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Supplementary Information for

Autophagy genes in myeloid cells counteract IFNγ-induced TNF-mediated cell death and fatal TNF-induced shock.

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Other supplementary materials for this manuscript include the following:

www.pnas.org/cgi/doi/10.1073/pnas.1822157116

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Supplementary Information Text

DETAILED MATERIALS AND METHODS.

Mammalian cells and treatments. BV2 microglial cells were cultured in D10 (DMEM (Gibco), 10% fetal bovine serum, and 1% HEPES (Gibco)). Primary bone marrow derived macrophages were grown in DMEM with 10% FBS, 10% CMG14-12, 1mM sodium pyruvate, and 1% GlutaMAX (Gibco). BMDMs were treated for experiments on D9 post differentiation (48 h after seeding in wells), and were derived from freshly isolated bone marrow, D0 frozen bone marrow, or from frozen D7 differentiated BMDM's (in 5% DMSO/ FBS), with similar results. All double knockout BV2 cell lines were on the *Atg5*KO clone 1 background, and this clone was used for *Atg5*KO experiments, unless otherwise noted. Recombinant cytokines, chemicals, and antibodies were obtained from the following sources and prepared as follows: murine IFNγ (Biolegend) and murine TNF (Peprotech) reconstituted in PBS (Gibco)/ 0.1% BSA (Fisher); Necrostatin-1 (Cayman Chemical), Necrostatin-2/ Nec-1s (Cayman Chemical), Ac-DEVD-CHO (BD Biosciences), and bafilomycin A1 (Sigma) solubilized in DMSO; TNF neutralizing antibody (clone TN3-19.2) or isotype control antibody (PIP) (Leinco).Addition of inhibitors and antibodies was just prior to addition of cytokines.

Retroviruses and lentiviruses. Gene blocks (IDT) containing N-terminal 3×Ty1 tag and murine *Becn1* was cloned into the pMXs vector using PCR and the Gibson reaction (New England Biolabs). Gene blocks containing N-terminal 3×Ty1 tag with murine *Atg16l1* or *Atg14* cDNA were cloned into pCDH-MCS-T2A-Puro-MSCV lentivirus vector (System Biosciences, CD522A-1) using the

Gibson reaction. Deletion constructs encoding 3×Ty1-ATG16L1ΔAFID (Δaa13-42) or 3×Ty1-ATG14ΔCCD (Δaa71-180) were generated by Gibson reaction. Wildtype BV2 cells used for puromycin treatment control were generated with a version of pCDH vector in which T2A sequence was exchanged with IRES sequence. sgRNAs for targeted polyclonal cell populations were cloned into BsmBI site of lentiGuide-Puro (Addgene #52963). All sequences for vectors used are available on request. Retroviruses and lentiviruses were produced in Plat-E (Cell Biolabs, Inc.) or 293T (ATCC) cells, respectively.

CRISPRko screening.

Primary screen: Cas9 activity was confirmed in stably expressing BV2 cells (BV2-Cas9) using pXPR_011 (Addgene #59702) EGFP expression on a FACSCalibur instrument (BD Biosciences). Library infectivity was determined by transducing variable volumes of lentivirus (0, 10, 20, 40, 80, or 160 µl) with a fixed volume of cells (3×10^6 cells in 2mL) in 50mL conical tubes by "spinoculation" (120 min × 1000 rcf at 30 °C), and a standard curve was then interpolated to determine volume needed to obtain desired infectivity. For library production, BV2-Cas9 cells were spinoculated at an MOI of 0.25 with Brie library (Addgene #73633), the number of live cells to achieve 500× coverage of the library (+10%) were plated in 15cm dishes, and media with puromycin exchanged after 48 hours. Cells were passaged to expand and on second passage cells were seeded at density of 1×10^7 cells in T-175 flasks to maintain 500× coverage per replicate. Twenty-four hours later, medium was exchanged with D10/ Puro ("mock") or D10/ Puro with 10 U/ mL IFNγ ("treated"). Mock samples were harvested after 24 h when flasks were confluent. Medium was exchanged with D10/ Puro in treated plates to remove dead cells after 48 h, and exchanged again after an additional 48 h period, then cells were harvested by trypsinization after further 72 h recovery.

