The Journal of Immunology

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

Jun Ohshima, Youngae Lee, Miwa Sasai, Tatsuya Saitoh, Ji Su Ma, Naganori Kamiyama, Yoshiharu Matsuura, Suh Pann-Ghill, Mikako Hayashi, Shigeyuki Ebisu, Kiyoshi Takeda, Shizuo Akira and Masahiro Yamamoto

J Immunol 2014; 192:3328-3335; Prepublished online 21 February 2014;
doi: 10.4049/jimmunol.1302822
http://www.jimmunol.org/content/192/7/3328

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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

Jun Ohshima,[*,†,‡,§,1] Youngae Lee,[*,†,1] Miwa Sasai,[*,†] Tatsuya Saitoh,[¶,‖] Ji Su Ma,[*,†,‡] Naganori Kamiyama,[*,†,‡] Yoshiharu Matsuura,[#] Suh Pann-Ghill,[**] Mikako Hayashi,[§] Shigeyuki Ebisu,[§] Kiyoshi Takeda,[‡,††,‡‡] Shizuo Akira,[¶,‖] and Masahiro Yamamoto[*,†]

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 Journal of Immunology*, 2014, 192: 3328–3335.**

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 latent infection, reactivation of this pathogen in immunocompromised individuals suffering from AIDS or being treated by chemotherapy often leads to lethal encephalitis (4). Eradication of *T. gondii* requires IFN-γ (5), which indirectly stimulates anti–*T. gondii* immune responses by robust induction of nearly 2000 genes known as IFN-γ–inducible genes (6, 7). IFN-γ activates antiparasite programs involving IFN-γ–inducible GTPases such as p47 IFN-γ–regulated GTPases (IRGs) and p65 guanylate-binding protein (GBPs) (8). Mice deficient in IRGs such as LRG-47 (also known as Irgm1), IGTP (Irgm3) or IIGP1 (Irga6), or GBPs displayed loss of resistance to *T. gondii* infection (9–13).

Immunity-related GTPases have been implicated in cellular immunity against bacteria via autophagy (14, 15). Autophagy is a homeostatic fundamental cellular process in which cytoplasmic cargos included in double-membrane structures, called "autophagosomes," are transported to lysosomes (16). The autophagic process involves

*Department of Immunoparasitology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan; †Laboratory of Immunoparasitology, World Premier International Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871, Japan; ‡Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan; §Department of Restorative Dentistry and Endodontology, Graduate School of Dentistry, Osaka University, Suita, Osaka 565-0871, Japan; ¶Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan; ‖Laboratory of Host Defense, World Premier International Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871, Japan; #Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan; **School of Nano-Bioscience and Chemical Engineering, Ulsan National Institute of Science and Technology, Ulsan 689-798, Republic of Korea; ††Laboratory of Mucosal Immunology, World Premier International Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871, Japan; and ‡‡Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan

[1]J.O. and Y.L. contributed equally to this work.

Received for publication October 22, 2013. Accepted for publication January 26, 2014.

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, the Japan Science and Technology Agency, the Senri Life Science Foundation, the Tokyo Biochemical Research Foundation, the Research Foundation for Microbial Diseases of Osaka University, the Nakajima Foundation, the Asahi Glass Foundation, the Osaka Foundation for Promotion of Clinical Immunology, the Sumitomo Foundation, the Sagawa Foundation of Promotion of Cancer Research, the Suzuken Memorial Foundation, the Osaka Cancer Research Foundation, the Daiichi-Sankyo Foundation of Life Science, the Uehara Memorial Foundation, the Mochida Foundation for Medical and Pharmaceutical Research, the Ichiro Kanehara Foundation, the Kanae Foundation for the Promotion of Medical Science, and the Japan Intractable Disease Research Foundation.

Address correspondence and reprint requests to Prof. Masahiro Yamamoto, Department of Immunoparasitology, Research Institute for Microbial Diseases, Laboratory of Immunoparasitology, World Premier International Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871, Japan. E-mail address: myamamoto@biken.osaka-u.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this article: GBP, p65 guanylate-binding protein; gRNA, guided RNA; IRG, p47 IFN-γ-regulated GTPases; LC3, microtubule-associated L chain 3; MEF, murine embryonic fibroblast; moi, multiplicity of infection; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302822

a series of autophagy proteins, among which Atg5 was recently linked to the IFN-γ–mediated parasiticidal 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 nondegradative 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 Bioscience), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Nacalai Tesque) (12). MEFs were maintained in DMEM (Nacalai Tesque) supplemented with 10% heat-inactivated FCS (JRH Bioscience), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells lacking Atg7, Atg16L1, Atg9a, or Atg14 were described previously (22–25). Human haploid HAP1 cells were described previously (26) and maintained in IMDM (Nacalai Tesque) supplemented with 10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Animal experiments were conducted with the approval of the Research Institute for Microbial Diseases, Osaka University.

Reagents

Abs against Irgb6 (TGTP; sc-11079), GBP1-5 (sc-166960), and actin (sc-8432) were purchased from Santa Cruz. Abs against LC3 (PL036 for Fig. 5E) and ATG16L1 (PM040) were purchased from MBL. Ab against LC3B (NB600-1384 for Western blotting) was purchased from Novus. Chloroquine was purchased from Wako. Anti-GAP45 rabbit Ab to stain *T. gondii* was a kind gift of Dr. D. Soldati-Favre. Recombinant mouse and human IFN-γ were obtained from PeproTech.

