent experiments. Graphs represent the proportion of total *Toxoplasma* PVs colocalized with the indicated effector IRG as the mean of at least three independent experiments plus or minus standard error of the mean (SEM). Statistical significance was determined using one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$; n.s., not significant.

pan-*Irgm*$^{-/-}$ MEFs (Fig. 4A and B). In contrast, a smaller proportion of PVs was heavily targeted by GABARAP in wild-type MEFs, but few to no PVs were heavily targeted in *Irgm2*$^{-/-}$ or pan-*Irgm*$^{-/-}$ MEFs (Fig. 4A and C). Quantitative image analysis confirmed these phenotypes of punctate and heavy GABARAP targeting in wild-type, *Irgm2*$^{-/-}$, and pan-*Irgm*$^{-/-}$ MEFs (see Fig. S3 in the supplemental material). Therefore, while

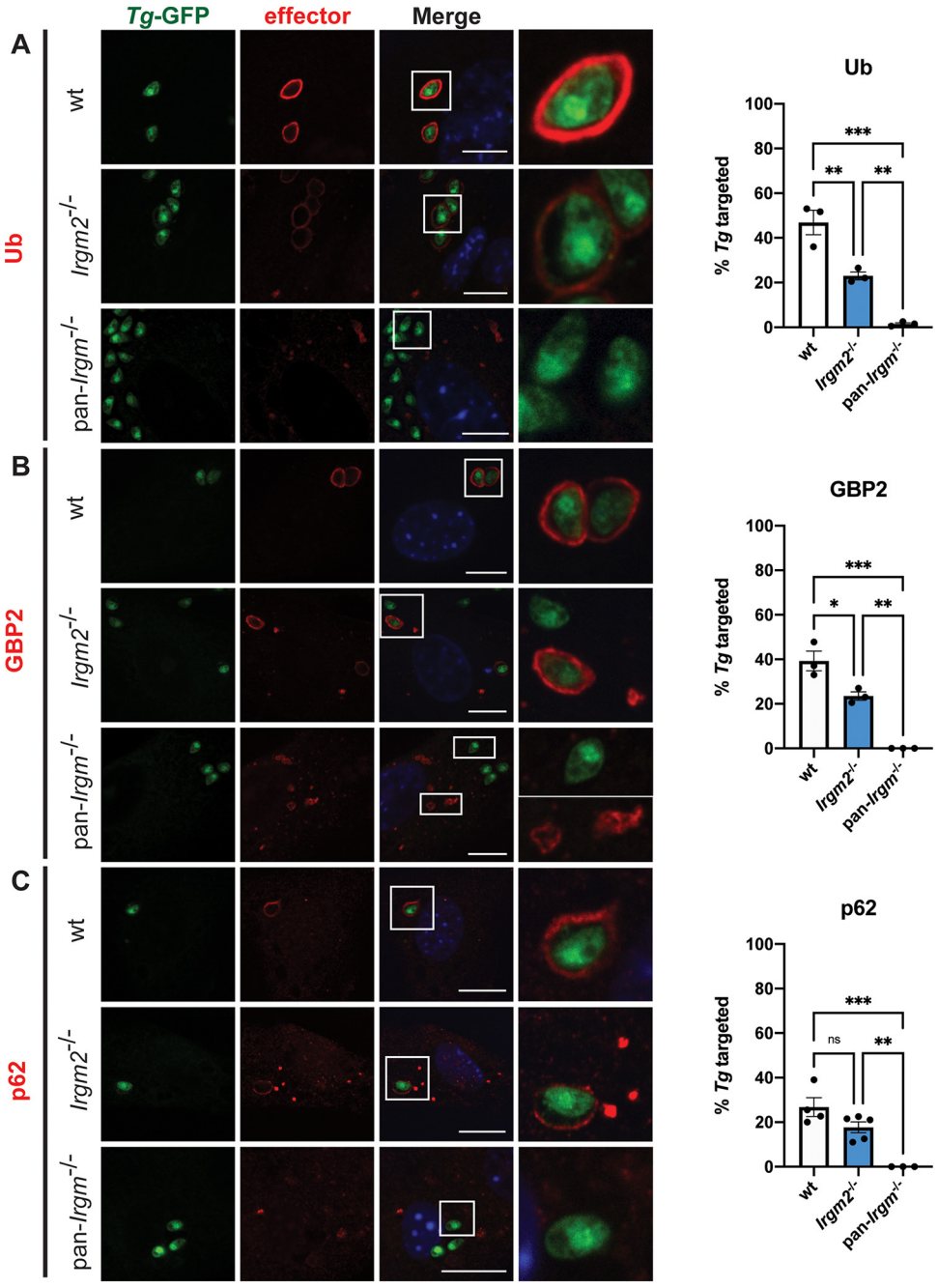

FIG 2 Lack of all Irgm paralogs, but not deletion of Irgm2 alone, eliminates recruitment of ubiquitin, Gbp2, and p62 to *Toxoplasma* PVs in Irgm-deficient MEFs. Wild-type, *Irgm2*$^{-/-}$, and pan-*Irgm*$^{-/-}$ MEFs were primed with IFN-γ overnight and infected with GFP-*Toxoplasma* for 1 h prior to fixation and immunofluorescent staining for ubiquitin (A), Gbp2 (B), or p62 (C) (red). The rightmost column of images shows magnified views of the areas in white boxes. Bars, 10 μm. Images are representative of at least three independent experiments. Graphs represent the proportion of total *Toxoplasma* PVs colocalized with