*Atg5*KO suppressor screen: *Atg5*KO-Cas9 cells were confirmed to exhibit equivalent IFNγ hypersensitivity as the parental *Atg5*KO line. Cas9 activity was assessed as for WT cells and the Brie library was transduced into *Atg5*KO clone 1 (which had Cas9 activity most similar to WT). Given the greater magnitude of selective pressure for equivalent IFNγ doses, an additional 48 h was allowed for *Atg5*KO cells to recover before harvesting for genomic DNA (gDNA) preparation.

NGS sequencing and data analysis: Total sequencing results were as follows: WT screen, 46.7M reads (Mock) and 4.6M reads (10U IFN treated); *Atg5*KO screen, 38.6M reads (Mock) and 17.8M reads (10 U IFNγ treated). Correlation of scores for all sgRNAs between each sequencing sample in each condition were determined to evaluate for outliers and no sequencing samples were discarded. Scores were then averaged across all samples for each condition, and untreated average was subtracted from treated average to achieve the $\log_2$ fold changes for each sgRNA. Mean $\log_2$ fold change was then used to determine the hypergeometric distribution and p-values for volcano plots (github.com/mhegde/volcano_plots, hypergeom_2.3.1), and analyzed using STARS (v1.2) program to obtain ranked scores and FDR values. Hits with a STARS FDR < 0.1 were considered significant for further analysis.

Viability and cytotoxicity analyses. Cells were seeded in 96-well plates, treated with cytokines, compounds, and/or DMSO 18-24 h later, and assayed for viability after an additional 24 h. Cell viability experiments were performed in white opaque 96 well plates (Nunc) using CellTiter-Glo assay (Promega), and luminescence intensity quantitated with luminescence fiber on a Cytation 5 with Gen5 software (v2.09.1). Cytotoxicity was determined by the addition of Sytox Orange (ThermoFisher) or propidium iodide (Thermo) where indicated. Caspase 3 activity was measured with NucView 530 reagent (1 µM final) (Biotium). Live cell imaging was performed in optical bottom µClear plates (Greiner) with viability dyes, compounds, or vehicle solutions added at the

time IFNγ except where noted. Treatments were performed ~6 h after cell seeding for BV2 cells, and ~48 h for BMDMs. Images were acquired every 2-4 h on a Cytation 5 (Biotek) 2 × 2 montage with 4x objective, and analyzed with Gen5 Image+ software (v3.04) (Biotek). For BMDM experiments, cells were treated with IFNγ 8 h prior to addition of TNF, and imaging started at time of TNF addition. Area under curve measurements for cytotoxicity were performed in Prism with Y = 0 as baseline and from X = 0 to last time point.

Western Blot. Soluble lysates were prepared in RIPA buffer, combined with equal volumes Laemmli buffer (BioRad) and 5% 2-Mercaptoethanol. Samples were separated on Any KD Stain-Free gels (BioRad), transferred to low-fluorescence PVDF membrane, followed by blocking in Odyssey blocking buffer (TBS) (Li-Cor). Membranes were imaged on a ChemiDoc MP system with Image Lab software (v2.0.0.27) (BioRad). Raw 16-bit images were quantitated using ImageJ/Fiji (v1.0) with normalization of band intensities to either total transferred protein using background subtracted stain-free signal ("Tot. prot." in figures) in entire lane, or to actin intensity, as indicated for each experiment.

RNA-seq. WT or *Atg5*KO clone 2 cells were treated with media or IFNγ (1 U/ mL), and 24 h later cells were trypsinized, counted, and equal numbers of viable cells pelleted. Cell pellets were gently lysed in 100 µL buffer RLN (per Qiagen RY25 protocol: 50 mM Tris-Cl (pH 8.0), 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40 Substitute (USB)) per 8×10^5 cells for 10 min on ice, and then centrifuged briefly at 16k rcf to remove nuclei and incompletely lysed cells. Total RNA from cytosolic lysates was extracted with 1 mL TRI reagent (Sigma) per 200 µl lysate with isopropanol precipitation per manufacturer's instructions. Each biological replicate for library construction was generated independently. One µg of total purified RNA from cytoplasmic