Generation of ATG16L1- or GBP-deficient HAP1 cells

The cDNA encoding the Cas9 nuclease for mammalian expression were obtained from Addgene (Plasmid 41815). The Cas9 nuclease cDNA and polyA fragments were recovered and inserted into pEF6 vector harboring the puromycin resistance cassette to generate pEF6-hCas9-Puro vector. The insert fragments of guided RNA (gRNA)1, gRNA2, gRNA3, and gRNA4 were amplified using KODFXNEO (Toyobo) and primers (Supplemental Table I): ATG16L1_gRNA1_F and ATG16L1_gRNA1_R for gRNA1, ATG16L1_gRNA2_F and ATG16L2_gRNA1_R for gRNA2, GBPs_gRNA3_F and GBPs_gRNA3_R for gRNA3, and GBPs_gRNA4_F and GBPs_gRNA4_R for gRNA4. The fragments for gRNA1, gRNA2, gRNA3, and gRNA4 were inserted into the gRNA cloning vector (Plasmid 41824) using Gibson Assembly mix (New England BioLabs) to generate the gRNA-expressing plasmids pgRNA1, pgRNA2, pgRNA3, and pgRNA4, respectively. HAP1 cells were cotransfected with the pEF6-hCas9-Puro vector and pgRNA1/2 (for generation of ATG16L1-deficient cells) or pgRNA3/4 (GBP-deficient cells) using Lipofectamine 2000 (Life Technologies) and cultured in IMDM containing 0.5 μg/ml puromycin for 4 d. Limiting dilution of the surviving cells was performed, and the resultant single colonies were expanded and lysed for PCR analysis to detect the deletion using primers (Supplemental Table I): ATG16L1_indel_F and ATG16L1_indel_R for ATG16L1-deficient cells and GBPs_indel_F and GBPs_indel_R for GBP-deficient cells. PCR^+ clones were subsequently tested using Western blotting to confirm the lack of protein expression.

Western blotting

MEFs (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 containing 1.0% Nonidet P-40, 150 mM NaCl, 20 mM Tris-Cl (pH 7.5), 5 mM EDTA, and protease inhibitor mixture (Roche). The cell lysates were separated by SDS-PAGE, transferred to polyvinyl difluoride membrane, and subjected to Western blotting using the indicated Abs.

Measurement of T. gondii *numbers by luciferase assay*

MEFs (5×10^5) or HAP1 cells (2×10^6) were left untreated or were treated with 10 ng/ml IFN-γ for 24 h, followed by infection with luciferase-expressing *T. gondii* (multiplicity of infection [moi] = 0.5) for 24 h. To measure *T. gondii* titers, the infected cells were harvested and lysed with 100 μl lysis buffer (Promega) with sonication. After centrifugation at 15,000 rpm at 4°C, the luciferase activity of 5 μl the supernatant was measured using the Dual-Luciferase Reporter Assay System and a GLOMAX 20/20 luminometer (both from Promega). The percentages of the activities in IFN-γ–stimulated or unstimulated cells over those in unstimulated cells are shown.

Immunofluorescence

MEFs (2.5×10^5) or HAP1 cells (1×10^6) infected with *T. gondii* (moi = 2) were fixed for 10 min in PBS containing 3.7% formaldehyde. Cells were permeabilized with PBS containing 0.1% Triton X-100 and then blocked with 8% FCS in PBS. Subsequently, cells were incubated with anti-Irgb6 goat Ab (1:50), anti–Gbp1-5 mouse Ab (1:200), anti-GAP45 rabbit Ab (1:1000) (Figs. 1–3, 4, 6), anti-LC3 rabbit Ab (1:500) (Fig. 5), anti-GAP45 rabbit Ab (1:1000), and anti-Irgb6 goat Ab (1:50), for 1 h at 37°C, followed by incubation with donkey IgG Abs (1:10000): Alexa Flour 488–conjugated anti-rabbit IgG, Alexa Fluor 594–conjugated anti-goat, or Alexa Fluor 647– or Alexa Fluor 594–conjugated anti-mouse (Molecular Probes) for 1 h at 37°C in the dark. Finally, the immunostained cells were mounted with PermaFluor (Thermo Scientific) on glass slides and analyzed using a confocal laser microscope (FV1200 IX-83; Olympus); the images were analyzed using FLUOVIEW software (Olympus).

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) using the Go-Taq qPCR Master mix (Promega). The values 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.

Atg16L1 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.

