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IFN-γ Elicits Macrophage Autophagy via the p38 MAPK Signaling Pathway

Takeshi Matsuzawa,* Bae-Hoon Kim,† Avinash R. Shenoy,† Shigeki Kamitani,‡ Masami Miyake,* and John D. MacMicking†

Autophagy is a major innate immune defense pathway in both plants and animals. In mammals, this cascade can be elicited by cytokines (IFN-γ) or pattern recognition receptors (TLRs and nucleotide-binding oligomerization domain-like receptors). Many signaling components in TLR- and nucleotide-binding oligomerization domain-like receptor-induced autophagy are now known; however, those involved in activating autophagy via IFN-γ remain to be elucidated. In this study, we engineered macrophages encoding a tandem fluorescently tagged LC3b (tflC3) autophagosome reporter along with stably integrated short hairpin RNAs to demonstrate IFN-γ–induced autophagy required JAK 1/2, PI3K, and p38 MAPK but not STAT1. Moreover, the autophagy-related guanosine triphosphatase Irgm1 proved dispensable in both stable tflC3-expressing RAW 264.7 and tflC3-transduced Irgm1−/− primary macrophages, revealing a novel p38 MAPK-dependent, STAT1-independent autophagy pathway that bypasses Irgm1.

Macrophages are involved in many essential host functions including clearance of infection, tissue homeostasis and repair, and resolution of inflammation (1). The type II IFN, IFN-γ, remains the prototypic macrophage-activating factor following its secretion by proliferating type 1 Th cells and NK cells (2). IFN-γ initiates its signal transduction cascade via tyrosine phosphorylation of STAT1 by JAK1 and JAK2 (3–5). Subsequently, STAT1 dimers bind to IFN-stimulated response elements and induce the transcription of several hundred IFN-stimulated genes (3, 4). In addition to the JAK–STAT1 pathway, IFN-γ induces gene expression via STAT1-independent pathways (5–7) and modulates various signaling cascades, including MyD88 (8), p38 MAPK (9), PI3K (10), and protein kinase C (11, 12). In macrophages, the major immune target genes of IFN-γ include MHC class I and II, inflammatory and pyrogenic cytokines (TNF-α and IL-6), chemokines, and antimicrobial proteins such as inducible NO synthase 2 (NOS2), phagocyte oxidase and immune guanosine triphosphatases (GTPases) (3–5, 13, 14).

Notably, IFN-γ is critical for cell-autonomous innate immunity against bacteria, protozoa, viruses, and fungi (14–16). Autophagy has emerged as a major immune defense pathway in both plant and animal kingdoms (17, 18). In mammals, this cascade can be elicited by host-derived cytokines, such as IFN-γ, or pattern recognition receptors, including TLRs and nucleotide-binding oligomerization domain-like receptors (18–24). IFN-γ–induced autophagy is also posited to control intracellular pathogens via several members of the immunity-related GTPase family (IRG proteins, formerly known as p47 GTPases) (19, 20). The mouse IRG family consists of 17–20 Irg genes, and their expression is driven by IFN-stimulated response sequences and γ-activated sequences (30). One IRG protein in particular, Irgm1, has been touted to exert critical functions in IFN-γ–induced autophagy (20). A human ortholog of Irgm1, IRGM (30), reportedly shares similar biologic duties, which may include mitophagy (20, 28). Unlike its mouse counterpart, however, human IRGM is not elicited by IFN-γ. Instead, its expression is driven by the endogenous retrovirus element ERV9 (30, 31). This raises the possibility of an IRGM-independent signaling pathway for IFN-γ–induced autophagy.

In this study, we generated macrophages harboring integrated chromosomal copies of a tandem red fluorescent protein (RFP)-GFP–tagged LC3b (tflC3) autophagosome reporter (32). This reporter exploits the loss of fluorescence of GFP (pKₐ 6.0) but not RFP (pKₐ 4.5) in the acidic environment of lysosomes to label autophagic compartments during their maturation to autolysosomes, which form by fusing sequentially or independently with endosomes and lysosomes (18). Moreover, introduction of the tandem fluorescent reporter into primary bone-marrow derived macrophages (BMMs) from Irgm1-deficient mice (Irgm1−/−) also demonstrated that this GTPase is dispensable during the early stages of autophagosome biogenesis. Our results suggest that IFN-γ accelerates not only the formation but also the maturation of autophagosomes and does so via a pathway different to the one implicating Irgm1 described above. This novel pathway uses JAK1/2 and p38 MAPK signaling but does not require STAT1.
Our findings open up the possibility of searching for new STAT1- and Irgm1-independent genes during IFN-γ–induced autophagic control of infection.

