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Role of Mouse and Human Autophagy Proteins in IFN-γ–Induced Cell-Autonomous Responses against Toxoplasma gondii

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IFN-γ mediates cellular innate immunity against an intracellular parasite, Toxoplasma gondii, by inducing immunity-related GTPases such as p47 IFN-γ–regulated GTPases (IRGs) and p65 guanylate-binding proteins (GBPs), which also participate in antibacterial responses via autophagy. An essential autophagy protein, Atg5, was previously shown to play a critical role in anti-T. gondii cell-autonomous immunity. However, the involvement of other autophagy proteins remains unknown. In this study, we show that essential autophagy proteins differentially participate in anti-T. gondii cellular immunity by recruiting IFN-γ-inducible GTPases. IFN-γ–induced suppression of T. gondii proliferation and recruitment of an IRG Irgb6 and GBPs are profoundly impaired in Atg7- or Atg16L1-deficient cells. In contrast, cells lacking other essential autophagy proteins, Atg9a and Atg14, are capable of mediating the anti-T. gondii response and recruiting Irgb6 and GBPs to the parasites. Although IFN-γ also stimulates anti-T. gondii cellular immunity in humans, whether this response requires GBPs and human autophagy proteins remains to be seen. To analyze the role of human ATG16L1 and GBPs in IFN-γ–mediated anti-T. gondii responses, human cells lacking ATG16L1 or GBPs were generated by the Cas9/CRISPR genome-editing technique. Although both ATG16L1 and GBPs are dispensable for IFN-γ–induced inhibition of T. gondii proliferation in the human cells, human ATG16L1 is also required for the recruitment of GBPs. Taken together, human ATG16L1 and mouse autophagy components Atg7 and Atg16L1, but not Atg9a and Atg14, participate in the IFN-γ–induced recruitment of the immunity-related GTPases to the intracellular pathogen.


The host immune system produces inflammatory cytokines in response to infection of an intracellular protozoan pathogen Toxoplasma gondii (1). T. gondii is an obligatory intracellular protozoan parasite and the causative agent of toxoplasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3). Although T. gondii infection in healthy animals largely results in opportunistic and plasmosis in humans and animals (2, 3).
a series of autophagy proteins, among which Atg5 was recently linked to the IFN-γ-mediated parasitoidal effect against T. gondii (17). Myeloid-specific ablation of Atg5 in mice culminated in decreased recruitment of an IRG to T. gondii in IFN-γ-activated macrophages and led to high parasite susceptibility in vivo. Furthermore, other autophagy proteins, such as Atg7 and Atg16L1, were recently shown to play a nondegradable role in the IFN-γ-mediated antiviral programs against murine norovirus (18), prompting us to explore the functions of autophagy proteins other than Atg5 in the IFN-γ-mediated anti-T. gondii cellular innate immune responses.

In this study, we characterized the role of essential autophagy proteins, Atg7, Atg16L1, Atg9a, and Atg14 in IFN-γ-mediated inhibition of T. gondii proliferation using mouse embryonic fibroblasts (MEFs) lacking these proteins. MEFs lacking Atg7 or Atg16L1 exhibit defects in the reduction of T. gondii by IFN-γ treatment and recruitment of an IRG (Irgb6) and GBPs to the infected parasites. In sharp contrast, Atg9a- or Atg14-deficient cells show normal suppression of T. gondii growth and the accumulation of immunity-related GTPases at levels comparable to wild-type (WT) cells in response to IFN-γ stimulation, indicating differential participation of autophagy regulators in the mouse system. IFN-γ stimulation leads to strong inhibition of T. gondii proliferation in human cells (19). However, the contribution of human immunity-related GTPases and autophagy proteins to the inhibition remains unknown. To analyze the role of ATG16L1 and GBPs in human cells, we generated ATG16L1- or GBP-deficient human cells using Cas9/CRISPR-induced genome editing (20, 21). Although IFN-γ-induced inhibition of T. gondii proliferation is normal in human cells devoid of ATG16L1 or GBPs, IFN-γ-induced recruitment of GBPs to the parasites is defective in ATG16L1-deficient human cells. Thus, these results demonstrate that IFN-γ–mediated recruitment of IRGs and GBPs to T. gondii is differentially regulated by autophagy proteins in mice, and the mechanism for ATG16L1-dependent GBP recruitment to the parasite may be conserved in humans.