fractions was enriched for poly-A-containing transcripts using Dynabeads mRNA DIRECT Purification kit (Life Technologies). mRNA was fragmented thermo-chemically in RT buffer (Agilent) in the presence of custom barcoded oligo-dT primer/adapters (IDT) at 94 C for exactly 4.5 min and immediately eluted from beads. Primers were allowed to anneal to transcripts at room temperature and cDNA was generated using AffinityScript Reverse Transcriptase (Agilent). Samples were normalized and pooled by relative levels of mActB transcripts. RNA from cDNA hybrids was degraded with 1M NaOH at 70 °C for 12 min and neutralized with 0.5 M acetic acid. 3' barcodes were removed by Agencourt AMPure XP beads (Beckman Coulter). Following ligation of a pre-adenylated 5' adapter (IDT) using Thermostable T4 ligase (NEB), and barcode cleanup, fragments were amplified using 12 cycles of enrichment with Fusion High-Fidelity Master Mix (NEB) with primers compatible with Illumina sequencing. Library quantity and quality was confirmed using High Sensitivity D1000 ScreenTape on a 2200 TapeStation (Agilent). Libraries were sequenced on an Illumina HiSeq 2500 instrument with a 2 × 101 run, demultiplexed using fastq-multx (v1.3.0) allowing 2 mismatches, and sequence quality was assessed using FastQC (v0.11.2). Trimming for poly-T sequences and quality was performed with bbduk (BBmap package) using quality score of 20 and minlen of 25. For read pairs where reads from poly-T end failed trimming threshold, the read mate that passed trimming criteria was analyzed as a single-end read. Read mapping was performed with STAR (v20201) to the GRCm38 genome with the following options: --outFilterMultimapNmax 15 --outFilterMismatchNmax 6. Reads were quantified using HTSeq (v0.6.1) with default parameters. Counts for mate pairs were analyzed as paired-end, and those for which a mate was analyzed as single end were combined in final counts file for differential expression. Annotations for which there were no reads in any sample were removed. Differential expression was assessed using DESeq2 (v1.10.1) in the R software package

(v3.2.3). An adjusted p-value < 0.1 was considered significant. Analyses and pathway comparisons were performed on normalized read counts.

Figure S1

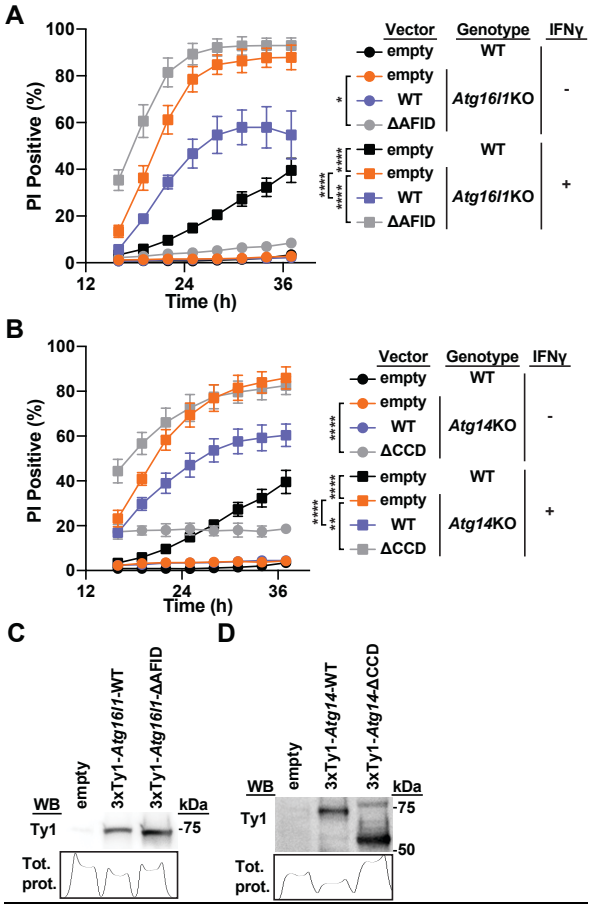


Fig. S1 (relates to Fig. 2). *Atg14* and *Atg16l1* protect against IFNγ cytotoxicity. Cytotoxicity of *Atg16l1*KO (A) or *Atg14*KO (B) compared to wildtype ("WT") BV2 cells transduced with "empty" vector or construct indicated ("WT", wildtype construct for mutated gene; "ΔAFID", ATG5 Interacting Domain deletion; "ΔCCD", Coiled-Coiled Domain deletion) with or without IFNγ (3U/mL). C) and D) western blot (WB) for 3×Ty1-tagged proteins as indicated in KO cell lines from (A) and (B), respectively. "Tot. prot." in (C) and (D) reflects intensity profile of total protein in each lane on membrane for p62 blot shown, which was used for loading control and the area under curve used for normalization in quantitation. p-values: *, <0.05; **, <0.01; ****, <0.0001; significant comparisons shown vs. KO line with empty vector for each IFNγ treatment by 2-way ANOVA with Dunnett's post-test. Data in (A) and (B) represent mean with SD of 4 technical replicates, with similar results observed in at least 3 independent experiments.

