Cell Host & Microbe

Supplemental Information

Nondegradative Role of Atg5-Atg12/ Atg16L1 Autophagy Protein Complex in Antiviral Activity of Interferon Gamma

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Supplemental Experimental Procedures

Mice and Cells. Mice expressing the Cre recombinase from within the lysozyme M locus (*LysMcre* mice) were purchased from the Jackson Laboratory (Strain # 004781). *Atg5*$^{flox/flox}$ and *Atg5*$^{flox/flox}$*+LysMcre* mice with two different backgrounds (Figures 1C and 1D from mixed 129-C57BL/6 mice and all the others from C57BL/6 mice) were used for this study and consistent phenotypes between different backgrounds were observed.

To generate bone marrow derived macrophages (BMDM), mouse bone marrows were isolated and cultured on non-tissue-culture treated plate for 7 days in BMDM media (Dulbeco's Modified Eagle Medium, 10% fetal bovine serum, 5% horse serum, 10% CMG14-12 (Takeshita et al., 2000), 1x MEM nonessential amino acids, 1mM sodium pyruvate, 2 mM L-glutamine). At day 7, adherent BMDMs were dissociated from the plate and set up for experiment. At day 10, media were changed to fresh media and IFNγ were treated if necessary. At day 11, cells were used for MNV infection or autophagy analysis. Depending on the deletion status of floxed gene (e.g. *Atg7*$^{flox/flox}$*+LysMcre*), BMDMs were cultured up to 21 days and used for infection study. For EMCV and MHV, BMDMs were prepared as previously described (Zhao et al., 2007).

Microarray. Transcript levels in control and Atg5-deficient macrophages with and without IFNγ treatment were compared using microarrays. Fold-change induced between treated and untreated conditions were computed separately for control and Atg5-deficient macrophages (N=2). Briefly, RNA collected from IFNγ treated and untreated control and Atg5 deficient bone marrow macrophages was profiled using Affymetrix Mouse M430 2.0 (Figure 2A). The data were normalized using GC Robust Multi-array Average normalization routine in Matlab. Multiple filters were applied to remove non-informative genes and genes with low absolute values or small profile variance. The IFNγ treated datasets, from both control and Atg5-deficient macrophages, were normalized to the respective data from untreated macrophages. An unpaired two-sample T-test was used to identify genes with significant differences in fold-change induction between control and Atg5-deficient macrophages (P-value < 0.05). To estimate the number of false positives in multiple hypotheses testing, a false discovery rate

(FDR) was computed using the Storey-Tibshirani procedure. FDR-adjusted p-values were computed using the linear step-up procedure introduced by Benjamini and Hochberg.

Viruses and Infection. All MNV experiments were performed with strain MNV-1.CW3 (Thackray et al., 2007). Virus stocks were generated using adherent RAW 264.7 cells in VP-SFM media (Gibco) as previously described (Chachu et al., 2008). Day 7 BMDMs were plated in 24-well plate (1 x 10^5 cells/well) for growth analysis by plaque assay or protein analysis by western blot and in 6-well plate (5 x 10^5 cells/well) for replication analysis by flow cytometry. Cells were treated with drugs or IFNs for 12 hours and then inoculated with viruses for 30 min to 1 hr at either room temperature (for MNV, EMCV, and MHV) or 37°C (for WNV). After inoculation, cells were washed twice and incubated at 37°C for 12 or 24 hours. The infected cells were harvested by fixation (for flow cytometry and immunofluorescence), by freezing (for growth analysis by plaque assay), or by lysing with sample buffer (for western blot).

Growth Analysis by Plaque assay. The titer of MNV was measured by plaque assay as previously described (Chachu et al., 2008) except for modification of the overlay media (MEM, 10% FBS, 1% Pen/Strep, and 1% methyl cellulose) and staining with 20% ethanol/1% crystal violet solution. For the titration of encephalomyocarditis virus (K strain), L929 cells were used and assayed similarly to MNV as described. Plaque assay for the Murine Hepatitis virus (A59 strain) was performed as previously described (Zhao et al., 2007). For West Nile virus (North American isolate, lineage 1 WNV strain 3000.0259), Vero cells were used for titration as previously described (Diamond et al., 2003).

Drugs. Drugs were purchased from the following company and used as indicated in figure legends: rapamycin (sc-3504, 5 mM in DMSO), wortmannin (sc-3505, 5 mM in DMSO), bafilomycin A_1 (sc-201550, 100 uM in DMSO), E64D (sc-201280, 5 mg/ml in DMSO), pepstatin A (sc-45036, 5 mg/ml in DMSO) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); chloroquine (C6628, 20 mM in water) from Sigma; LY294002 (#9901, 10 mM in DMSO) from Cell Signaling; mouse IFNγ (485MI/CF, 200 U/ul (100 ng/ul) in PBS/0.1%BSA) from R&D Systems; mouse IFNβ (12400-1, 100 U/ul in PBS/0.1%BSA) from PBL; N-ACETYL-L-CYSTEINE (A15409, 100 mg/ml (612 mM) in water) from Alfa Aesar.