Materials and Methods
Cells, mice, and parasites

ME49 expressing luciferase of T. gondii was maintained in Vero cells by biweekly passage in RPMI 1640 (Nacalai Tesque) supplemented with 2% heat-inactivated FCS (JRH Biosciences) and 10% heat-inactivated FCS (JRH Biosciences) and 10 U/ml penicillin, 100 U/ml streptomycin (Nacalai Tesque) supplemented with 10% heat-inactivated FCS (JRH Biosciences), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Nacalai Tesque) supplemented with 10% heat-inactivated FCS (JRH Biosciences), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells lacking Atg7, Atg16L1, Atg9a, or Atg14 were described previously (22–25). Human MEFs (2.5 × 10^5) were left untreated or were treated with 10 ng/ml IFN-γ for 24 h. The cells were lysed in lysis buffer (Promega). Real-time PCR was performed with a CFX Connect qPCR Master mix (Bio-Rad/Lab). The Go-Taq qPCR Master mix (Promega) were normalized to the amount of GAPDH in each sample. The following primer sets were used: GBP1_qpF and GBP1_qpR for GBP1, GBP2_qpF and GBP2_qpR for GBP2, GBP3_qpF and GBP3_qpR for GBP3, GBP4_qpF and GBP4_qpR for GBP4, and GBP5_qpF and GBP5_qpR for GBP5. The primer sequences are listed in Supplemental Table I.

Quantitative real-time PCR

Total RNA was extracted, and cDNA was synthesized using MMLV RT (Promega). Real-time PCR was performed with a CFX Connect qPCR Master mix (Bio-Rad/Lab) using the Go-Taq qPCR Master mix (Promega). The primer sequences were normalized to the amount of GAPDH in each sample. The following primer sets were used: GBP1_qpF and GBP1_qpR for GBP1, GBP2_qpF and GBP2_qpR for GBP2, GBP3_qpF and GBP3_qpR for GBP3, GBP4_qpF and GBP4_qpR for GBP4, and GBP5_qpF and GBP5_qpR for GBP5. The primer sequences are listed in Supplemental Table I.

Statistical analysis

The unpaired Student t test was used to determine the statistical significance of the experimental data.

Results

Atg7 is required for IFN-γ–dependent recruitment of immunity-related GTPases to T. gondii

Cells lacking an autophagy protein, Atg5, are defective in IFN-γ–dependent host defense against T. gondii (17). A critical step in the regulation of autophagy is the modification of a ubiquitin-like
protein called “microtubule-associated L chain 3” (LC3). This modification is carried out by a protein complex nucleated by the conjugation of Atg12 to Atg5. Because Atg7 is an essential enzyme for this conjugation and formation of LC3 puncta in starvation-induced autophagy (27, 28) (Supplemental Fig. 1), we tested whether Atg7 is involved in the IFN-γ-dependent cellular host defense against T. gondii. MEFs from WT and Atg7-deficient mice were stimulated or not with IFN-γ for 24 h and infected with luciferase-expressing type II (ME49) or type I (RH) T. gondii, which are susceptible or resistant to IFN-γ–dependent IRG-induced killing activity, respectively. Twenty-four hours postinfection, parasite number was assessed by the luciferase counts emitted. Despite a significant reduction in the type II parasite load in Atg7-deficient MEFs after IFN-γ stimulation, the extent of the parasite reduction was markedly less than that in WT cells (Fig. 1A). In contrast, a difference in type I T. gondii numbers between WT and Atg7-deficient MEFs was not observed, even after IFN-γ stimulation (Fig. 1A). The recruitment of GBPs and IRGs, such as Irgb6, is a hallmark of IFN-γ–dependent clearance of T. gondii in IFN-γ–activated cells (6). Because Atg7-deficient cells expressed similar levels of Irgb6 and GBPs in response to IFN-γ (Fig. 1B), we next tested the recruitment of Irgb6 and GBPs to parasites in IFN-γ–stimulated cells by indirect immunofluorescence (Fig. 1C–F). In cells lacking Atg7, IFN-γ–dependent Irgb6 and GBP accumulation are significantly reduced relative to WT cells, indicating that Atg7 plays an important role in anti–T. gondii cellular host defense.