the indicated host effector protein as the mean of at least three independent experiments plus or minus SEM. Statistical significance was determined using one-way ANOVA with Tukey's multiple-comparison test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; n.s., not significant.

Irgm2-deficient MEFs retained substantial targeting of many other effectors to *Toxoplasma* PVs, they demonstrated a severe defect in heavy GABARAP targeting, with GABARAP instead forming cytoplasmic aggregates.

While effector IRGs, Gbp2, ubiquitin, p62 and LC3 display a ring-like targeting pattern seemingly decorating the entirety of the PVM (Fig. 1 and 3), the GABARAP

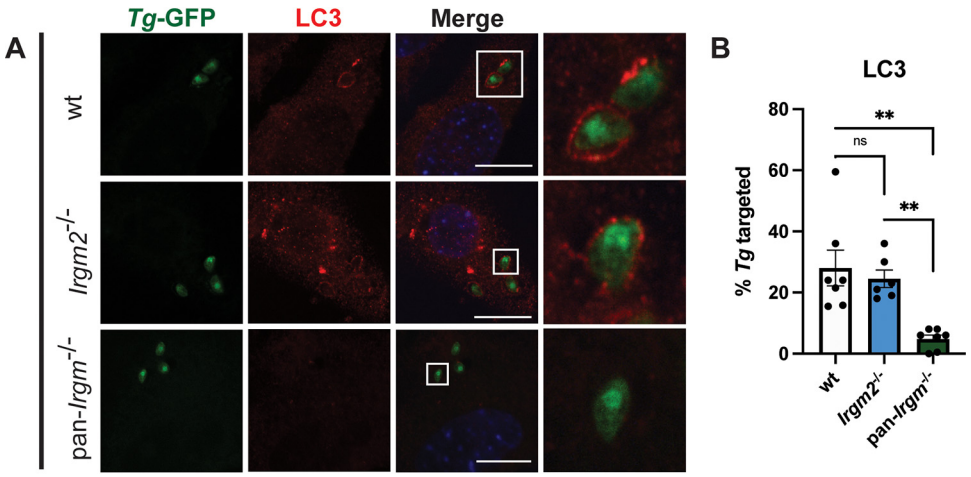

FIG 3 LC3 decoration of the *Toxoplasma* PV is reduced in pan-*Irgm*$^{-/-}$ but not *Irgm2*$^{-/-}$ MEFs. Wild-type, *Irgm2*$^{-/-}$, and pan-*Irgm*$^{-/-}$ MEFs were primed with IFN-γ overnight and infected with GFP-*Toxoplasma* for 1 h prior to fixation and immunofluorescent staining for LC3 (red) (A). The rightmost column of images shows magnified views of the areas in white boxes. Bars, 10 μm. Images are representative of at least three independent experiments. Graphs represent the proportion of total *Toxoplasma* PVs colocalized with LC3 as the mean of at least three independent experiments plus or minus SEM (B). Statistical significance was determined using one-way ANOVA with Tukey's multiple-comparison test. **, $P < 0.005$; n.s., not significant.