Protein analysis by western blot and immunoprecipitation. To detect MNV antigen expression in infected cells, a high-titer hyperimmune serum specific for recombinant MNV ProPol protein (Sosnovtsev et al., 2006) was generated in rabbits. Briefly, rabbits were immunized with three sequential doses of purified recombinant ProPol (250 ug per boost). Rabbit antibody against MNV capsid was described previously (Wobus et al., 2004). Commercial antibodies used in this study are as follows: Atg4B (Sigma, A2981), Atg5 (Sigma, A2859), Atg7 (Sigma, A2856), Atg16L (Sigma, A7356), Beta-actin (Sigma, A5316), FLAG (Sigma, F3165 and F7425), LC3B (Sigma, L7543), p62 (Sigma, P0067), Rab7 (Cell Signaling, Danvers, MA, #9367) and V5 (Invitrogen, R960-25).

Flow cytometry. Infected BMDMs were fixed at 12 hpi using 4% formaldehyde (Ted Pella, CA; 18505) for 10 min at room temperature (RT) and permeabilized with PBS/0.2% TritionX-100 (PBSTX) for overnight at 4C. Cells were stained with rabbit anti-propol antibody at 1:1000 dilution in flow blocking buffer (PBS/0.2% TritionX-100/1% normal goat serum/1% normal mouse serum) at RT for 1 hr. Cells were then washed with PBSTX, stained with DyLight 649 Donkey anti-rabbit IgG (Biolegend, CA; 406406) at RT for 1 hr, and washed with PBSTX before data acquisition. Flow data was acquired using FACSCalibur flow cytometer (BD Biosciences, CA) and analyzed using FlowJo software (Tree Star, OR). Uninfected/stained samples were used as gating controls; infected/stained samples without treatment were used to calculate relative infection for IFN-treated samples.

Immunofluorescence analysis. BMDM cells (Figure 7F) were grown on cover glass (Fisher Scientific, PA; 12-545-80) and fixed/permeabilized as described above. Cells were then stained with rabbit anti-propol antibody in blocking buffer (PBS/0.2% TritionX-100/1% normal goat serum/10% normal mouse serum) at RT for 1 hr and then stained with Alexa Fluor 555 goat anti-rabbit antibody. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Due to the lack of specific antibodies to detect the localization of endogenous proteins in macrophages, plasmids expressing FLAG-tagged Atg5, Atg12, and Atg16L1 were transfected with a modified MNV reverse genetic construct (Ward et al., 2007) into mouse embryonic fibroblast (MEF) (Figure 6). At 24 hrs after transfection, the cells were fixed/permeabilized as described above. Cells were then stained with mouse anti-FLAG and rabbit anti-propol antibody in blocking buffer (PBS/0.2% TritionX-100/1% normal goat serum) at RT for 1 hr and then stained with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit antibodies. MNV reverse genetic construct was modified to express ORF1 (polyprotein for MNV replication complex) of MNV-1.CW3 (Strong *et al.*, submitted). In this MEF system, we confirmed that Atg5 is also required for IFNγ to suppress MNV replication (Figure S5A). Images were obtained and analyzed using Olympus BX51 (Olympus America, PA) and SPOT Advanced software (Diagnostic Instruments, MI).

STORM Setup and Imaging. The STORM microscope was constructed around a Nikon Ti-E inverted microscope operating in wide-field epifluorescence mode and using the Nikon Perfect Focus system for focus stabilization. The activation/imaging lasers (CUBE 405nm-50, Sapphire 561nm-150, Sapphire 460-10 from Coherent and Stradus 642nm-110 from Vortran Laser) were individually shuttered by a mechanical shutter (Uniblitz LS3ZM2, Vincent Associates) and an acousto-optical tunable filter (AOTF, PCAOM, Crystal technology). Laser lines were combined, expanded and collimated before being focused at the back focal plane of a 100x objective (100X UPlanSApo NA 1.4, Olympus). A quad band dichroic mirror (zt405/488/561/640rpc from Chroma) and a band-pass filter (ET705/70m, Chroma) separated the fluorescence signal collected by the same objective from the excitation light. Images were recorded with an EMCCD camera (iXon Plus DU897, Andor) and a Dual View system (Photometrics DV2) inserted between the microscope and the camera as an image relay. The Dual View splitting cassette was replaced with a cylindrical lens (1M focal length) to create the astigmatism for 3D imaging. MEF cells were plated on a No. 1.5 coverglass, transfected and processed for immunofluorecense as described above. Secondary antibodies for STORM were purchased from Jackson Immunoresearch and custom conjugated with photoswitchable dye pairs as described before (Bates et al., 2007). Imaging was performed by inverting coverslips onto a glass slide with imaging buffer (80:10:10:1 ratio of DPBS, 1 M mercaptoethylamine (pH 8.5),

50% glucose solution, and an antibeaching oxygen scavenger system (10 mg of glucose oxidase + 25 ml of catalase and 100 ul of DPBS). Excess buffer was drained and edges of coverglass were sealed with nailpolish before image acquisition. Astigmatism STORM imaging (Huang et al., 2008) allowed us to observe the distribution of these proteins in 3D.