**Astg6L1 plays a critical role in IFN-γ–mediated suppression of T. gondii growth and accumulation of GTPases on the parasites**

In addition to Atg7, Atg16L1 participates in the modification of LC3 in autophagy and is included in a complex with Atg5 (27) (Supplemental Fig. 1). Therefore, we next assessed whether Atg16L1 is also implicated in the IFN-γ–dependent cellular immunity against type I or type II T. gondii. IFN-γ–stimulated Atg16L1-deficient MEFs decreased the type II T. gondii numbers to a significantly lesser degree than did WT cells (Fig. 2A). However, the extent of type I parasite reduction in IFN-γ–stimulated Atg16L1-deficient cells was comparable to that in WT cells (Fig. 2A). Although the expression levels of Irgb6 and GBPs were comparable between WT and Atg16L1-deficient cells (Fig. 2B), the recruitment of both proteins to parasites was significantly lower in Atg16L1-deficient cells (Fig. 2C–F). Thus, Atg16L1, as well as Atg7, is required for the IFN-γ–induced anti–T. gondii cellular host defense.

**Astg9a and Atg14 are dispensable for IFN-γ–mediated anti–T. gondii cellular responses**

Atg9a is another essential autophagy protein that plays an important role in the generation of autophagosome membranes. Indeed, starvation-induced formation of LC3 puncta was barely detected in Atg9a-deficient cells, as previously reported (24). We next analyzed whether Atg9a is involved in IFN-γ–dependent responses to T. gondii in MEFs. In sharp contrast to Atg7 and Atg16L1, Atg9a-deficient cells were competent in the IFN-γ–mediated accumulation of Irgb6 and GBPs on parasites (Fig. 3A–D). Consistent with the localization of GTPases, IFN-γ–mediated reduction of parasite numbers in Atg9a-deficient cells was similar to that in WT cells (Fig. 3E), indicating that Atg9a is dispensable for the IFN-γ–induced cellular host defense.

Atg14 is also a key player in orchestrating autophagy, participating in a PI3K complex that phosphorylates phosphatidylinositol (29). We used Atg14-deficient MEFs to assess the role of Atg14 in the IFN-γ–dependent recruitment of GTPases to parasites. Al-