targeting pattern was distinct, consisting of linear staining along portions of the PVM, association of large GABARAP aggregates with the PVM, or encircling of the PVM with GABARAP-positive lamellar structures (Fig. 4A). We further observed that PVs that were heavily targeted by GABARAP in wild-type MEFs were invariably ubiquitinated but not always targeted by Irga6 (Fig. 4E; see also Fig. S1A and B in the supplemental material), suggesting that PV ubiquitination is a prerequisite for GABARAP targeting. Confirming previous reports (17, 33), we observed that Irgm2 was unique among the clade M IRGs in its ability to target the *Toxoplasma* PV (see Fig. S2 in the supplemental material), hinting at its potentially distinctive functional role in controlling the terminal steps of PV destruction. Collectively, these findings implicate Irgm2 specifically in the recruitment of GABARAP subfamily members to the *Toxoplasma* PV in a manner dependent on ubiquitin.

***Irgm2*$^{-/-}$ and pan-*Irgm*$^{-/-}$ mice and cells are severely compromised for host defense to *Toxoplasma*.** It has been shown that mice deficient for Irgm1 and/or Irgm3 are defective for host defense against many intracellular pathogens, including *Toxoplasma* (18, 30, 36–38). We hypothesized that the absence of effector IRGs and their ubiquitin-dependent downstream effectors on the *Toxoplasma* PV in pan-*Irgm*$^{-/-}$ MEFs would result in a failure of these cells to restrict *Toxoplasma* growth in an IFN-γ-dependent manner. Unsurprisingly, IFN-γ priming of pan-*Irgm*$^{-/-}$ MEFs conferred no restriction of *Toxoplasma* plaque formation (Fig. 5A), consistent with the notion that the targeting of host effector proteins to the *Toxoplasma* PVM is required for lysis of the PV and subsequent destruction of the parasite. GabarapL2 has also been shown to promote host restriction of *Toxoplasma* growth (47), and while *Toxoplasma* PVs in *Irgm2*$^{-/-}$ MEFs were decorated with effector IRGs, Gbps, ubiquitin, and p62, there was no heavy targeting of GABARAP in these Irgm2-deficient cells. We therefore asked whether the absence of heavy GABARAP targeting in Irgm2-deficient cells would correlate with a defect in killing of *Toxoplasma*. In support of this hypothesis, *Irgm2*$^{-/-}$ MEFs lacked the ability to restrict *Toxoplasma* growth upon IFN-γ priming (Fig. 5A). These findings demonstrate that Irgm2 is required for cell-autonomous immunity to *Toxoplasma*.

We next sought to determine if the *in vitro* phenotypes observed in *Irgm2*$^{-/-}$ and pan-*Irgm*$^{-/-}$ MEFs were relevant in an *in vivo* infection model. We performed intraperitoneal infection of wild-type, *Irgm2*$^{-/-}$, and pan-*Irgm*$^{-/-}$ mice with *Toxoplasma*