RNA analysis by northern blot and RNase protection assay. Purified RNA was subjected to electrophoresis on a 1% formaldehyde gel and transferred onto Nytran SPC membrane using TurboBlotter (GE Healthcare, 10416304). The nt 5,750 to 6,666 (major capsid protein VP1) of the MNV-1.CW3 genome and nt 446-596 of mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank: GU214026.1) were cloned into the pCRII-TOPO vector (Invitrogen; K4650-01). The resulting plasmid was linearized and *in vitro* transcribed with ^{32}P-UTP using MAXIscript T7 Kit (Applied biosystems; AM1312) to generate RNA transcript probes.

The synthesis and/or accumulation of negative strand RNA was analyzed using an two-cycle RNase protection assay (RPA) as previously described (Novak and Kirkegaard, 1991). For synthesis of RNA probes for two-cycle RNase protection assay (RPA), the nt 4,500 to 4,760 (polymerase) of the MNV-1.CW3 genome was cloned into the pCRII-TOPO vector (pCRII4500-4760). A 339 nt plus polarity RNA probe that protects a 261 nt fragment of the negative strand genomic RNA was synthesized with SP6 polymerase from Not I linearized pCRII4500-4760 (MNV probe). A 283 nt minus polarity RNA probe, that protects a 151 nt fragment of mouse GAPDH, was synthesized with T7 polymerase from Hind III linearized pCRII-GAPDH described above (GAPDH probe) and used as a control. Synthesized probes were gel extracted and ethanol precipitated before use and labeled probes were synthesized to a specific activity of ~ 1 x 10^8 cpm/ug (MNV probe) and 1 x 10^6 cpm/ug (GAPDH probe) and used within 2 days.

ATP concentration. Macrophages were incubated for 2 hrs with media alone or media containing 25mM 2-deoxyglucose + 3 μM antimycin to inhibit glycolysis and mitochondrial ATP generation, respectively. The cells were lysed on ice in ATP lysis buffer (50mM Tris pH 7.4, 300mM NaCl, 5mM EDTA, 0.5% Triton X-100, + complete protease inhibitors (Roche, Basel, Switzerland)). Lysates were plated in duplicate in a white, opaque 96-well flat-bottom plate and mixed 1:1 with CellTiter-Glo reagent (Promega, Madison, WI) for 2 minutes on an orbital shaker before being read on a luminometer. An ATP standard curve was determined by diluted concentrated ATP directly into ATP lysis buffer.

Cycloheximide chase. Control and Atg5 deficient bone marrow derived macrophages were infected at MOI=5 after pre-treatment of none or 10 U/ml of IFNγ. At 8 hrs post infection, cells were treated with 40μM cycloheximide and 10μM anisomycin and harvested at 0, 2, 3 and 4 hrs after addition of translation inhibitors for western blot. To test effectiveness of translation inhibitors, control macrophages were treated with decreasing concentrations of cycloheximide and anisomycin for 30mins. Cells were pulsed with [^{35}S]-cysteine-methionine for 2hrs and lysates were run on SDS-PAGE gel and analyzed by autoradiography.