*Atg9a 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-

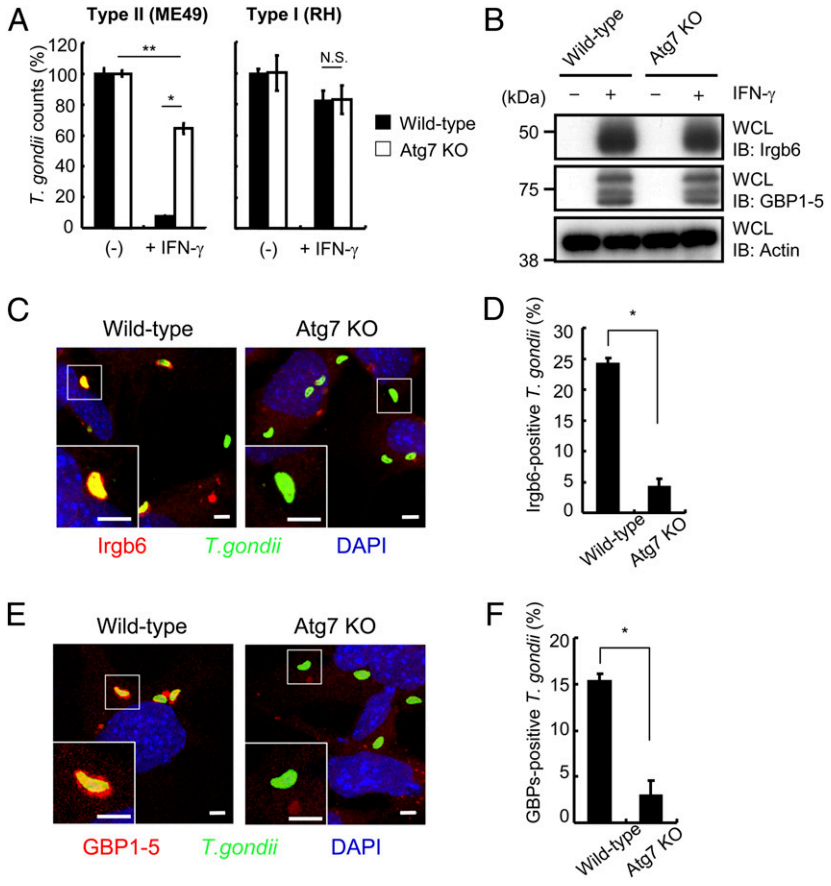

FIGURE 1. Atg7 is required for anti–*T. gondii* cellular immunity. (**A**) WT and Atg7-deficient MEFs were untreated or treated with 10 ng/ml IFN-γ for 24 h. Untreated or IFN-γ–treated cells were infected with ME49 (*left panel*) or RH (*right panel*) *T. gondii* expressing luciferase (moi = 0.5) and harvested at 24 h postinfection. The number of total parasites was monitored by luciferase activity using the lysates. Data are mean ± SD of triplicates. $*p < 0.002$. $**p < 0.007$. (**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.

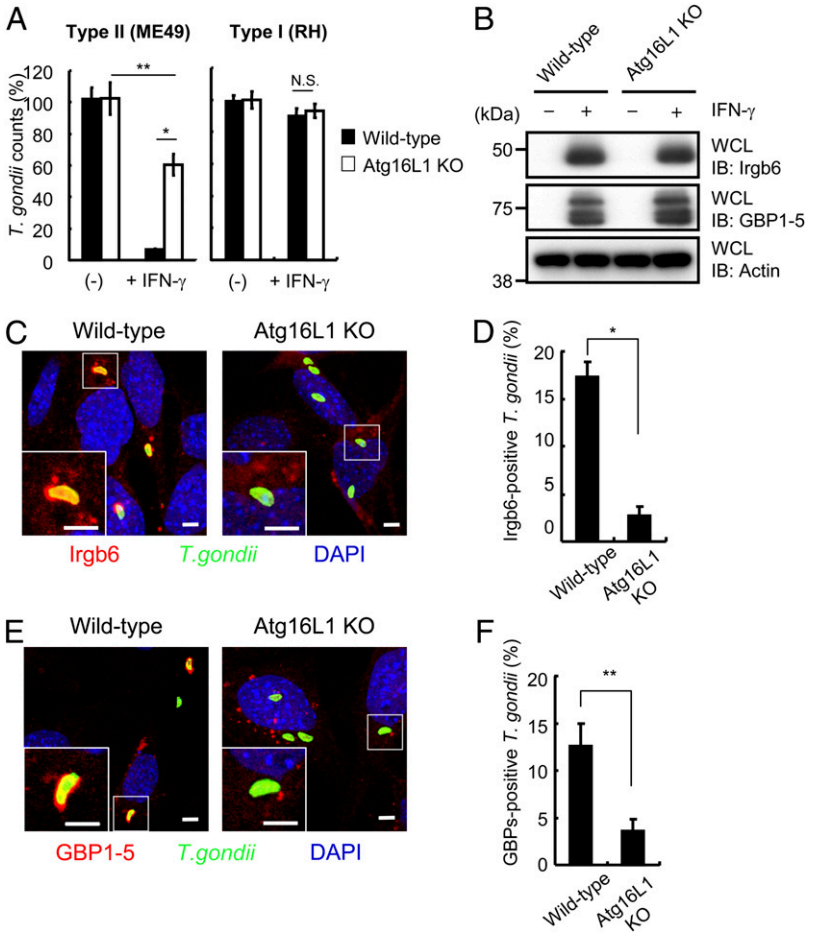

FIGURE 2. Atg16L1 plays a critical role in anti–*T. gondii* cellular immunity. (**A**) WT and Atg16L1-deficient MEFs were left untreated or treated with 10 ng/ml IFN-γ for 24 h. Untreated or IFN-γ–treated cells were infected with ME49 (*left panel*) or RH (*right panel*) *T. gondii* expressing luciferase (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; (**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 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* using indirect immunofluorescence (Fig. 6A). In unstimulated conditions, almost no parasites were stained with anti–GBP1-5. In contrast, IFN-γ significantly induced accumulation of GBPs on *T. gondii* in HAP1 cells (Fig. 6A, 6B, Supplemental Fig. 3A, 3B). Next, we analyzed whether the IFN-γ–dependent recruitment of human GBPs to *T. gondii* is dependent on ATG16L1. In contrast to WT cells, cells lacking ATG16L1 showed a marked defect in the