Materials and Methods

Mice

Wild-type (WT), STAT1−/−, and Irgm1−/− mice were bred at Yale University School of Medicine, and primary BMMs were derived from the mice as reported previously (25). All animal experiments were undertaken according to Yale institutional guidelines for the care and handling of such mice.

Plasmids and tLC3 macrophage cell lines

mRFP-GFP-LC3b plasmid (a gift from Dr. T. Yoshimori, Osaka University, Osaka, Japan) was transfected to RAW 264.7 murine macrophages or primary BMMs using Lipofectamine LTX (Invitrogen, Carlsbad, CA) or Osakamed (Osaka, Japan) was transfected into RAW 264.7 murine macrophages or primary BMMs using Lipofectamine LTX (Invitrogen, Carlsbad, CA) or Nucleofector system (Amaza Biosystems, Cologne, Germany) to stably express tLC3. To generate pLKD.neo, a lentiviral vector to express short hairpin (sh)RNA, a fragment of the human U6 promoter was PCR amplified with the primers 5′-CGGAATTTCTCTAGACTTGGCTGAGAC-3′ and 5′-TTCTGCCAGCTCTTTCCAC-3′ and 5′-TTAAAGCTAAGGTT-3′ with the primers 5′-GAGAC-3′ and 3′-TTCTGTCACGGTTCGATAATG-3′. The amplicon was digested with NruI and EcoRI (Takara Bio, Tokyo, Japan) before cloning into pLKO.1-puro/shG4s (Sigma-Aldrich, St. Louis, MO) as the template. The amplicon was digested with NruI and EcoRI (Takara Bio, Tokyo, Japan) before cloning into pLKO.1-puro/shG4s to generate the pLKD.neo vector. The neomycin resistance gene was PCR amplified with the primers 5′-GGGGTACCTCAGAAGAACTCGTCAAGAAG-3′ and 5′-GGGTGTTTACGTAGAGACGATTCA-3′ and pLKD.neo vector. Subsequently, pLKD.pur or pLKD.neo lentiviruses were constructed using the Addgene pLKO.1 protocol (http://www.addgene.org). The RNA interference sequences were as follows: Atg7, 5′-TTCTGTCAGCCTGTTAAGATGTG-3′; IFN-γR1, 5′-GCGAGCTCTTAAAGCTAAGGTT-3′; IFN-γR1-1 and 5′-CCACCAATAGATACGCATTTA-3′; deoxy (shIFN-γR1-2); STAT1, 5′-CCGAGAAGACCTAAGGTT-3′ and 5′-GCGAGCTCTTAAAGCTAAGGTT-3′ (shIFN-γR1-1) and 5′-CCACCAATAGATACGCATTTA-3′; deoxy (shIFN-γR1-2); IFN-γR1, 5′-CCGAGAAGACCTAAGGTT-3′ and 5′-GCGAGCTCTTAAAGCTAAGGTT-3′ (shIFN-γR1-1) and 5′-CCACCAATAGATACGCATTTA-3′; deoxy (shIFN-γR1-2); Irgm1, 5′-TGAATTCTCGACCTC-3′ and 5′-TGAATTCTCGACCTC-3′ (shIrgm1); and 5′-TGAATTCTCGACCTC-3′ (shIrgm1). TheVirapower Lentiviral Expression system (Invitrogen) was used to cotransfect the viral vector into 293FT cells (Invitrogen) to produce lentiviruses. The resulting lentiviral particles were subjected to SDS-PAGE before transferring to polyvinylidene fluoride (PVDF) membranes. Western blotting was performed with the following Abs: rabbit anti-Atg7 polyclonal Ab (GeneTex, Irvine, CA), mouse anti-LC3b mAb (2G6; Nano Tools, Hamburg, Germany), rabbit anti-NOS2 rabbit pAb (Sigma-Aldrich, St. Louis, MO) against the core autophagic adpot protein Atg7, confirming fidelity of the bipartite reporter (Fig. 1B–E). This assay allows us to determine whether STAT1 and Irgm1 promote the formation of autophagosomes as well as their maturation to autolysosomes via canonical Atg pathways. Western blotting with an anti-LC3b mAb confirmed that the activation of LC3b-I to LC3b-II in IFN-γ–treated RAW 264.7 cells on treatment with the lysosomal protease inhibitors E64d and pepstatin A to prevent LC3b degradation within autolysosomes (Fig. 1J). This also peaked between 2 and 4 h after IFN-γ treatment. Hence, we measured the IFN-γ–induced formation and maturation of autophagosomes at this time in genetic and pharmacologic loss-of-function experiments described below.