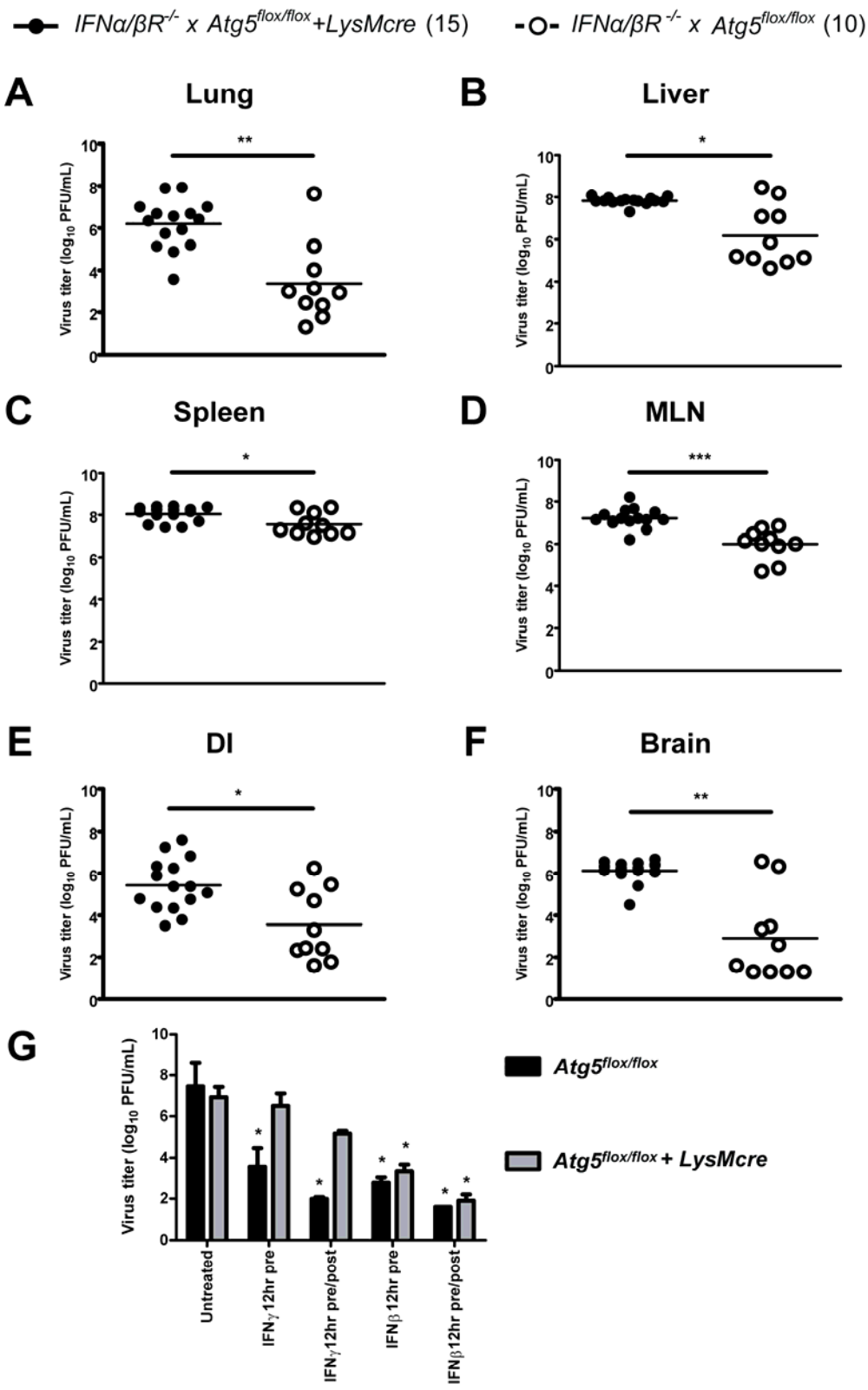

Figure S1. Hwang et al.

Figure S1. Atg5 was required for the control of MNV by IFNγ *in vivo* and *in vitro*, related to Figure 1. (A-E) Tissue viral burden in mice 5 days after inoculation with 3 x 10^4 pfu of MNV - lung (A), liver (B), spleen (C), mesenteric lymph node (MLN, D), distal ileum (DI, E), and brain (E). Atg5-deficient mice were compared to control mice. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$). (F) Growth analysis of MNV in control and Atg5-deficient macrophages (N=2) at 24hpi (MOI=0.05) treated with 100 U/ml of IFNγ or IFNβ either 12 hr before inoculation (**pre**) or before/after inoculation (**pre/post**). Mean viral titer ± SEM are shown. * indicates statistically significant difference ($p<0.05$) compared to untreated.