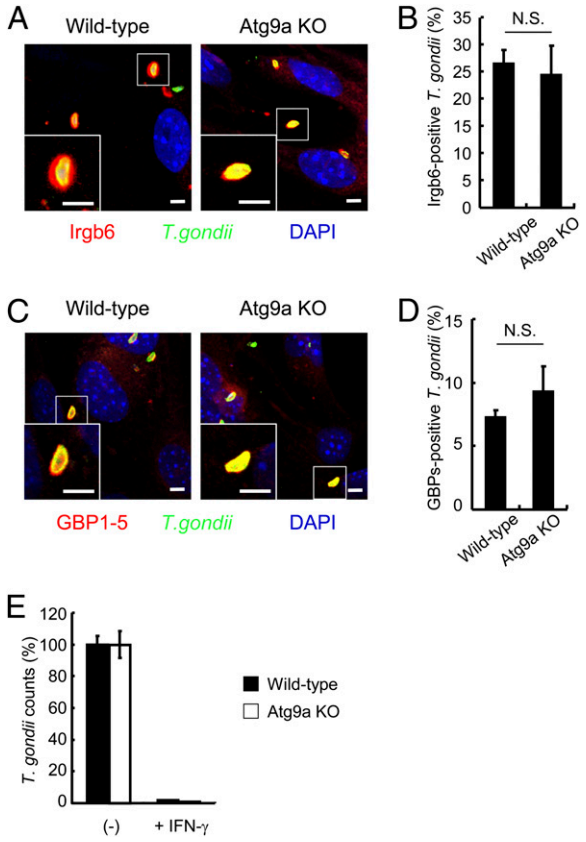

FIGURE 3. Atg9a is dispensable for anti–*T. gondii* cellular immunity. WT and Atg9a-deficient MEFs, treated with 10 ng/ml IFN-γ for 24 h, were infected with ME49 *T. gondii* (moi = 2), fixed at 6 h postinfection, and incubated with rabbit anti–*T. gondii* (green), goat anti-Irgb6 [red; (**A**)], or mouse anti-GBP1-5 [red; (**C**)], and DAPI (blue). Scale bars, 5 μm. Percentage of parasites positive for Irgb6 (**B**) or GBP1-5 (**D**) staining at 6 h postinfection in IFN-γ–stimulated WT and Atg9a-deficient MEFs. 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 the 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.

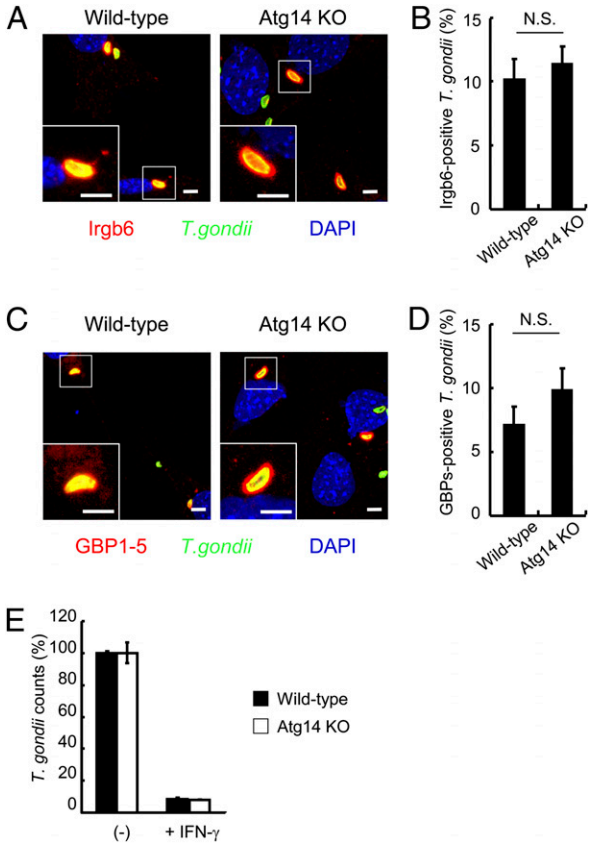

FIGURE 4. Atg14 is not involved in anti–*T. gondii* cellular immunity. WT and Atg14-deficient MEFs, treated with 10 ng/ml IFN-γ for 24 h, were infected with ME49 *T. gondii* (moi = 2), fixed at 6 h postinfection, and incubated with rabbit anti–*T. gondii* (green), goat anti-Irgb6 [red; (**A**)] or mouse anti-GBP1-5 [red; (**C**)], and DAPI (blue). Scale bars, 5 μm. Percentage of parasites positive for Irgb6 (**B**) or GBP1-5 (**D**) staining at 6 h postinfection in IFN-γ-stimulated WT and Atg14-deficient MEFs. Data are mean ± SD of triplicates. (**E**) WT and Atg14-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 luciferase activity using the lysates. Data are mean ± SD of triplicates. All data are representative of three independent experiments.