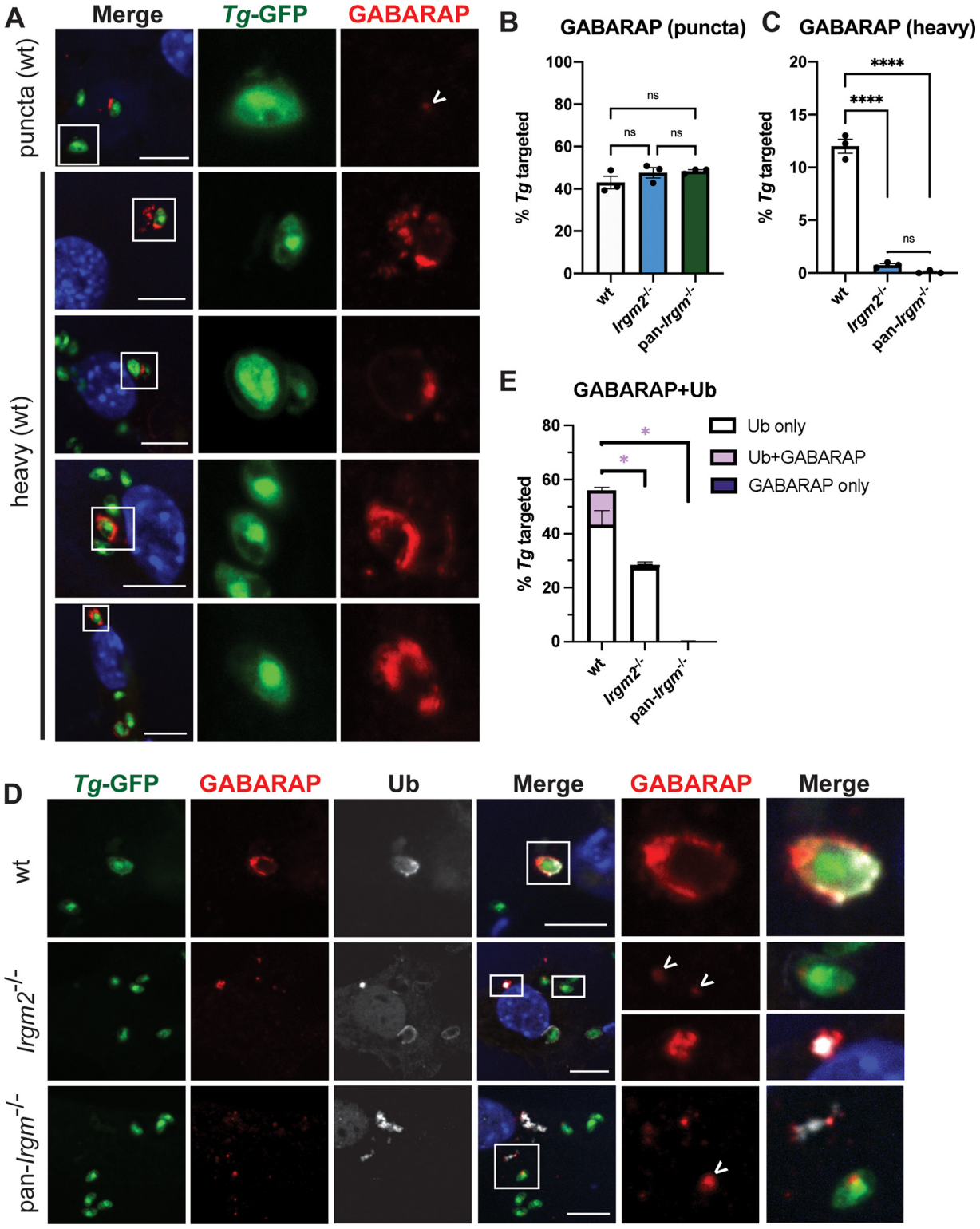

FIG 4 Heavy GABARAP targeting of *Toxoplasma* PVs requires Irgm2. Wild-type, *Irgm2*$^{-/-}$, and pan-*Irgm*$^{-/-}$ MEFs were primed with IFN-γ overnight and infected with GFP-*Toxoplasma* for 1 h prior to fixation and immunofluorescent staining for GABARAP (red) (A) or for GABARAP (red) and ubiquitin (white) (B). White arrowheads represent examples of GABARAP puncta associated with *Toxoplasma*. Several representative examples of GABARAP heavy targeting are shown (A). The rightmost column of images shows magnified views of the areas in white boxes. Bars, 10 μm. Images are representative of at least three independent experiments. Graphs represent the proportion of total *Toxoplasma* PVs associated with GABARAP puncta only (C); heavily targeted by GABARAP (D); or targeted by ubiquitin only (white), by GABARAP only (dark purple), or by both ubiquitin and GABARAP (lilac) (E), and show the mean of at least three independent experiments plus or minus SEM. Statistical significance was determined using one-way ANOVA with Tukey's multiple-comparison test (C, D) or two-way ANOVA (E). *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$; n.s., not significant.