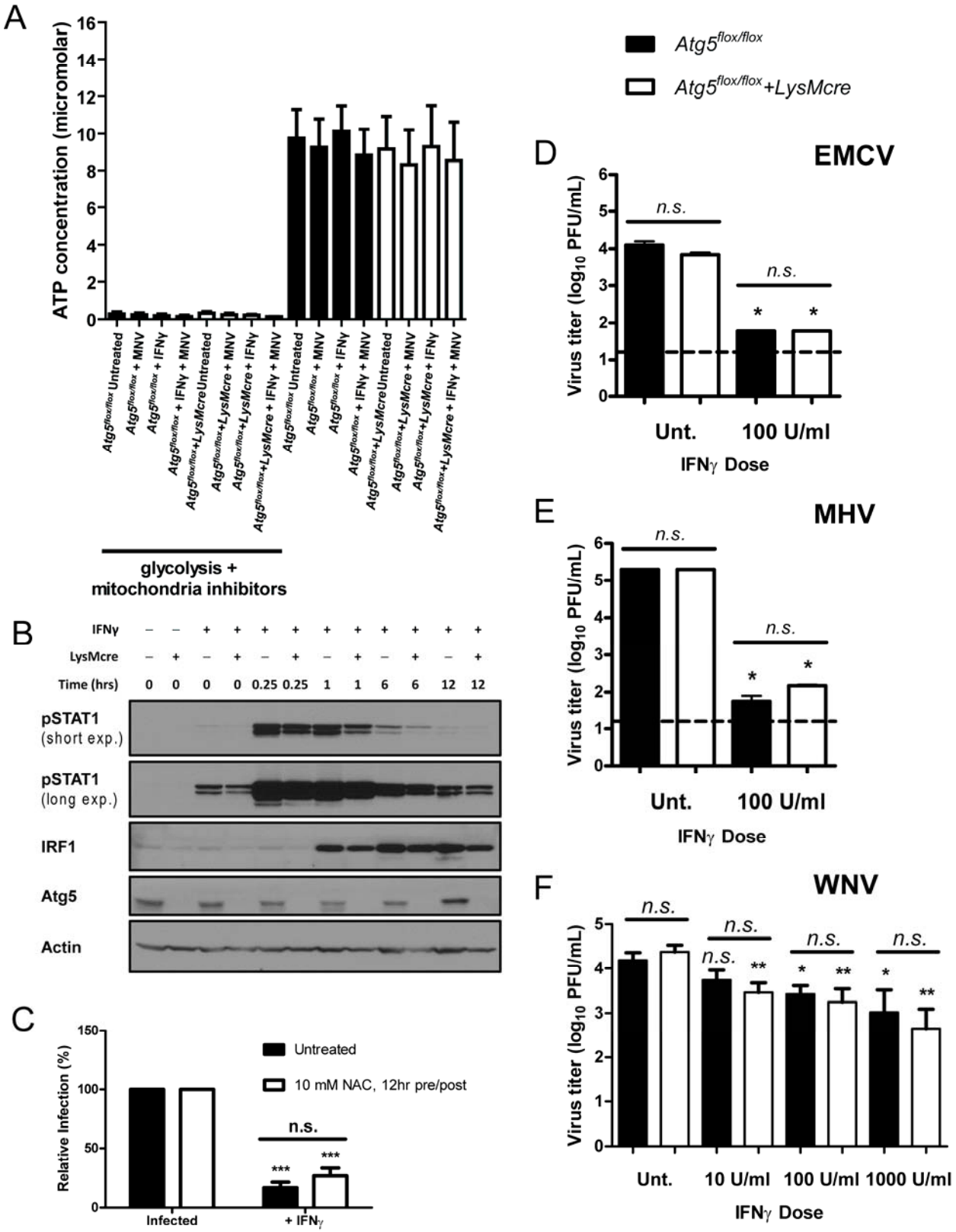

Figure S2. Hwang et al.

Figure S2. Atg5 is dispensable for cell homeostasis and IFNγ signaling in primary macrophages, related to Figure 2. (A) ATP concentration of untreated, IFNγ treated (10 U/ml for 12hrs), MNV inoculated, and IFNγ treated + MNV inoculated control (*Atg5*$^{flox/flox}$) and Atg5-deficient (*Atg5*$^{flox/flox}$*+LysMcre*) macrophages. The first eight bars represent macrophages treated for 2 hrs with 2-deoxyglucose and antimycin to inhibit glycolysis and mitochondrial ATP generation, respectively. ATP levels between control and Atg5 deficient macrophages were not statistically different (N=3). (B) A representative western blot (N=2) of the phosphorylation of STAT1 and the induction of IRF1 after treatment with 10 U/ml IFNγ for the time indicated. (C) Lack of a role for ROS in IFNγ-mediated control of MNV replication. Macrophages were treated with 10 mM of NAC (N-ACETYL-L-CYSTEINE) for 12 hours before and during MNV infection (total 24 hours) and MNV replication was analyzed by flow cytometry (N=3). (D-F) Growth analysis of encephalomyocarditis virus (EMCV, D), murine hepatitis virus (MHV, E), and West Nile virus (WMV, F) in control and Atg5-deficient macrophages (N=2). Macrophages were untreated or pre-treated with the indicated dose of IFNγ for 12 hr before infection and then inoculated at low (0.05) MOI. At 24 hpi, virus titers were measured by plaque assay. In all graphs, data represent mean ± SEM.