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. 2B). 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* infection under unstimulated or IFN-γ–stimulated conditions. Both types of cells showed comparable IFN-γ–induced suppression of *T. gondii* numbers that was dose dependent (Fig. 7E). Taken together, although IFN-γ–induced GBP recruitment does not negatively affect *T. gondii* numbers in infected cells, the accumulation in humans is regulated by ATG16L1.

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).

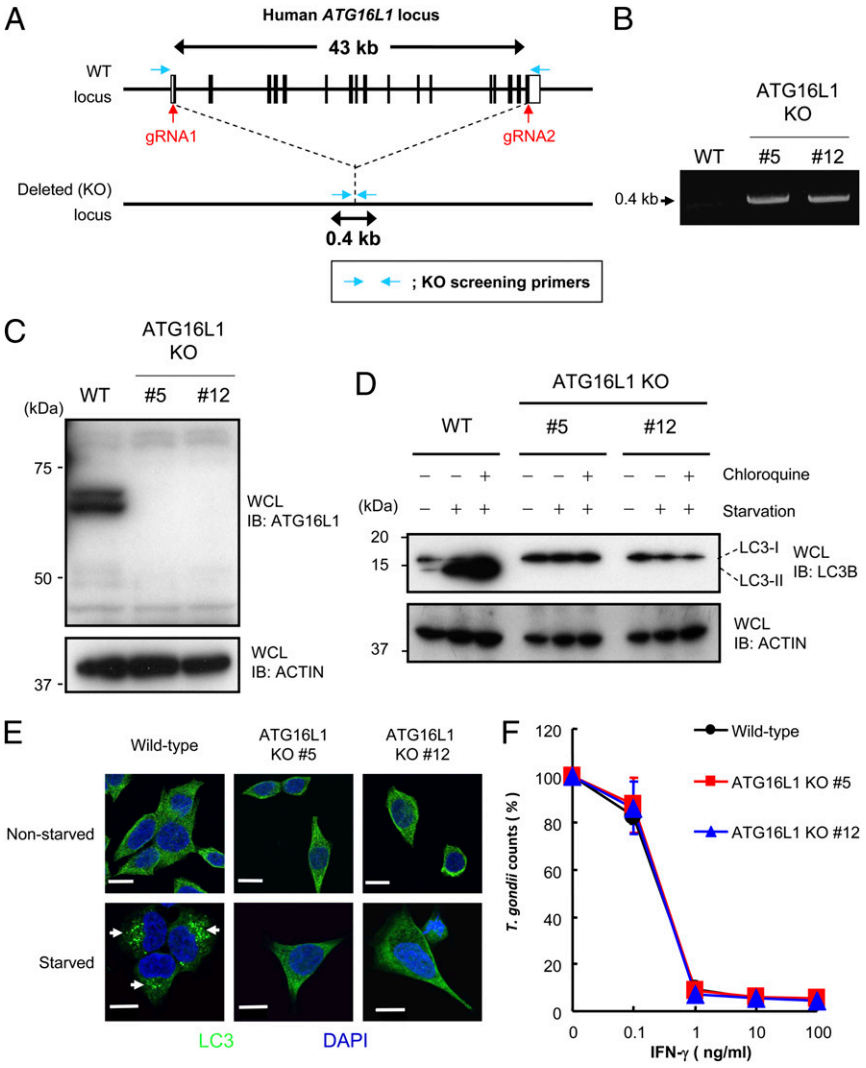

FIGURE 5. Human ATG16L1 is required for starvation-induced autophagy but not for IFN-γ–induced inhibition of *T. gondii* growth in HAP1 cells. (**A**) The gene-targeting strategy for human ATG16L1 locus by Cas9-mediated genome editing. (**B**) PCR detection of cells with deletion of human ATG16L1 locus. 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 stimulation 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 Atg9 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*