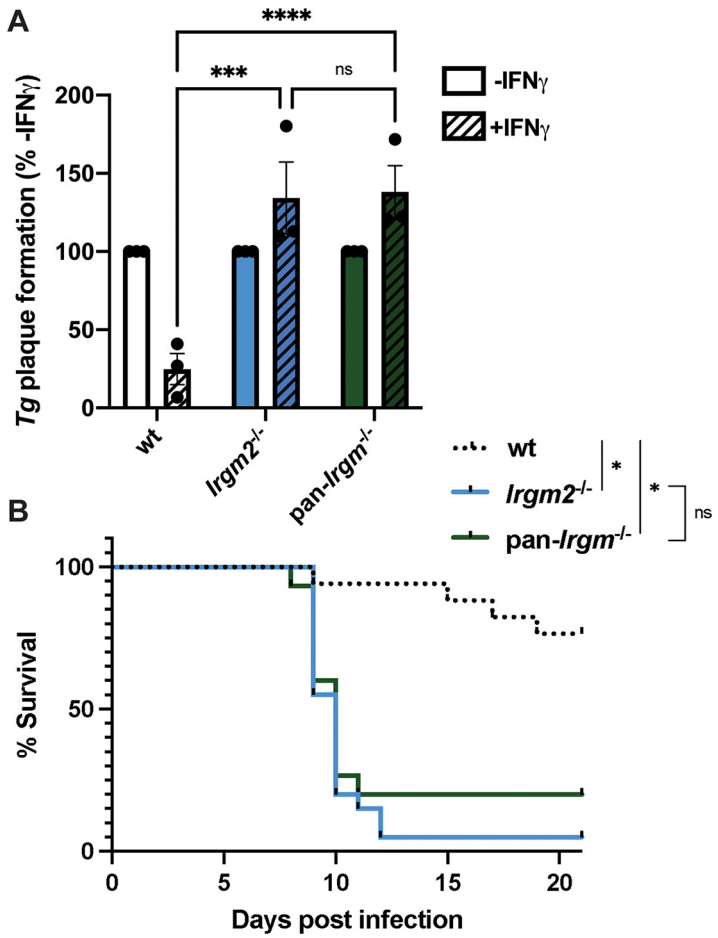

FIG 5 *Irgm2*$^{-/-}$ and pan-*Irgm*$^{-/-}$ mice are defective for immunity to *Toxoplasma in vitro* and *in vivo*. Wild-type, *Irgm2*$^{-/-}$, and pan-*Irgm*$^{-/-}$ MEFs were primed with IFN-γ overnight and infected with GFP-*Toxoplasma* for 72 h prior to quantification of plaque formation. Graphs represent plaque numbers in IFN-γ-primed cells relative to those in unprimed cells of the same genotype (mean ± SEM for at least three independent experiments). Statistical significance was determined using one-way ANOVA with Tukey's multiple-comparison test, comparing normalized plaque formation percentages of IFN-γ-treated samples. (A). Wild-type, *Irgm2*$^{-/-}$, and pan-*Irgm*$^{-/-}$ mice were infected intraperitoneally with *Toxoplasma*, and survival was monitored over 21 days. Wild-type $n = 17$; *Irgm2*$^{-/-}$ $n = 20$; pan-*Irgm*$^{-/-}$ $n = 15$. Graph represents combined data from two independent experiments. Statistical significance was determined using the log-rank test (B). *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$; n.s., not significant.