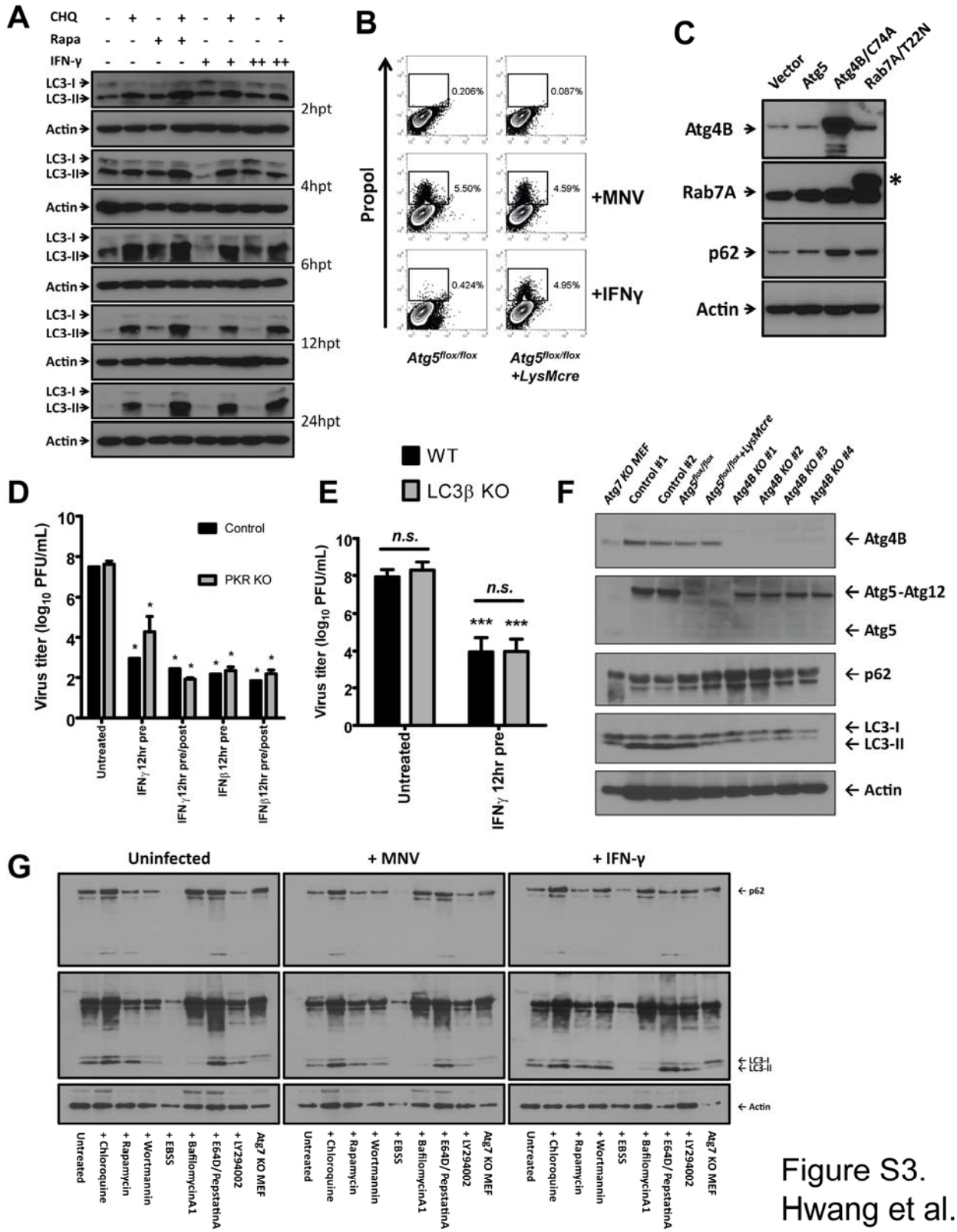

Figure S3.
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Figure S3. Catabolic autophagy was not required for the IFNγ-mediated suppression of MNV replication, related to Figure 3. (A) The effect of IFNγ on the autophagy pathway. A representative western blot (N=3) of lipidation status of LC3 (LC3-II) after treatment with chloroquine (CHQ, 20 uM), rapamycin (Rapa, 10 uM), or IFNγ (+: 1 U/ml, ++: 100 U/ml of IFNγ). Actin was detected as loading control. (B) Propol expression as marker for MNV replication. Control and Atg5-deficient macrophages were fixed and stained with anti-propol antibody at 12 hpi (MOI=5). Mock (left), inoculated (+MNV, middle), and inoculated after 12 hr pre-treatment with 100 U/ml IFNγ (+IFNγ, right). (C) A representative western blot (N=3) of Atg4B, Rab7A, p62 in macrophages transduced with lentivirus control vector or expressing Atg4B/C74A or Rab7A/T22N. Actin was detected as loading control. * indicates Rab7A-T2A. (D) Growth analysis of MNV (N=2) in control and PKR-deficient macrophages at 24 hpi (MOI=0.05). 100 U/ml of IFNγ or IFNβ were treated either 12 hr before inoculation (**pre**) or before/after inoculation (**pre/post**). * indicates statistically significant difference ($p<0.05$) compared to untreated. Mean ± SEM. (E) Growth analysis of MNV (N=4) in wild type (WT) and LC3β deficient (LC3β KO) macrophages at 24hpi (MOI=0.05) treated for 12 hr before inoculation with 10 unit/ml of IFNγ. *** ($p<0.001$). Mean ± SEM. (F) A representative western blot (N=2) of Atg4B, Atg5, p62, and LC3 in macrophages from control and Atg4B KO mice. Actin as loading control. (G) The effect of drug treatment on the autophagy in mock, MNV-inoculated, and IFNγ-treated/MNV-inoculated macrophages from C57BL/6 mice. A representative western blot (N=2) of p62 level and lipidation status of LC3. Drugs (20 uM chloroquine, 10 uM rapamycin, 50 nM wortmannin, 100 uM bafilomycinA_1, 10 ug/ml of E64D/PepstatinA, 10 uM LY294002 or EBSS for starvation) were treated with or without 100 U/ml of IFNγ for 12 hrs before inoculation. After inoculation of MNV (MOI=5), drugs were added again. At 12 hpi, total cellular proteins were harvested and subjected to western blot. Actin was detected as loading control. Atg7 deficient MEF (Atg7 KO MEF) cell lysate was used as an autophagy-deficient control.