Figure S2

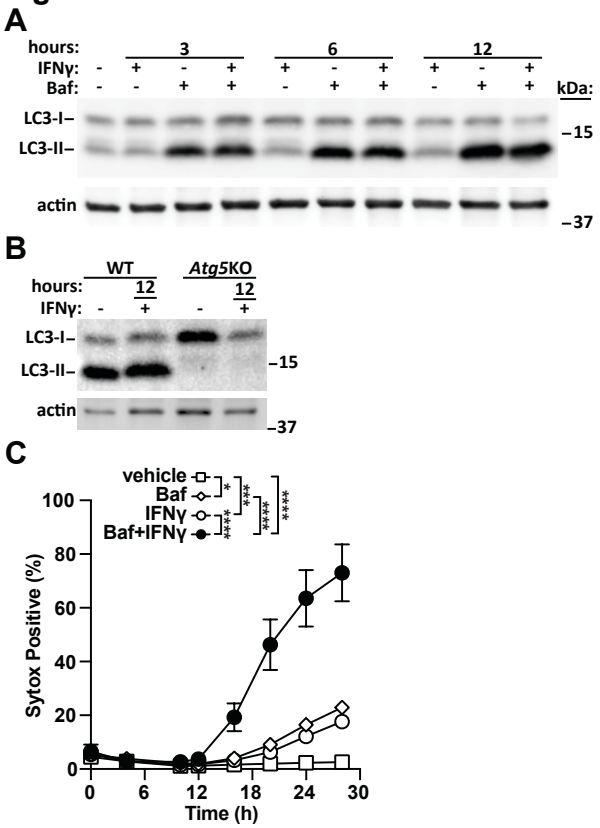
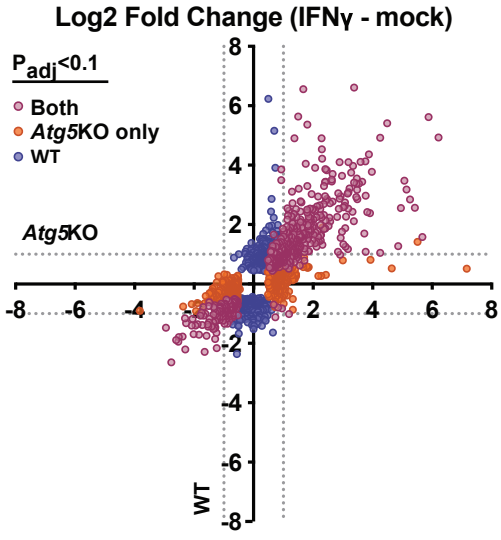
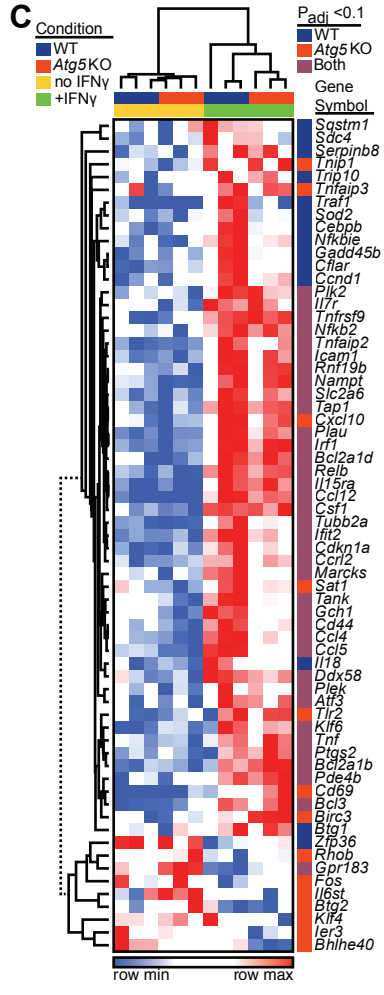
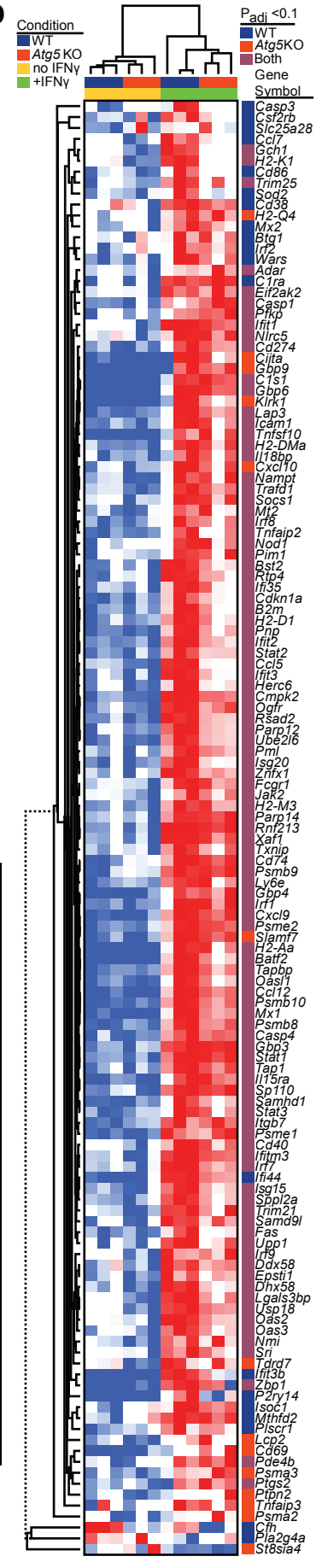