**FIGURE 1. Atg7 is required for anti–T. gondii cellular immunity.** (A) WT and Atg7-deficient MEFs were untreated or treated with 10 ng/ml IFN-γ for 24 h. Untreated or IFN-γ–treated cells were infected with ME49 (left panel) or RH (right panel) T. gondii expressing luciferase ( moi = 0.5) and harvested at 24 h postinfection. The number of total parasites was monitored by luciferase activity using the lysates. Data are mean ± SD of triplicates. *p < 0.002. **p < 0.0007. (B) WT and Atg7-deficient MEFs, treated with 10 ng/ml IFN-γ, for 24 h were lysed. Lysates were detected by Western blot with the indicated Abs. WT and Atg7-deficient MEFs, treated with 10 ng/ml IFN-γ for 24 h, were infected with ME49 T. gondii ( moi = 2), fixed at 6 h postinfection, and incubated with rabbit anti–T. gondii (green), goat anti-Irgb6 [red; (C)], or mouse anti-GBP1-5 [red; (E)], and DAPI (blue). Scale bars, 5 μm. Percentage of parasites positive for Irgb6 (D) or GBP1-5 (F) staining at 6 h postinfection in IFN-γ–stimulated WT and Atg7-deficient MEFs. Data are mean ± SD of triplicates. *p < 0.001. Data are representative of two (B) or three (A, C, D, E, and F) independent experiments.
FIGURE 2. Atg16L1 plays a critical role in anti–T. gondii cellular immunity. (A) WT and Atg16L1-deficient MEFs were left untreated or treated with 10 ng/ml IFN-γ for 24 h. Untreated or IFN-γ–treated cells were infected with ME49 (left panel) or RH (right panel) T. gondii expressing luciferase (moi = 0.5) and harvested at 24 h postinfection. The number of total parasites was monitored by luciferase activity using the lysates. Data are mean ± SD of triplicates. *p < 0.003, **p < 0.002. (B) WT and Atg16L1-deficient MEFs, treated with 10 ng/ml IFN-γ for 24 h, were lysed. The lysates were detected by Western blot with the indicated Abs. WT and Atg16L1-deficient MEFs, treated with 10 ng/ml IFN-γ for 24 h, were infected with ME49 T. gondii (moi = 2), fixed at 6 h postinfection, and incubated with rabbit anti–T. gondii (green), goat anti-Irgb6 [red; (C)] or mouse anti-GBP1-5 [red; (D)], and DAPI (blue). Scale bars, 5 μm. Percentage of parasites positive for Irgb6 (D) or GBP1-5 (F) staining at 6 h postinfection in IFN-γ–stimulated WT and Atg16L1-deficient MEFs. Data are mean ± SD of triplicates. *p < 0.001, **p < 0.002. Data are representative of two (B) or three (A, C, D, E, and F) independent experiments.

though Atg14-deficient MEFs were defective in LC3 puncta formation in the serum-free starved condition (Supplemental Fig. 1), Irgb6 and GBPs were recruited to the parasites in IFN-γ–stimulated Atg14-deficient cells, as well as in WT cells (Fig. 4A–D). In addition, Atg14-deficient MEFs were capable of reducing parasite numbers in response to IFN-γ (Fig. 4E), suggesting that Atg14 function is nonessential for IFN-γ–mediated anti–T. gondii cellular defense. These results demonstrate that some autophagy-related proteins, but not all, are used in the IFN-γ–induced anti–T. gondii machinery.

Human ATG16L1 is essential for starvation-induced autophagy but not for IFN-γ–induced suppression of T. gondii growth in HAP1 cells

The data showing that the Atg5–Atg7–Atg16L1 axis plays a critical role in the IFN-γ–induced anti–T. gondii program in the mouse system prompted us to examine whether the axis is crucial for anti–T. gondii cellular immunity in the human system. To examine the role of ATG16L1 in human cells, we took advantage of the CRISPR/Cas9 system, which provides an efficient gene-targeting technique that facilitates multiplexed gene targeting (20, 21), and generated haploid human HAP1 cells devoid of ATG16L1 using Cas9 nuclease and single-guided RNAs targeting the open reading frame of the human ATG16L1 gene (Fig. 5A, Supplemental Fig. 2A). We succeeded in obtaining several clones of ATG16L1-deficient cells, in which deletion of the genomic region and proteins for ATG16L1 were analyzed by PCR and Western blotting, respectively (Fig. 5B, 5C). We first analyzed whether human ATG16L1 is required for autophagy induced by nutrient starvation, in which processing of LC3 and formation of the puncta are shown to be observed (30) (Fig. 5D, 5E). The nutrient starvation in ATG16L1-deficient cells completely failed to induce efficient LC3 conjugation to PE, a critical process for autophagosome formation (Fig. 5D) (30). Moreover, the starvation-induced formation of LC3 puncta was not observed in ATG16L1-deficient human cells (Fig. 5E), suggesting an essential role for human ATG16L1 in autophagy. Then, we tested whether human ATG16L1 is involved in the IFN-γ–induced anti–T. gondii response in human cells. IFN-γ suppressed T. gondii proliferation in human WT cells as efficiently as in mouse cells (Fig. 5F). Moreover, T. gondii proliferation was inhibited in IFN-γ–stimulated ATG16L1-deficient human cells in a dose-dependent fashion (Fig. 5F), suggesting that ATG16L1 in the human cell line is dispensable for the IFN-γ–mediated anti–T. gondii response.