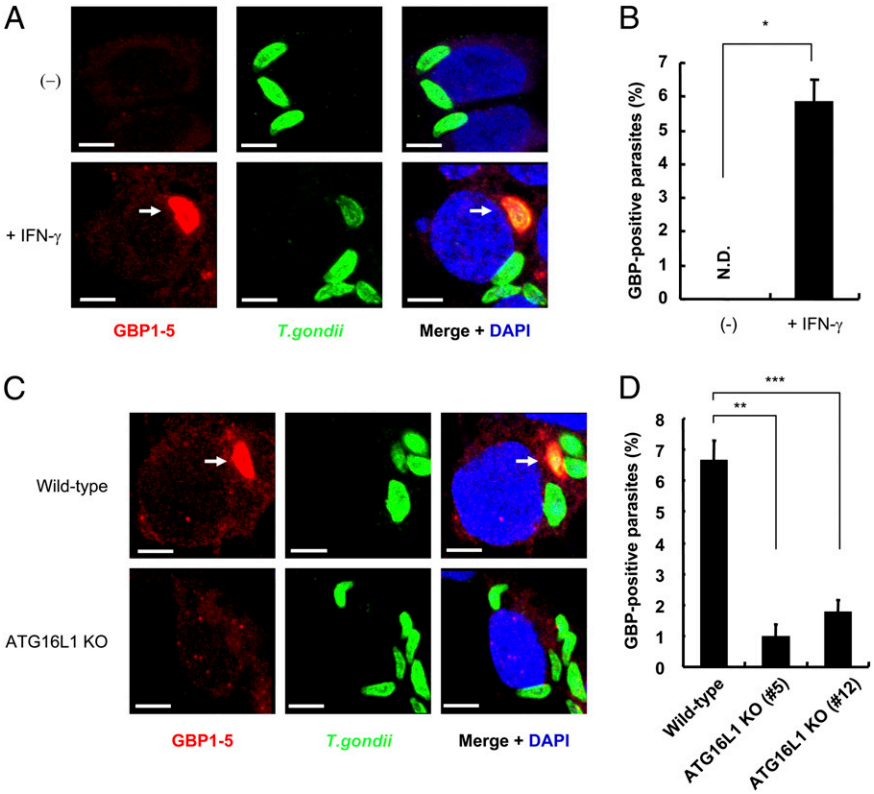

FIGURE 6. GBPs are recruited to *T. gondii* in an ATG16L1-dependent fashion. (**A**) WT HAP1 cells, which were left untreated or treated with 10 ng/ml IFN-γ for 24 h, were infected with ME49 *T. gondii* (moi = 2), fixed at 6 h postinfection, and incubated with rabbit anti–*T. gondii* (green), mouse anti-GBP1-5 (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.

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

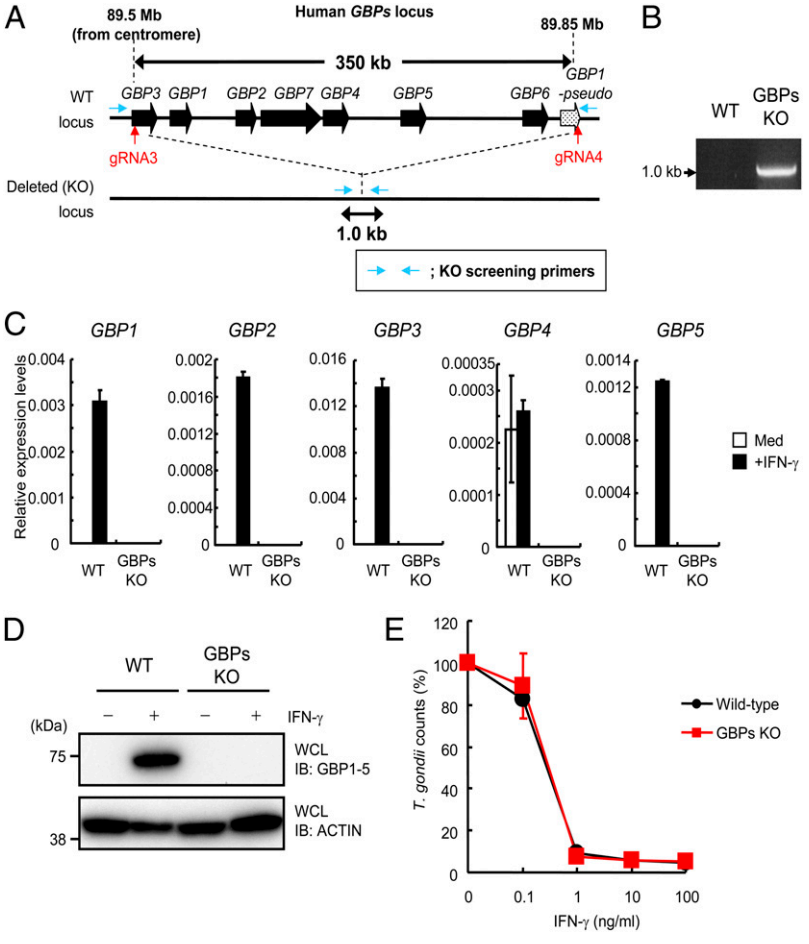

FIGURE 7. Normal IFN-γ–induced suppression of parasite proliferation in HAP1 cells lacking human GBPs. (**A**) Gene-targeting strategy for the entire human GBP locus using Cas9-mediated genome editing. (**B**) PCR detection of cells with deletion of entire human GBP locus. Primers used are denoted in (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.

Acknowledgments

We thank M. Enomoto and Dr. D.M. Standley for secretarial and technical assistance and reading of the manuscript, respectively. We also thank Drs. M. Komatsu, D. Soldati-Favre, and T.R. Brummelkamp for providing Atg7-deficient mice, anti–*T. gondii* Ab, and HAP1 cells, respectively.

Disclosures

The authors have no financial conflicts of interest.