tachyzoites and monitored mice for survival over 21 days. As expected, most of the wild-type mice survived the infection, while the vast majority of pan-*Irgm*$^{-/-}$ mice died by 12 days postinfection (dpi) (Fig. 5B). Remarkably, *Irgm2*$^{-/-}$ mice succumbed to the infection at a rate comparable to that of pan-*Irgm*$^{-/-}$ mice. Mice deficient for Irgm1 have demonstrated generalized lymphomyeloid collapse upon infection with intracellular pathogens such as *Mycobacterium avium* (59, 60), but we found robust numbers of CD4^{+} T cells in the peritoneal exudates and spleens of *Toxoplasma*-infected *Irgm2*$^{-/-}$ and pan-*Irgm*$^{-/-}$ mice (see Fig. S4 in the supplemental material), indicating that these mice do not succumb to *Toxoplasma* infection due to lymphopenia. These findings suggest that the observed cell-autonomous immunity defect in *Irgm2*$^{-/-}$ cells corresponds with a similar defect in immunity *in vivo*.

DISCUSSION

While Irgm1 and Irgm3 are known to be crucial in the assembly of host effector proteins on the *Toxoplasma* PVM and subsequent PV lysis, the role for Irgm2 in this

context had not been previously characterized. Using *Irgm2*$^{-/-}$ cells and mice, we show that although Irgm2 is not absolutely required for the recruitment of Irga6, Irgb6, Irgb10, Gbp2, ubiquitin, p62, or LC3 to the PVM, it is required for IFN-γ-mediated clearance of *Toxoplasma* infection. This suggests a critical role for Irgm2 in host defense outside of the recruitment of these important effectors. We show that this role may well involve GABARAP. Prior work has demonstrated that mice deficient for GabarapL2 display defective IFN-γ-induced killing of *Toxoplasma* (47) and that Irgm2 physically interacts with GabarapL2 (51). Additionally, a recent study reported after the initial submission of this study corroborated our finding that Irgm2 promotes cell-autonomous immunity to *T. gondii in vitro* and *in vivo*, although this study did not examine the role of GABARAP (61). Thus, our finding that Irgm2 deficiency decreases heavy GABARAP targeting to *Toxoplasma* PVs allows us to postulate that Irgm2 mediates host defense via recruitment of GABARAP to the PVM. It remains possible that Irgm2 is also involved in the recruitment of other effectors, such as specific Gbps or IRGs, that remain to be identified.

The role of GABARAP in cell-autonomous immunity is currently unclear. Deficiency in the Atg8 lipidation machinery or GabarapL2 itself results in mislocalization of effector IRGs and Gbps and reduced PV ubiquitination (39, 47), suggesting that Atg8 lipidation acts upstream of recruitment of effector IRGs. It is possible that direct insertion of LC3 or GABARAP family proteins into the PVM acts as a signal soliciting the recruitment of effector IRGs, initiating the cell-autonomous immune pathway. However, our data suggest a different model, in which heavy GABARAP targeting occurs downstream of PV ubiquitination and represents a terminal step in destruction of the PV. Ubiquitination of the PV and recruitment of ubiquitin-binding proteins such as p62 have been shown to be crucial for cell-autonomous immunity to *Toxoplasma* (24, 27, 55). p62 can act as an adaptor protein targeting ubiquitinated substrates to Atg8-positive autophagosomes for selective autophagy (62, 63). p62 also directs intracellular bacteria toward autophagosomal degradation (64, 65). We found that heavy GABARAP targeting occurs exclusively on ubiquitinated *Toxoplasma* PVs. Furthermore, only 12% of PVs were heavily targeted by GABARAP in wild-type cells, compared to 47% of PVs targeted by ubiquitin. These findings support a model in which PV ubiquitination drives heavy GABARAP targeting, possibly via ubiquitin-binding proteins, which are demonstrably important for cell-autonomous immunity to *Toxoplasma* (49, 50).