Human GBPs are recruited to T. gondii in an ATG16L1-dependent fashion

Murine GBPs play a critical role in anti–T. gondii cell-autonomous immunity (10, 12, 13). In addition, murine GBPs were recruited to the parasites in an Atg7- and Atg16L1-dependent manner (Figs. 1A, 2A). Human GBPs were shown to play a role in controlling intracellular pathogens (31). Therefore, we first assessed whether endogenous human GBPs are recruited to T. gondii in an ATG16L1-dependent fashion (Fig. 5F), suggesting that ATG16L1 in the human cell line is dispensable for the IFN-γ–mediated anti–T. gondii response.
recruitment of GBPs to the parasites (Fig. 6C, 6D, Supplemental Fig. 3A–D), indicating the conserved important role of ATG16L1 in the recruitment of GTPases to T. gondii in humans and mice.

To elucidate the role of GBPs in anti-T. gondii cellular immunity directly, we generated HAP1 cells lacking all human GBPs using the CRISPR/Cas9 genome-editing technique (Fig. 7A, Supplemental Fig. 7B–D). These cells completely lacked mRNA and protein expression of GBPs, as confirmed by quantitative real-time PCR and Western blotting, respectively (Fig. 7B–D). Then, we challenged WT and GBP-deficient human cells with T. gondii expressing luciferase (moi = 0.5) and harvested at 24 h postinfection. The number of total parasites was monitored by the luciferase activity using the lysates. Data are mean ± SD of triplicates. (E) WT and Atg9a-deficient MEFs were left untreated or treated with 10 ng/ml IFN-γ for 24 h. Untreated or IFN-γ-treated cells were infected with ME49 T. gondii expressing luciferase (moi = 0.5) and harvested at 24 h postinfection. The number of total parasites was monitored by the luciferase activity using the lysates. Data are mean ± SD of triplicates. All data are representative of three independent experiments.

Discussion

In the current study, we investigated the role of autophagy proteins in IFN-γ–mediated anti-T. gondii cellular immunity using MEFs deficient in some of the essential components. Among them, Atg7 and Atg16L1, but not Atg9a and Atg14, are critically involved in the recruitment of immunity-related GTPases in the mouse system. In addition, we demonstrated that human ATG16L1 is also required for IFN-γ–mediated recruitment of human GBPs to the parasite.