Fig. S2. Analysis of autophagic flux in BV2 cells during IFNγ treatment. A) Immunoblot for LC3 in WT BV2 cells treated with IFNγ (10 U/mL), bafilomycin A (Baf) (1 μM), and/or DMSO vehicle for time indicated. B) Immunoblot for LC3 in BV2 cells of genotype indicated treated with IFNγ (10 U/mL) for 12 h. G) Cytotoxicity of WT BV2 cells treated as indicated with DMSO (vehicle), Baf (50 nM), and/or IFNγ (10 U/mL). p-values: *, <0.05; ***, <0.001; ****, <0.0001; significant comparisons of area under the curve measurements shown between all conditions by 2-way ANOVA with Sidaks's post-test. Data in (C) represent mean with SD of 4 technical replicates, with similar results observed in at least 3 independent experiments.

Figure S3







B

Description	# Genes in Gene Set (K)	Both # genes	Both FDR	WT-only # genes	WT-only FDR	*Atg5* KO-only # genes	*Atg5* KO-only FDR
Genes up-regulated in response to alpha interferon proteins.	97	56	8E-85	11	1E-07	4	0.032
Genes up-regulated in response to IFNG [GeneID=3458].	200	97	4E-138	20	9E-12	14	3E-07
Genes up-regulated by IL6 [GeneID=3569] via STAT3 [GeneID=6774]	87	17	2E-16	7	2E-04	5	0.005
Genes regulated by NF-kB in response to TNF [GeneID=7124].	200	36	8E-33	15	1E-07	14	3E-07
Genes mediating programmed cell death (apoptosis) by activation of caspases.	161	24	5E-20	11	9E-06	12	1E-06
Genes involved in cholesterol homeostasis.	74	12	5E-11	8	9E-06	n.s.	n.s.
Genes up-regulated through activation of mTORC1 complex.	200	19	1E-12	18	5E-10	17	3E-09
Genes defining inflammatory response.	200	38	2E-35	6	0.04	10	2E-04
Genes up-regulated during transplant rejection.	200	37	4E-34	8	0.004	9	8E-04
Genes involved in p53 pathways and networks.	200	26	4E-20	9	0.001	16	9E-09
Genes encoding components of the complement system	200	25	5E-19	14	6E-07	9	8E-04
Genes up-regulated in response to ultraviolet (UV) radiation.	158	11	2E-06	11	9E-06	10	4E-05
Genes up-regulated by STAT5 in response to IL2 stimulation.	200	19	1E-12	13	3E-06	8	0.003
Genes up-regulated by KRAS activation.	200	15	1E-08	12	1E-05	13	2E-06
Genes up-regulated in response to low oxygen levels (hypoxia).	200	14	7E-08	9	0.001	16	9E-09
Genes involved in the G2/M checkpoint, as in progression through the cell division cycle.	200	12	3E-06	15	1E-07	8	0.003
Genes encoding proteins involved in glycolysis and gluconeogenesis.	200	10	8E-05	12	1E-05	12	1E-05
Genes encoding cell cycle related targets of E2F transcription factors.	200	7	0.0064	17	3E-09	7	0.01
Genes defining early response to estrogen.	200	11	2E-05	9	0.001	11	5E-05
A subgroup of genes regulated by MYC - version 1 (v1).	200	n.s.	n.s.	13	3E-06	8	0.003
Genes encoding proteins involved in processing of drugs and other xenobiotics.	200	21	1E-14	n.s.	n.s.	n.s.	n.s.