JAK2 is required for autophagy early after IFN-γ stimulation

IFN-γ engagement of its receptor results in transactivation of JAK1 and JAK2, phosphorylation and translocation to STAT1 molecules to the nucleus where they regulate gene transcription (5). To test whether JAKs are required for IFN-γ–induced autophagy, we treated IFN-γ–activated RAW-tfLC3 cells with the JAK inhibitor I, 2-(1,1-dimethylethyl)-9-fluro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one. This inhibitor is highly and selectively potent for JAK1–3 (IC50, ~1–15 nM) as well as for the tyrosine kinase, Tyk2, which operates downstream of the type I IFN receptor IFN-αR. It completely abolished autophagosome formation and maturation together with expression of the antimicrobial enzyme, inducible NOS2, a known JAK1/2 target gene that served as a positive control (Fig. 2). Thus, JAK1/2 appear obligate for the early signaling cascade downstream of IFN-γ that promotes autophagic induction.

**Results**

IFN-γ enhances autophagosome biogenesis in tLC3 reporter macrophages

Using clonally derived RAW 264.7 macrophage cells expressing tLC3 (RAW-tLC3), we found both the number and size of autophagosomes increased in a dose-dependent manner following treatment with IFN-γ (0.1–300 U/ml) for 24 h (Fig. 1A, 1B, 1F). Furthermore, time-course analysis revealed that autophagosome formation began within ~60 min of IFN-γ stimulation (200 U/ml) and peaked between 2 and 6 h after cytokine treatment (Fig. 1H). Similar analyses revealed that IFN-γ promoted the maturation of autophagosomes to autolysosomes (detected only as RFP vacuoles), a process that began 2 h after IFN-γ stimulation and peaked slightly later, between 4 and 8 h after stimulation (Fig. 1B, 1G, 1I). Both processes could be blocked by provision of shRNA against the core autophagic adpot protein Atg7, confirming fidelity of the bipartite reporter (Fig. 1B–E). These results suggest that IFN-γ promotes the formation of autophagosomes as well as their maturation to autolysosomes via canonical Atg pathways. Western blotting with an anti-LC3b mAb confirmed that the activation of LC3b-I to LC3b-II in IFN-γ–treated RAW 264.7 cells on treatment with the lysosomal protease inhibitors E64d and pepstatin A to prevent LC3b degradation within autolysosomes (Fig. 1J). This also peaked between 2 and 4 h after IFN-γ treatment. Hence, we measured the IFN-γ–induced formation and maturation of autophagosomes at this time in genetic and pharmacologic loss-of-function experiments described below.

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**STAT1 and Irgm1 are dispensable for autophagy activation by IFN-γ stimulation**

In the classical IFN-γ signal cascade, JAK1/2 activation leads to STAT1 phosphorylation and translocation to the nucleus to activate target genes such as Irgm1 (25), the latter of which has been implicated as a major component in stimulating the autophagic response (19, 20). We therefore took a loss-of-function approach by introducing lentiviral pLKD shRNAs into the RAW-tfLC3 reporter cell line to derive stable macrophages that not only express tLC3 but are also silenced for either STAT1 or Irgm1. In addition, we generated a third set of stable RAW-tfLC3 macrophage clones lacking IFN-γR1 as a robust negative control.

This assay allows us to determine whether STAT1 and Irgm1 are functionally involved in eliciting autophagy under conditions where the host cell is not transiently activated via TLR or nucleotide-binding oligomerization domain-like receptor sensing of transfected siRNA pools (29). IFN-γ–stimulated formation
and maturation of autophagosomes was inhibited only in IFN-\(\gamma\)R1–silenced cells; neither STAT1 nor Irgm1 silencing had any effect on autophagosomal induction (Fig. 3A–E). In contrast, expression of endogenous Irgm1 was completely blocked in IFN-\(\gamma\), STAT1, and Irgm1 shRNA-expressing RAW-tfLC3 macrophage cell lines (Fig. 3F). Identical results were obtained with two independent shRNA-expressing RAW-tfLC3 clones for each of the three shRNA-targeted genes. Similar outcomes were also observed using primary BMMs from C57BL/6 WT, STAT1\(^{-/-}\), and Irgm1\(^{-/-}\) mice that had been nucleoporated with the tfLC3 plasmid (Fig. 3G–I). In this study, both autophagosome formation and maturation within the first 4–6 h was identical between genotypes, as was conversion of endogenous LC3b-I to LC3b-II (Figs. 3J, 4E). Thus, our genetic loss-of-function results indicate that IFN-\(\gamma\) can activate macrophage autophagy in an STAT1- and Irgm1-independent manner.