References

1. Yarovinsky, F., and A. Sher. 2006. Toll-like receptor recognition of *Toxoplasma gondii*. *Int. J. Parasitol.* 36: 255–259.
2. Boothroyd, J. C. 2009. *Toxoplasma gondii*: 25 years and 25 major advances for the field. *Int. J. Parasitol.* 39: 935–946.
3. Hunter, C. A., and L. D. Sibley. 2012. Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. *Nat. Rev. Microbiol.* 10: 766–778.
4. Israelski, D. M., and J. S. Remington. 1993. Toxoplasmosis in patients with cancer. *Clin. Infect. Dis.* 17(Suppl. 2): S423–S435.
5. Suzuki, Y., M. A. Orellana, R. D. Schreiber, and J. S. Remington. 1988. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science* 240: 516–518.
6. Kim, B. H., A. R. Shenoy, P. Kumar, C. J. Bradfield, and J. D. MacMicking. 2012. IFN-inducible GTPases in host cell defense. *Cell Host Microbe* 12: 432–444.
7. Taylor, G. A., C. G. Feng, and A. Sher. 2004. p47 GTPases: regulators of immunity to intracellular pathogens. *Nat. Rev. Immunol.* 4: 100–109.
8. MacMicking, J. D. 2012. Interferon-inducible effector mechanisms in cell-autonomous immunity. *Nat. Rev. Immunol.* 12: 367–382.
9. Collazo, C. M., G. S. Yap, G. D. Sempowski, K. C. Lusby, L. Tessarollo, G. F. Vande Woude, A. Sher, and G. A. Taylor. 2001. Inactivation of LRG-47 and IRG-47 reveals a family of interferon gamma-inducible genes with essential, pathogen-specific roles in resistance to infection. *J. Exp. Med.* 194: 181–188.
10. Degrandi, D., E. Kravets, C. Konermann, C. Beuter-Gunia, V. Klümpers, S. Lahme, E. Wischmann, A. K. Mausberg, S. Beer-Hammer, and K. Pfeffer. 2013. Murine guanylate binding protein 2 (mGBP2) controls *Toxoplasma gondii* replication. *Proc. Natl. Acad. Sci. USA* 110: 294–299.
11. Taylor, G. A., C. M. Collazo, G. S. Yap, K. Nguyen, T. A. Gregorio, L. S. Taylor, B. Eagleson, L. Secrest, E. A. Southon, S. W. Reid, et al. 2000. Pathogen-specific loss of host resistance in mice lacking the IFN-gamma-inducible gene IGTP. *Proc. Natl. Acad. Sci. USA* 97: 751–755.
12. Yamamoto, M., M. Okuyama, J. S. Ma, T. Kimura, N. Kamiyama, H. Saiga, J. Ohshima, M. Sasai, H. Kayama, T. Okamoto, et al. 2012. A cluster of interferon-γ-inducible p65 GTPases plays a critical role in host defense against *Toxoplasma gondii*. *Immunity* 37: 302–313.
13. Selleck, E. M., S. J. Fentress, W. L. Beatty, D. Degrandi, K. Pfeffer, H. W. Virgin, IV, J. D. Macmicking, and L. D. Sibley. 2013. Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against *Toxoplasma gondii*. *PLoS Pathog.* 9: e1003320.
14. Deretic, V. 2012. Autophagy as an innate immunity paradigm: expanding the scope and repertoire of pattern recognition receptors. *Curr. Opin. Immunol.* 24: 21–31.
15. Kim, B. H., A. R. Shenoy, P. Kumar, R. Das, S. Tiwari, and J. D. MacMicking. 2011. A family of IFN-γ-inducible 65-kD GTPases protects against bacterial infection. *Science* 332: 717–721.
16. Mizushima, N., T. Yoshimori, and Y. Ohsumi. 2011. The role of Atg proteins in autophagosome formation. *Annu. Rev. Cell Dev. Biol.* 27: 107–132.
17. Zhao, Z., B. Fux, M. Goodwin, I. R. Dunay, D. Strong, B. C. Miller, K. Cadwell, M. A. Delgado, M. Ponpuak, K. G. Green, et al. 2008. Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. *Cell Host Microbe* 4: 458–469.
18. Hwang, S., N. S. Maloney, M. W. Bruinsma, G. Goel, E. Duan, L. Zhang, B. Shrestha, M. S. Diamond, A. Dani, S. V. Sosnovtsev, et al. 2012. Non-degradative role of Atg5-Atg12/Atg16L1 autophagy protein complex in antiviral activity of interferon gamma. *Cell Host Microbe* 11: 397–409.
19. Pfefferkorn, E. R., and P. M. Guyre. 1984. Inhibition of growth of *Toxoplasma gondii* in cultured fibroblasts by human recombinant gamma interferon. *Infect. Immun.* 44: 211–216.
20. Mali, P., L. Yang, K. M. Esvelt, J. Aach, M. Guell, J. E. DiCarlo, J. E. Norville, and G. M. Church. 2013. RNA-guided human genome engineering via Cas9. *Science* 339: 823–826.
21. Cong, L., F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. Wu, W. Jiang, L. A. Marraffini, and F. Zhang. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819–823.
22. Komatsu, M., S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, T. Ueno, M. Koike, Y. Uchiyama, E. Kominami, and K. Tanaka. 2006. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 441: 880–884.