genes: min – max

FDR: min – max

Fig. S3. Gene Set Enrichment Analysis of WT and *Atg5*KO cells IFNγ (relates to Fig. 4). A) Scatter plot showing correlation of fold-changes between WT and *Atg5*KO cells after IFNγ treatment with adjusted p-value < 0.1 in both or either line (only genes with P_{adj} < 0.1 in at least one line shown). Dashed lines indicate 2-fold change. B) GSEA results for differentially expressed genes in the three different groups indicated in (A) with FDR q-value < 0.05. Shown are unique pathways among top 10 for all three groups. Color scales encompass all three groups. Pathways ranked by ratio of sum of genes across the three groups to the number of genes in the gene set. C-D) Heat map of genes annotated in Hallmark "Genes regulated by NF-κB in response to TNF" (C) or "Interferon Gamma Response" (D) pathways, with unsupervised hierarchical clustering by 1 - Pearson's for all genes in pathway. Treatment "Condition" annotation indicated at top of each column. Significant change (adjusted p-value <= 0.1) for both or either genotype indicated in "P_{adj} < 0.1" annotation. Color intensity relative in each row. Branch between highest level of clusters in (C) and (D) cropped for space (dashed line).

Figure S4

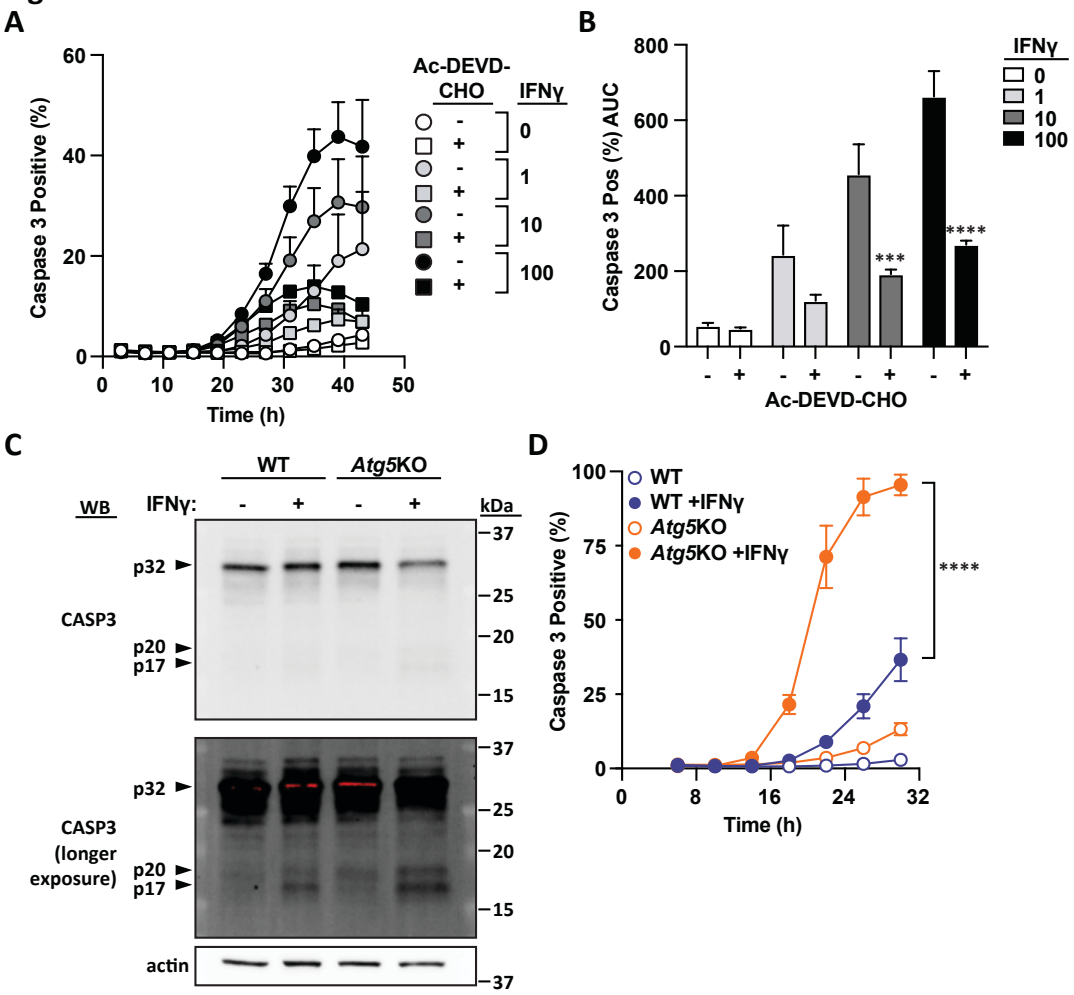
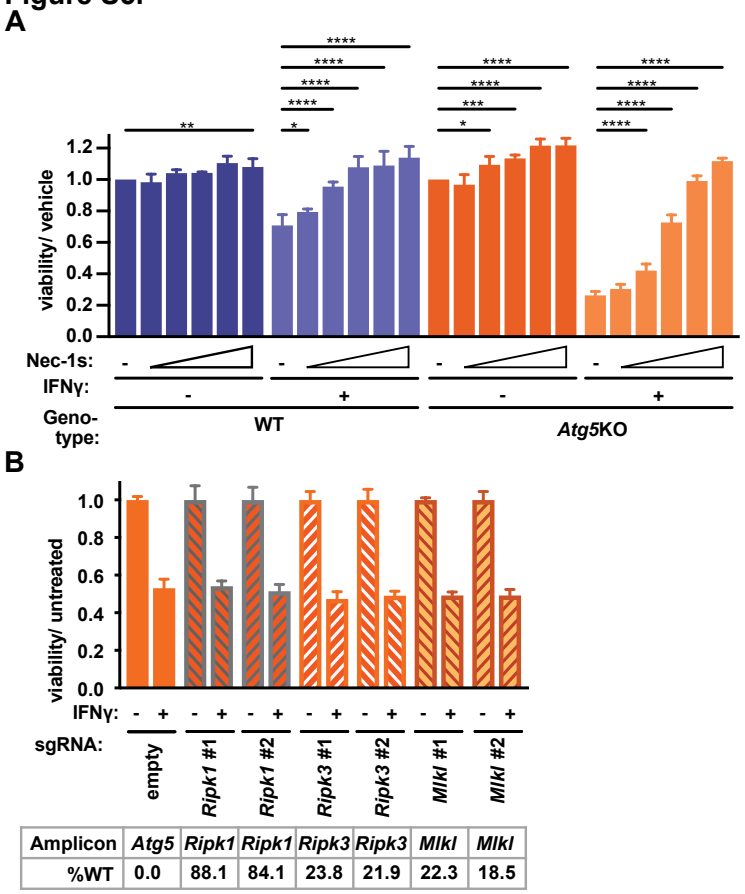


Fig. S4. Analysis of caspase 3 activity during IFNγ treatment. A) and B) NucView530 activity ("Caspase 3 Positive") of WT BV2 cells treated with dose of IFNγ indicated (U/mL) with (+) or without (-) Ac-DEVD-CHO (0.25 µM). Data in (B) are area under curve of data shown in (A). C) Immunoblot for caspase 3 in BV2 cell of genotype indicated treated with IFNγ (10 U/mL) for 24 h. D) NucView530 activity in BV2 cells of genotype indicated treated with IFNγ (10 U/mL). p-values: ***, <0.001; ****, <0.0001; significant comparisons of area under the curve measurements shown for Ac-DEVD-CHO (+ vs. –) for each dose of IFNγ in (B), and for WT vs. KO for each treatment in (D), by 2-way ANOVA with Sidaks's post-test. Data represent mean plus SD of 4 (A) or 6 (D) technical replicates. Similar results to (A-D) were observed in 3 independent experiments.