**PI3K and p38 MAPK are required for autophagy activation by IFN-\(\gamma\) stimulation**

The above findings suggested an unconventional pathway being responsible for IFN-\(\gamma\)-induced autophagy. Besides the classic JAK1/2-STAT1 signaling cascade, IFN-\(\gamma\) activates several other downstream pathways. These include p38 MAPK (9) and PI3K signaling cascades (6, 10). The latter cascade, in particular, encompasses some key regulators of autophagy, notably the serine/threonine kinase mammalian target of rapamycin (mTOR) and class I and III PI3Ks (33). Class I PI3Ks phosphorylate phosphatidylinositol (PtdIns)-4,5-bisphosphate at the plasma membrane to generate PtdIns-3,4,5-trisphosphate, which activates mTOR (33–35). VPS34, a class III PI3K, also contributes to mTOR activation when amino acids are plentiful. However, its role differs under starved or cytokine-activated conditions where it phosphorylates PtdIns in endomembranes to produce PtdIns-3-phosphate, which is required for recruiting Beclin-1/Atg6 to stimulate autophagy (33, 34). Two PI3K inhibitors, 3-methyladenine and LY294002, have been shown to inhibit autophagy (33), and LY294002 completely blocked IFN-\(\gamma\)-induced autophagy in our RAW-tfLC3 macrophages as well (Fig. 4A–C). Interestingly, however, it did not alter IFN-\(\gamma\)-induced NOS2 or Irgm1 expression (Fig. 4D), suggesting functional bifurcation of these pathways.

This bifurcation was further reinforced upon examination of p38 MAPK activity. A specific inhibitor of p38 MAPK, 4-(4-fluo-
rophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine, completely blocked autophagy but had no effect on NOS2 and Irgm1 expression following IFN-\(\gamma\) activation (Fig. 4, A–D). Such divergence was again underscored in WT, STAT1\(^{−/−}\), and Irgm1\(^{−/−}\) BMMs (Fig. 4E, 4F). Early IFN-\(\gamma\)-induced phosphorylation of p38 as well as other MAPK members, Erk1/2 and SAPK/JNK, was still

FIGURE 2. JAK1/2 is required for autophagy early after IFN-\(\gamma\) stimulation. (A–C) RAW-tfLC3 was retreated with JAK inhibitor I or left untreated for 2 h, and then, the cells were stimulated with 200 U/ml IFN-\(\gamma\) for 4 h. The cells were fixed and stained with DAPI to detect nuclei. Representative fluorescent images are shown, and an arrowhead or an arrow indicates autophagosome or autolysosome, respectively (A). Scale bar, 20 \(\mu\)m. The proportions of RAW 264.7 cells that formed autophagosomes (B) and autolysosomes (C) are shown. The proportions were calculated using at least 300 cells, and the values are expressed as the mean ± SD from three independent experiments. (D) The cells were lysed in a cell lysis buffer with a protease inhibitor mixture, and then, the lysates were subjected to SDS-PAGE analysis and Western blotting. Densitometric phosphorylated STAT1/STAT1 ratios are shown underneath the blot.

FIGURE 3. STAT1 and Irgm1 are dispensable for autophagy activation by IFN-\(\gamma\) stimulation. (A–C and G–I) RAW 264.7 cells, stable knockdown cells, and BMMs that expressed tfLC3 were stimulated with 200 U/ml IFN-\(\gamma\) for 4 h. The cells were fixed and stained with DAPI to detect nuclei. Representative fluorescent images are shown, and arrowheads or arrows indicate autophagosomes or autolysosomes, respectively (A, G). Scale bars, 20 \(\mu\)m. The proportions of RAW 264.7 cells that formed autophagosomes (B, H) and autolysosomes (C, I) are shown. The proportions were calculated using at least 300 cells, and the values are expressed as the mean ± SD from three independent experiments. (D–F and J) The cells were lysed in a cell lysis buffer with a protease inhibitor mixture, and then, the lysates were subjected to SDS-PAGE analysis and Western blotting. Densitometric LC3b-II/GAPDH ratios are shown underneath the blot.

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in intact Irgm1−/− BMMs, which coincided with normal autophagosome and autolysosome formation (Figs. 3G–J, 4E). These results reveal both PI3K and p38 MAPK are required for the activation of autophagy in an Irgm1-independent manner.