Previous work showed that the IFN-γ–induced antiparasite response requires Atg5, which, together with Atg7 and Atg16L1, modifies LC3 in autophagy (17, 27). Furthermore, Atg7 and Atg16L1 are essential for the IFN-γ–induced nonautophagic antiviral response (18). Given that GBPs, another group of IFN-γ–inducible GTPases, critically control IFN-γ–mediated intracellular elimination of T. gondii in cooperation with Irgb6 in mice (12), Atg7 and Atg16L1, in addition to Atg5, may be integral to the recruitment of Irgb6 and GBPs to T. gondii for clearance. Conversely, we demonstrated that other essential autophagy proteins, including Atg9a and Atg14, are dispensable for the IFN-γ–dependent cellular response. In autophagy, Atg9a and Atg14 are critically involved in autophagosome formation (32–34). However, IFN-γ–mediated Atg5-dependent anti-T. gondii clearance is reported to be independent of autophagosome formation (17).
FIGURE 5. Human ATG16L1 is required for starvation-induced autophagy but not for IFN-γ–induced inhibition of T. gondii growth in HAP1 cells. (A) The gene-targeting strategy for human ATG16L1 locus by Cas9-mediated genome editing. (B) PCR detection of cells with deletion of human ATG16L1 locus. Primers used are denoted in (A). Also see Supplemental Fig. 2A for the targeting sequences to design gRNA1 and gRNA2, as well as assessment of the deletions by sequencing the PCR products detected in (B). (C) WT and ATG16L1-deficient HAP1 cells were lysed, and the lysates were detected by Western blot with the indicated Abs. (D) WT and ATG16L1-deficient HAP1 cells, with or without serum-starvation for 6 h in the absence or presence of 80 μM chloroquine for 6 h, were lysed. The lysates were detected by Western blot with the indicated Abs. (E) WT and ATG16L1-deficient HAP1 cells cultured in serum-free media (lower panels) to be starved for 6 h or in serum-containing media (upper panels) were fixed and incubated with rabbit anti-LC3 (green) and DAPI (blue). Arrows indicate puncta of LC3. Scale bar, 10 μm. (F) WT and ATG16L1-deficient HAP1 cells were left untreated or treated with the indicated concentrations of IFN-γ for 24 h. Untreated or IFN-γ–treated cells were infected with ME49 T. gondii expressing luciferase ( moi = 0.5) and harvested at 24 h postinfection. The number of total parasites was monitored by luciferase activity using the lysates. Data are mean ± SD of triplicates and are representative of three independent experiments.

Atg5 and Atg7, but not Atg9a and Atg14, are required for recruitment of LC3 in autophagy against bacteria, whereas they are all essential to suppress the growth of intracellular bacteria by autophagy (25), indicating that Atg5-, Atg7-, and Atg16L1-dependent action is separable from Atg9a and/or Atg14-dependent membrane formation in autophagy against bacteria. Although it remains controversial whether autophagy is involved in the IFN-γ–mediated anti-T. gondii cellular response (17, 35), the recruitment of the immunity-related GTPases to the parasite is regulated independently of at least Atg9a and Atg14, which are key players in autophagosome formation in autophagy. Thus, our current study expands the notion that autophagy proteins differentially participate in mouse IFN-γ–induced anti–T. gondii activity, which originally was proposed by the previous pioneer findings about the essential, but autophagosome-independent, function of Atg5 and the dispensable role of another essential autophagy regulator, Beclin1, in IFN-γ–induced anti-parasite activity (17, 36).

In human cells, IFN-γ stimulation also leads to several anti–T. gondii effector mechanisms involving tryptophan degradation (37, 38) and iron depletion (39, 40). In this study, we show that ATG16L1 also plays an important role in the recruitment of GBPs to the parasite in a human cell line. Although we demonstrate that IFN-γ–stimulated recruitment remains effective to reduce the number of T. gondii in human ATG16L1- or GBP-deficient cells, even at the lower concentrations of IFN-γ, whether the human IFN-γ–dependent immunity to T. gondii is independent of the ATG16L1–GBP axis should be examined carefully in the future. Given the anti-T. gondii function of GBPs in the recruitment or retention of IRGs onto the parasites in mouse (12, 13), the reason why human cells do not require the ATG16L1–GBP system for the IFN-γ–dependent suppression of parasite numbers might be explained, in part, by the fact that humans do not possess the large variety of IRGs found in the mouse system (41). Alternatively, other effector mechanisms described above might be dominant in humans or the human HAP1 cell line, eventually masking the effect of loss of ATG16L1/GBPs in cell-autonomous in vitro immunity. Indeed, IFN-γ–stimulated human fibroblasts were shown to induce cell death and early egress of the parasite, downregulating the parasite numbers (42). Furthermore, given that IFN-γ stimulates accumulation of human GBPs on the parasites in an ATG16L1-dependent fashion, the ATG16L1–GBP axis might play an in vivo role in the anti–T. gondii program involving GBP5 in the NALP3-dependent inflammasome activation that leads to IL-1/IL-18 production (43). To elucidate the physiological role of human ATG16L1 or GBPs in various settings and cell types, the use of human embryonic stem cells or induced pluripotent stem cells lacking these proteins would be of interest.