Figure S5.



Amplicon	*Atg5*	*Ripk1*	*Ripk1*	*Ripk3*	*Ripk3*	*Mlkl*	*Mlkl*
%WT	0.0	88.1	84.1	23.8	21.9	22.3	18.5

Fig. S5. Role of necroptosis mediators in IFNγ-induced death of *Atg5*KO cells (related to Fig. 5). A) Viability of cells of genotype indicated treated with increasing doses of necrostatin-1s (Nec-1s: 31, 63, 125, 250, or 500 nM) or DMSO (-) with or without IFNγ (1 U/mL). B) Viability of *Atg5*KO-Cas9 cells stably-expressing indicated sgRNA and treated with IFNγ (10 U/mL) proportional to untreated condition for each stable line. "%WT" in (B) indicates percent alleles with wildtype sequence based on NGS of amplicon that encompasses the target site in gene indicated (or parental clone mutation site for *Atg5*). p-values: *, <0.05; ***, <0.001; ****, <0.0001; in (A), comparison to DMSO for each IFNγ condition, in (B) each stable line to empty vector per IFNγ treatment; 2-way ANOVA with Dunnett's post-test. Data represent mean with SD of 3-4 technical replicates, and similar results were observed in at least 3 independent experiments.

Figure S6

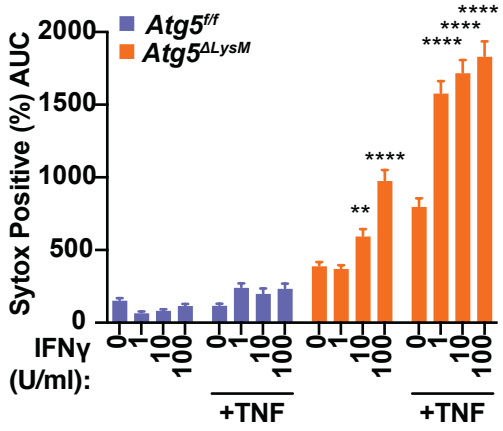


Fig. S6. IFNγ-induced cytotoxicity in primary BMDM's. Cytotoxicity in BMDM's from mice of the genotype indicated pretreated with dose of IFNγ indicated for 8 h prior to addition of TNF (20 ng/mL). p-values: **, <0.01; ****, <0.0001; comparison to "0 IFNγ" sample within each group; 2-way ANOVA with Dunnett's post-test. Data represent mean with SD of area under the curve for 46 h period after addition of TNF. Similar results were observed in 3 independent experiments.

Table S1 (separate file). CRISPRko screen results for WT and *Atg5*KO. For each screen (WT or *Atg5*KO) the average log_2 fold change ("LFC") for each gene and hypergeometric distribution with calculated p-value noted. STARS results for positive ("Pos") enrichment. Autophagy_GO pathway annotations are indicated for each set and the pathway is listed on a separate worksheet in Table S1.

Table S2 (separate file). RNA-seq results for WT and *Atg5*KO cells. DeSeq2 summary results for each cell line. Sequence files, normalized counts, and metadata for this analysis have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE132739 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132739).

Supplemental Dataset1 (separate file). Tab delimited file containing the log_2 normalized scores for each sgRNA in WT BV2 screen for IFNγ induced cell death. Biological replicates were averaged, and the difference of average IFNγ scores from average Mock scores was used as input for STARs analysis. Raw sequencing data is available upon request.

Supplemental Dataset1 Key (separate file). Tab delimited file with metadata for samples in Supplemental Dataset1 indicating the number of biological replicates per condition, gDNA technical preparations per biological replicate, and sequencing technical replicates for each gDNA sample. Scores used in normalization were the sum of all sequencing replicates for each gDNA sample.

Supplemental Dataset2 (separate file). Tab delimited file containing the log_2 normalized scores for each sgRNA in *Atg5*KO BV2 screen for IFNγ induced cell death. Biological replicates were

averaged, and the difference of average IFNγ scores from average Mock scores was used as input for STARs analysis. Raw sequencing data is available upon request.

Supplemental Dataset2 Key (separate file). Tab delimited file with metadata for samples in Supplemental Dataset1 indicating the number of biological replicates per condition, gDNA technical preparations per biological replicate, and sequencing technical replicates for each gDNA sample. Scores used in normalization were the sum of all sequencing replicates for each gDNA sample.