In *Irgm2*$^{-/-}$ cells, PVs are ubiquitinated and targeted by p62 but are neither heavily targeted by GABARAP nor destroyed. This key finding has three major implications. First, the recruitment of Irga6, Irgb6, Irgb10, ubiquitin, Gbp2, and p62 is not sufficient for PV lysis. Although these factors play important roles in host-directed vacuole processing, one or more Irgm2-dependent additional steps are necessary. Second, ubiquitin-dependent heavy GABARAP targeting is mediated by Irgm2. Third, GABARAP recruitment may represent a required and terminal step in PV lysis. For example, GABARAP proteins may play a direct role in vesiculation of the PVM, or GABARAP may mediate the delivery of unidentified host factors that directly rupture the PVM. Taking this into consideration, the defect in cell-autonomous immunity to *Toxoplasma* in *Irgm1*$^{-/-}$ *Irgm3*$^{-/-}$ cells, which display a failure of PV ubiquitination and p62 targeting (24, 35), may derive from a failure of ubiquitin-dependent GABARAP recruitment.

Our studies provide increased clarity regarding the respective roles of host effector proteins in the IFN-γ-elicited destruction of *Toxoplasma*. We found that Irgm2 is required for host defense but performs a function distinct from those of Irgm1 and Irgm3. We propose a model in which Irgm1 and Irgm3 regulate the targeting of effector IRGs to PVs, which leads to ubiquitination and recruitment of p62 and Gbps. We have shown that these steps are not sufficient for PV lysis, and we propose that Irgm2 mediates the subsequent ubiquitin-dependent recruitment of GABARAP, marking the PV for destruction. Future studies should be directed at elucidating the mechanism by which Irgm2 and GABARAP mediate host defense to *Toxoplasma* and whether these mechanisms are important in defense against other intracellular pathogens.

MATERIALS AND METHODS

Mouse embryonic fibroblast and *Toxoplasma* culture and infections. Primary MEFs were derived from wild-type C57BL6/J, *Irgm2*$^{-/-}$, and pan-*Irgm*$^{-/-}$ mice as described previously (52). MEFs and African green monkey kidney Vero cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100U/ml), and streptomycin (100 μg/ml). *Toxoplasma gondii* type II A7 Prugniaud strain tachyzoites expressing green fluorescent protein (GFP) (66) were propagated in Vero cells as described previously (40). For *Toxoplasma* infections, MEFs were incubated overnight with or without 100 U/ml IFN-γ prior to infection with tachyzoites for 1 h prior to fixation (for immunofluorescent staining) or for 72 h prior to plaque quantification (for burden assay).

Immunofluorescent staining. Primary MEFs were plated in 24-well plates on coverslips and incubated with 100 U/ml IFN-γ overnight prior to infection with *Toxoplasma* tachyzoites at a multiplicity of infection of 3:1 to 5:1. At 1 h postinfection, cells were washed three times with phosphate-buffered saline (PBS; pH 7.4) prior to fixation in 0.025% glutaraldehyde (vol/vol) and 3% formaldehyde (vol/vol) at room temperature. Samples stained for GABARAP were fixed in glutaraldehyde-formaldehyde for 15 min, washed three times with PBS, and then additionally fixed in ice-cold 100% methanol for 1 min. All other samples were simply fixed in glutaraldehyde-formaldehyde for 20 min. After fixation, coverslips were washed three times in PBS and then blocked for 30 min at room temperature with 2.5% (wt/vol) bovine serum albumin (BSA) in PBS. Cells were stained with the indicated primary antibodies, followed by Alexa Fluor-conjugated secondary antibodies (Molecular Probes/Invitrogen) as well as Hoechst 33258 for staining of nucleic acids. For cells fixed in glutaraldehyde-formaldehyde only, 0.05% saponin (wt/vol) was included with BSA for permeabilization. Stained cells were washed with PBS and then mounted on slides with 0.12 g/ml Mowiol (Sigma) and 0.01% (wt/vol) paraphenylenediamine (Sigma). Samples were imaged using an Axio Observer Z1 780 inverted confocal fluorescence microscope (Zeiss). Colocalization of *Toxoplasma* PVs with proteins was quantified in at least three independent experiments using MEFs derived from separate embryos. Graphs represent pooled data from all experiments. In each experiment, at least 100 PVs were randomly selected in a blind fashion for each of two technical replicates per genotype. The fraction of targeted PVs was determined for each technical replicate by dividing the number of targeted PVs by the total number of PVs.