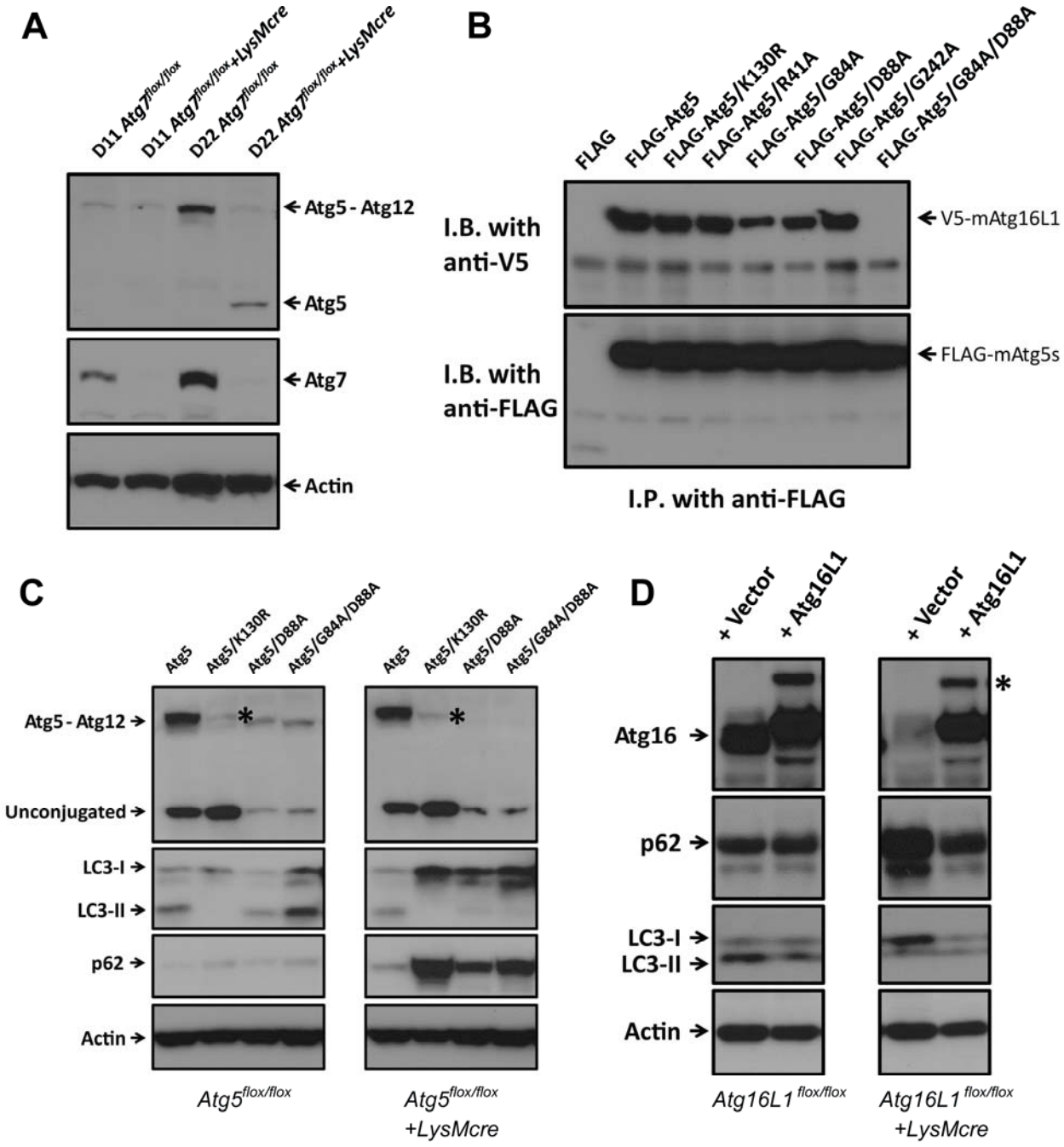

Figure S4. Hwang et al.

Figure S4. Atg5-12/Atg16L1 was required for IFNγ-mediated suppression of MNV replication, related to Figure 4 and 5. (A) Functional deletion of Atg7 in *Atg7*$^{flox/flox}$*+LysMcre* macrophages. A representative western blot (N=3) of Atg5 and Atg7 in control and Atg7-deficient macrophages 11 or 22 days in culture. Actin was detected as loading control. (B) Identification of amino acids in Atg5 critical for its binding to Atg16L1. Mouse Atg5 wild-type and mutants were cloned into a FLAG-tagging plasmid and mouse Atg16L1 was cloned into a V5-tagging plasmid using the GATEWAY cloning system (Invitrogen). At 48 hrs post-transfection of 293T cells with V5-mAtg16L1 and FLAG-mAtg5 mutant as indicated, the transfected cells were lysed and subject to immunoprecipitation using anti-FLAG antibody. Total immune-complexes were then subject to western blot (N=2) detecting FLAG (immunoprecipitated) and V5 (co-immunoprecipitated). (C) A representative western blot (N=3) of Atg5, LC3, and p62 in control and Atg5-deficient macrophages transduced with lentiviral vector expressing Atg5, Atg5/K130R, Atg5/D88A, or Atg5/G84A/D88A. Actin was detected as loading control. * indicates the uncleaved form of Atg5/K130R-T2A-copGFP. (D) A representative western blot (N=3) of Atg16L1, p62, and LC3 in control and Atg16L1-deficient macrophages transduced with lentivirus control vector or expressing Atg16L1. Actin was detected as loading control. * indicates the un-cleaved form of Atg16L1-T2A-copGFP.