Discussion

IFN-γ-induced autophagosome formation was previously reported to enlist Irgm1, based on short-term GFP-Irgm1 overexpression or transient siRNA silencing in RAW 264.7 cells (19, 20). By using dual-color tfLC3 reporter stably integrated into the chromosome of RAW 264.7 macrophages, combined with genetic or pharmacologic loss-of-function approaches, we found IFN-γ accelerates not only the formation but also the maturation of autophagosomes via JAK1/2 and p38 MAPK signaling pathway. In the current study, we found permanent loss-of-function approaches using primary BMMs from Irgm1−/− mice or RAW 264.7 macrophages stably expressing a dual tfLC3 autophagosome–autolysosome reporter together with Irgm1 shRNAs suggest this IRG protein is dispensable during the early stages of autophagy. Our findings raise the question of whether Irgm1 is a bona fide autophagy gene or whether it has additional lysosomal trafficking roles against pathogens such as Mycobacterium tuberculosis and Crohn’s disease-associated Escherichia coli as suggested from several studies (25, 28, 35). Recent evidence in Irgm1-deficient mice crossed with GFP-LC3b transgenic animals found induction of LC3b puncta in myeloid stem cell precursors was actually elevated rather than impaired compared with WT controls (36). Thus, the precise roles of Irgm1 as part of the IFN-γ-induced autophagic cascade need further clarification. In this respect it mirrors another member of the IRG family, Irga6, which targets and disrupts the Toxoplasma gondii parasitophorous vacuole with the help of autophagy protein Atg5 in an autophagosome-independent fashion (37). As such, the IRGs may fulfill multiple trafficking and signaling functions besides autophagy during infection and inflammatory settings.

It has been reported that members of the MAPK family can be activated by IFN stimulation (5, 9, 38, 39). Moreover, the activation of Erk2, and possibly other MAPKs, by IFN-γ is mediated by JAK2 and proline-rich tyrosine kinase 2 (40). Our analysis of IFN-γ-induced autophagy also indicated JAK1/2 and p38 MAPK dependence but STAT1 independence. Autophagy is used to degrade microorganisms that invade intracellularly (18, 41). Taken together, these results suggesting that IFN-γ-induced autophagy activation through JAK2/proline-rich tyrosine kinase 2/MAPK signaling pathway contributes to the host defense. In this study, as many as 100–150 genes can be expressed downstream of IFN-γ in STAT1−/− fibroblasts. Among these are caspase-7 such as Lice2, IL-1β, Daax, and the serine protease PIM-1 that coordinate apoptotic/pyroptotic responses possibly in response to autophagic stimuli (7). Likewise, other gene targets may proceed via STAT1

FIGURE 4. PI3K and p38 MAPK are required for autophagy activation by IFN-γ stimulation. (A–D) RAW-tfLC3 cells were pretreated with the indicated concentrations of a PI3K inhibitor or p38 MAPK inhibitor for 2 h, and then, the cells were stimulated with 200 U/ml IFN-γ for 4 h. The cells were fixed and stained with DAPI to detect nuclei. Representative fluorescent images are shown, and an arrowhead or an arrow indicates autophagosome or autolysosome, respectively (A). Scale bar, 20 μm. The proportions of RAW 264.7 cells that formed autophagosomes (B) and autolysosomes (C) are shown. The proportions were calculated using at least 300 cells, and the values are expressed as the mean ± SD from three independent experiments. The cells were lysed in a cell lysis buffer with a protease inhibitor mixture, and then, the cell lysates were subjected to SDS-PAGE analysis and Western blotting (D). (E and F) BMMs from WT, Irgm1−/−, or STAT1−/− mice were treated with 200 U/ml IFN-γ for 6 h or the indicated times. The cells were lysed in a cell lysis buffer with a protease inhibitor mixture, and then, the cell lysates were subjected to SDS-PAGE analysis and Western blotting. Densitometric LC3b-II/GAPDH and phosphorylation ratios are shown underneath the blot.
independent, PI3K-dependent signaling, as seen in human peripheral blood monocytes (6). The requirement for p38 MAPK is in accordance with recent observations that this pathway regulates binding of p38IP to Atg9 for trafficking to nascent autophagosomes (42). Hence, our findings may encourage efforts to focus on a subset of IFN-γ–induced target genes that can be transcribed in STAT1-independent fashion while engaging p38 MAPK en route to stimulating autophagosome formation. These efforts should have widespread implications for understanding autophagic and cytokine-based immunity to infection.

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Disclosures

The authors have no financial conflicts of interest.

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