23. Saitoh, T., N. Fujita, M. H. Jang, S. Uematsu, B. G. Yang, T. Satoh, H. Omori, T. Noda, N. Yamamoto, M. Komatsu, et al. 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* 456: 264–268.
24. Saitoh, T., N. Fujita, T. Hayashi, K. Takahara, T. Satoh, H. Lee, K. Matsunaga, S. Kageyama, H. Omori, T. Noda, et al. 2009. Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. *Proc. Natl. Acad. Sci. USA* 106: 20842–20846.
25. Kageyama, S., H. Omori, T. Saitoh, T. Sone, J. L. Guan, S. Akira, F. Imamoto, T. Noda, and T. Yoshimori. 2011. The LC3 recruitment mechanism is separate from Atg9L1-dependent membrane formation in the autophagic response against *Salmonella*. *Mol. Biol. Cell* 22: 2290–2300.
26. Carette, J. E., M. Raaben, A. C. Wong, A. S. Herbert, G. Obernosterer, N. Mulherkar, A. I. Kuehne, P. J. Kranzusch, A. M. Griffin, G. Ruthel, et al. 2011. Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature* 477: 340–343.
27. Fujita, N., T. Itoh, H. Omori, M. Fukuda, T. Noda, and T. Yoshimori. 2008. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol. Biol. Cell* 19: 2092–2100.
28. Weidberg, H., E. Shvets, T. Shpilka, F. Shimron, V. Shinder, and Z. Elazar. 2010. LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. *EMBO J.* 29: 1792–1802.
29. Itakura, E., and N. Mizushima. 2009. Atg14 and UVRAG: mutually exclusive subunits of mammalian Beclin 1-PI3K complexes. *Autophagy* 5: 534–536.
30. Kabeya, Y., N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, and T. Yoshimori. 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* 19: 5720–5728.
31. Nordmann, A., L. Wixler, Y. Boergeling, V. Wixler, and S. Ludwig. 2012. A new splice variant of the human guanylate-binding protein 3 mediates anti-influenza activity through inhibition of viral transcription and replication. *FASEB J.* 26: 1290–1300.
32. Matsunaga, K., E. Morita, T. Saitoh, S. Akira, N. T. Ktistakis, T. Izumi, T. Noda, and T. Yoshimori. 2010. Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L. *J. Cell Biol.* 190: 511–521.
33. Obara, K., and Y. Ohsumi. 2011. Atg14: a key player in orchestrating autophagy. *Int. J. Cell Biol.* 2011: 713435.
34. Orsi, A., M. Razi, H. C. Dooley, D. Robinson, A. E. Weston, L. M. Collinson, and S. A. Tooze. 2012. Dynamic and transient interactions of Atg9 with autophagosomes, but not membrane integration, are required for autophagy. *Mol. Biol. Cell* 23: 1860–1873.
35. Ling, Y. M., M. H. Shaw, C. Ayala, I. Coppens, G. A. Taylor, D. J. Ferguson, and G. S. Yap. 2006. Vacuolar and plasma membrane stripping and autophagic elimination of *Toxoplasma gondii* in primed effector macrophages. *J. Exp. Med.* 203: 2063–2071.
36. Portillo, J. A., G. Okenka, E. Reed, A. Subauste, J. Van Grol, K. Gentil, M. Komatsu, K. Tanaka, G. Landreth, B. Levine, and C. S. Subauste. 2010. The CD40-autophagy pathway is needed for host protection despite IFN-Γ-dependent immunity and CD40 induces autophagy via control of P21 levels. *PLoS ONE* 5: e14472.
37. Gupta, S. L., J. M. Carlin, P. Pyati, W. Dai, E. R. Pfefferkorn, and M. J. Murphy, Jr. 1994. Antiparasitic and antiproliferative effects of indoleamine 2,3-dioxygenase enzyme expression in human fibroblasts. *Infect. Immun.* 62: 2277–2284.
38. Pfefferkorn, E. R. 1984. Interferon gamma blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. *Proc. Natl. Acad. Sci. USA* 81: 908–912.
39. Dimier, I. H., and D. T. Bout. 1997. Inhibition of *Toxoplasma gondii* replication in IFN-gamma-activated human intestinal epithelial cells. *Immunol. Cell Biol.* 75: 511–514.
40. Dimier, I. H., and D. T. Bout. 1998. Interferon-gamma-activated primary enterocytes inhibit *Toxoplasma gondii* replication: a role for intracellular iron. *Immunology* 94: 488–495.
41. Könen-Waisman, S., and J. C. Howard. 2007. Cell-autonomous immunity to *Toxoplasma gondii* in mouse and man. *Microbes Infect.* 9: 1652–1661.
42. Niedelman, W., J. K. Sprokholt, B. Clough, E. M. Frickel, and J. P. Saeij. 2013. Cell death of gamma interferon-stimulated human fibroblasts upon *Toxoplasma gondii* infection induces early parasite egress and limits parasite replication. *Infect. Immun.* 81: 4341–4349.
43. Shenoy, A. R., D. A. Wellington, P. Kumar, H. Kassa, C. J. Booth, P. Cresswell, and J. D. MacMicking. 2012. GBP5 Promotes NLRP3 inflammasome assembly and immunity in mammals. *Science.* 336: 481–485.