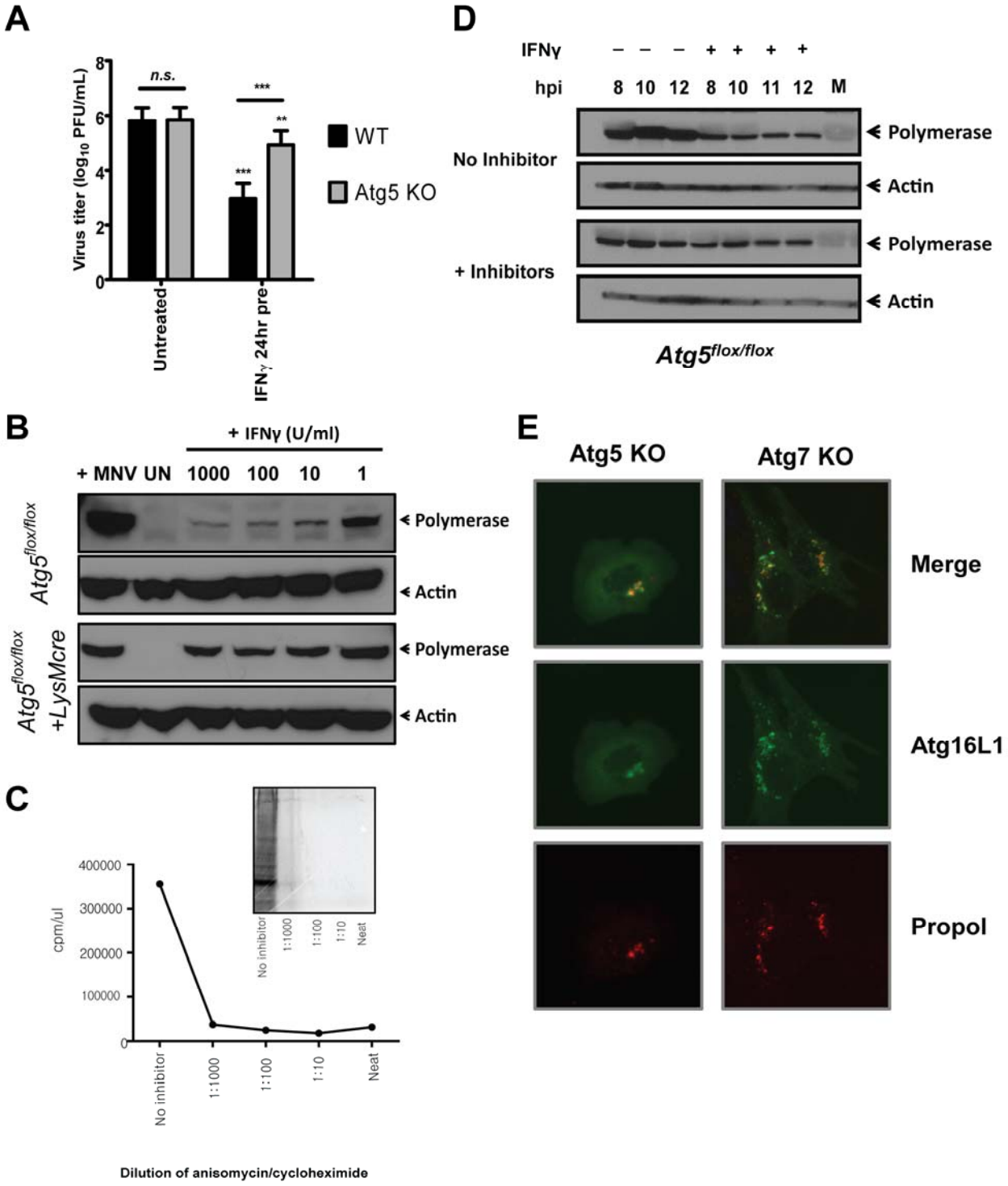

Figure S5. Hwang et al.

Figure S5. Atg5 is required for IFNγ-mediated suppression of MNV replication, related to Figure 6 and 7. (A) Infectious virus production of MNV (N=4) in viral RNA transfected wild type (WT) and Atg5 deficient (Atg5 KO) MEFs. MEFS were treated with 100 U/ml of IFNγ 24 hr before transfection of MNV viral RNA (viral RNA purified from virions). ** ($p<0.01$), *** ($p<0.001$). Data represent mean ± SEM. (B) A representative western blot (N=2) of the expression of non-structural MNV polymerase in control and Atg5-deficient macrophages at 12 hpi with MNV (MOI=5) after 1000, 100, 10, or 1 U/ml of IFNγ pre-treatment for 12 hrs. Actin was detected as loading control. (C) Control macrophages were treated with varying concentrations of anisomycin/cycloheximide and new protein synthesis was monitored by ^{35}S labeling. (D) Control and Atg5 deficient macrophages were treated with IFNγ (10 U/ml) or media then infected with MNV at MOI=5. Anisomycin/cycloheximide cocktail were added to the cells 8 hpi, at doses sufficient to inhibit cellular translation (Neat in C). Lysates were harvested at serial time points after addition of cocktail up to 12 hrs post inoculation. Representative picture from 2 independent experiments. M = mock infected. (E) Localization of FLAG-tagged Atg16L1 in relation to MNV replication complex as detected by staining for viral propol in transfected Atg5 or Atg7 knock-out (KO) MEFs.

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