For quantitative image analysis, images obtained as described above were analyzed using ImageJ software (see Fig. S3 in the supplemental material). Briefly, *Toxoplasma* tachyzoites were identified using the DNA channel and confirmed using the GFP channel. Extracellular parasites, parasites outside the focal plane, and parasites in regions of high background staining were excluded. The GABARAP channel was thresholded to limit background staining, and then the integrated density of GABARAP staining within the immediate vicinity of *Toxoplasma* parasites was calculated. We used an arbitrary cutoff to distinguish between punctate and heavy targeting that matched obvious examples of the two staining patterns.

Antibodies. The primary antibodies used for immunofluorescent staining include anti-Irga6 mouse monoclonal antibody 10D7 at 1:200 (26); anti-Irgb6 rabbit polyclonal antiserum at 1:1,000 (34); anti-Irgb10 rabbit polyclonal antiserum at 1:500 (67); anti-Gbp2 rabbit polyclonal antiserum at 1:500 (31); rabbit anti-p62/SQSTM1 (MBL International) at 1:500; rabbit anti-LC3 (MBL international) at 1:500; mouse anti-ubiquitin FK2 at 1:50 or UBCJ2 at 1:500 (Enzo); rabbit anti-Gabarap/GabarapL1/GabarapL2 (Abcam) at 1:500; and rabbit anti-Irgm2 polyclonal antiserum. We generated a custom rabbit anti-Irgm2 serum antibody against amino acids 2 to 17 of the Irgm2 protein sequence (peptide sequence EEAVESPEVKEFEYFS; Thermo Fisher).

Quantification of *Toxoplasma* replication. MEFs were plated in a 96-well plate and incubated overnight in the presence or absence of 100 U/ml IFN-γ prior to infection with tachyzoites at a multiplicity of infection of 0.05:1. At 72 h postinfection, 40 fields of view per condition were imaged using an Axio Observer Z1 inverted phase contrast fluorescence microscope (Zeiss), and the number of GFP-positive (GFP$^+$) *Toxoplasma* plaques were counted in a blind fashion. The number of plaques in IFN-γ-primed cells was divided by the number of plaques in the corresponding unprimed cells. Graphs represent pooled data from three independent experiments conducted using MEFs derived from different embryos.

Murine *in vivo* infection. *Toxoplasma gondii* type II A7 Prugniaud strain tachyzoites expressing GFP were propagated in Hs27 human foreskin fibroblasts in DMEM supplemented with 10% FBS, penicillin (100U/ml), and streptomycin (100 μg/ml). Mice (8 to 12 weeks old) were infected intraperitoneally with 200 tachyzoites in 100 μl sterile PBS and monitored for 21 days. Kaplan Meier survival curves represent combined data from two independent experiments.

Peritoneal exudate was collected by lavage with 10 ml PBS, followed by centrifugation. Spleens were macerated and filtered through 40-μm cell strainers, and cells were collected by centrifugation. After Fc blocking using anti-CD16/CD32 (BioLegend), cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; live/dead), CD3-phycoerythrin (PE)/Cy7 (145-2C11; BioLegend), CD4-PE (GK1.5; Invitrogen), and CD45.2-allophycocyanin (APC) (104; BioLegend). CD4$^+$ T-cell populations were clearly identified as CD3$^+$ CD45$^+$ CD4$^+$ live cells, and numbers were quantified using CountBright absolute counting beads (Invitrogen). Samples were run on an FACSCanto II flow cytometer with FACSDiva software (BD Biosciences) and analyzed using FlowJo.

Statistical analysis. Prism 9 software (GraphPad) was used to perform statistical analysis. One-way analysis of variance (ANOVA) with Tukey's multiple-comparison test was used to determine the significance of differences among group means. In experiments examining GABARAP targeting alongside ubiquitin or Irga6 targeting, two-way ANOVA was used. The log-rank test was used for determining

statistical significance for *in vivo* survival experiments. All graphs represent mean plus or minus standard error of the mean (SEM) of at least three independent experiments.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 14.4 MB.

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