In conclusion, some mouse autophagy proteins, such as Atg7 and Atg16L1, but not Atg9a and Atg14, are integral to IFN-γ–mediated anti–T. gondii cell-autonomous innate immune responses by controlling the recruitment of the immunity-related GTPases to the parasites. Although human ATG16L1, as well as GBPs, is dispensable for the IFN-γ–dependent cellular inhibition of T. gondii...
proliferation, the recruitment of GBPs requires ATG16L1. To uncover the underlying mechanism of IFN-γ-induced antiparasite responses, future studies will need to investigate how Atg7 and Atg16L1, together with Atg5, facilitate the anti-T. gondii events by

FIGURE 6. GBPs are recruited to T. gondii in an ATG16L1-dependent fashion. (A) WT HAP1 cells, which were left untreated or treated with 10 ng/ml IFN-γ for 24 h, were infected with ME49 T. gondii (moi = 2), fixed at 6 h postinfection, and incubated with rabbit anti-T. gondii (green), mouse anti-GBP1-5 (red), and DAPI (blue). Scale bar, 5 μm. Arrows indicate the colocalization of T. gondii and GBPs. (B) Percentage of parasites positive for GBP1-5 staining at 6 h postinfection in WT HAP1 cells that were left unstimulated or stimulated with 10 ng/ml IFN-γ. Data are mean ± SD of triplicates. *p < 0.001. (C) WT and ATG16L1-deficient HAP1 cells, treated with 10 ng/ml IFN-γ for 24 h, were infected with ME49 T. gondii (moi = 2), fixed at 6 h postinfection, and incubated with rabbit anti-T. gondii (green), mouse anti-GBP1-5 (red), and DAPI (blue). Scale bar, 5 μm. Arrows indicate the colocalization of T. gondii and GBPs. (D) Percentage of parasites positive for GBP1-5 staining at 6 h postinfection in IFN-γ-stimulated WT and ATG16L1-deficient HAP1 cells. Data are mean ± SD of triplicates. ***p < 0.001, ****p < 0.001. Data are representative of three independent experiments.

FIGURE 7. Normal IFN-γ–induced suppression of parasite proliferation in HAP1 cells lacking human GBPs. (A) Gene-targeting strategy for the entire human GBP locus using Cas9-mediated genome editing. (B) PCR detection of cells with deletion of entire human GBP locus. Primers used are denoted in (A). Also see Supplemental Fig. 2B for the targeting sequences to design gRNA3 and gRNA4, as well as assessment of the deletions by sequencing the PCR products detected in (B). (C) Quantitative PCR analysis of the expression of the indicated GBP mRNA in WT or GBP-deleted (GBPs KO) HAP1 cells that were left unstimulated or stimulated with 10 ng/ml IFN-γ. Data were normalized to the amount of GAPDH in each sample and are mean ± SD of triplicates. (D) WT and GBPs KO HAP1 cells, which were left unstimulated or stimulated with 10 ng/ml IFN-γ for 24 h, were lysed. The lysates were detected by Western blot with the indicated Abs. (E) WT and GBPs KO HAP1 cells were left untreated or treated with the indicated concentrations of IFN-γ for 24 h. Untreated or IFN-γ–treated cells were infected with ME49 T. gondii expressing luciferase (moi = 0.5) and harvested at 24 h postinfection. The number of total parasites was monitored by luciferase activity using the lysates. Data are mean ± SD of triplicates and are representative of three independent experiments.
elucidating unidentified regulatory circuits and their sequential programs.

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Disclosures

The authors have no financial conflicts of interest.

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