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Supplemental Information

Interleukin-22 Inhibits Respiratory Syncytial Virus Production by Blocking Virus-Mediated Subversion of Cellular Autophagy

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SUPPLEMENTAL DATA ITEMS

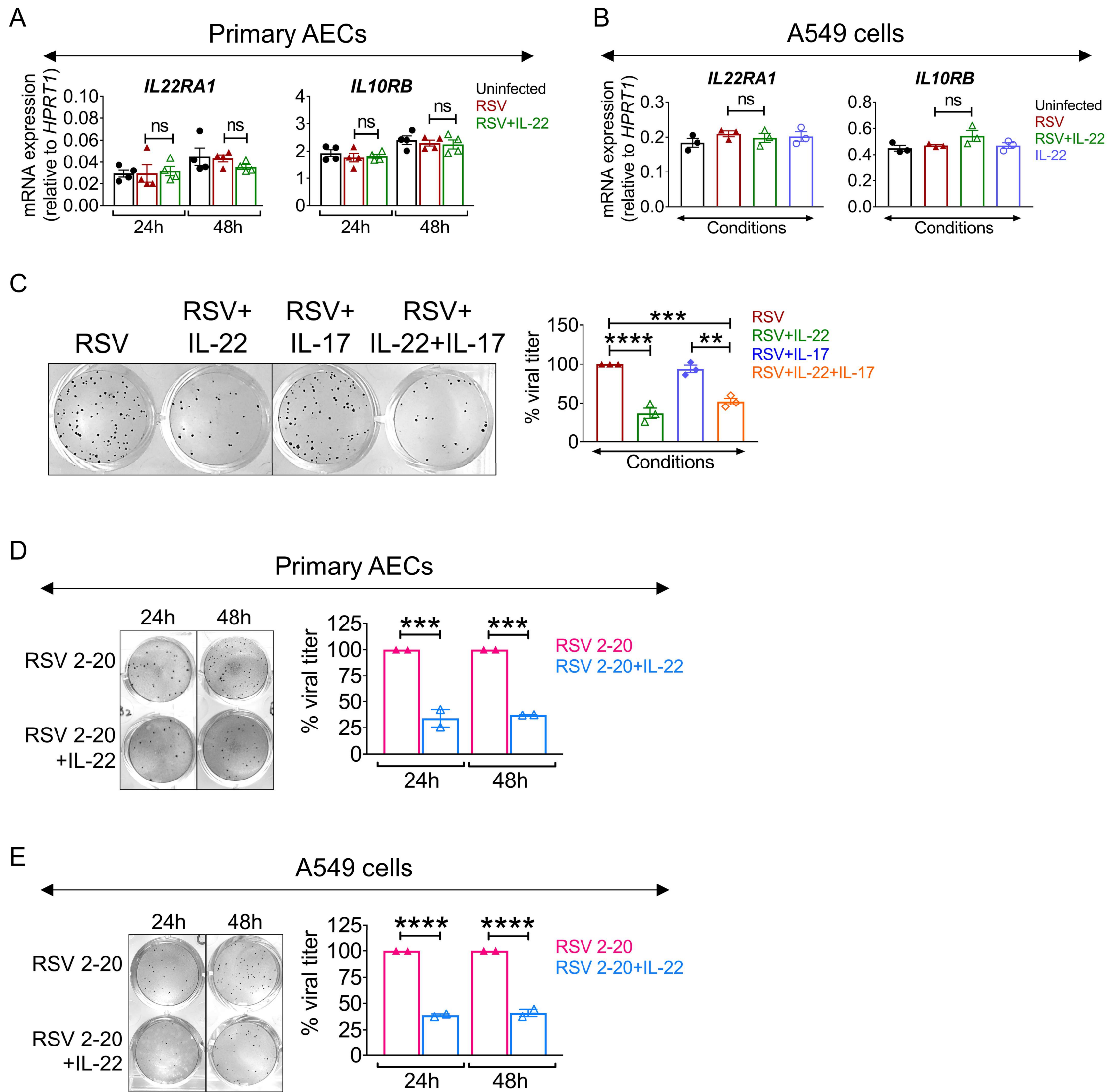

Figure S1. Similar levels of expression of IL-22 receptor subunits in AECs, inability of IL-17 to block RSV production, and IL-22-mediated inhibition of production of RSV strain 2-20. Related to Figure 1.

(A-B) mRNA expression of IL-22 receptors *IL22RA1* and *IL10RB* in **(A)** primary AECs (24 and 48 hours p.i.) and **(B)** A549 cells (24 hours p.i.) measured by quantitative RT-PCR after RSV infection ± IL-22 treatment. Data shown are mean ± SEM of 4 independent subjects (for primary AECs) and 3 independent experiments (for A549 cells). ns - non-significant.
(C) Representative viral plaques (left) and quantitated percent viral titer (right) in RSV-infected A549 cells with/without IL-22 and/or IL-17 treatment. Data shown are mean ± SEM of 3 independent experiments. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.
(D-E) Representative viral plaques (left) and quantitated viral titer (right) in RSV 2-20–infected **(D)** primary AECs and **(E)** A549 cells with/without IL-22 treatment. Percent viral titer for RSV 2-20 alone considered as 100% for each individual experiment. Data shown are mean ± SEM of 2 independent experiments. ***$P < 0.001$, ****$P < 0.0001$, ns - non-significant.
For statistical analysis, groups were compared using one-way ANOVA with Tukey post hoc test (A-E).

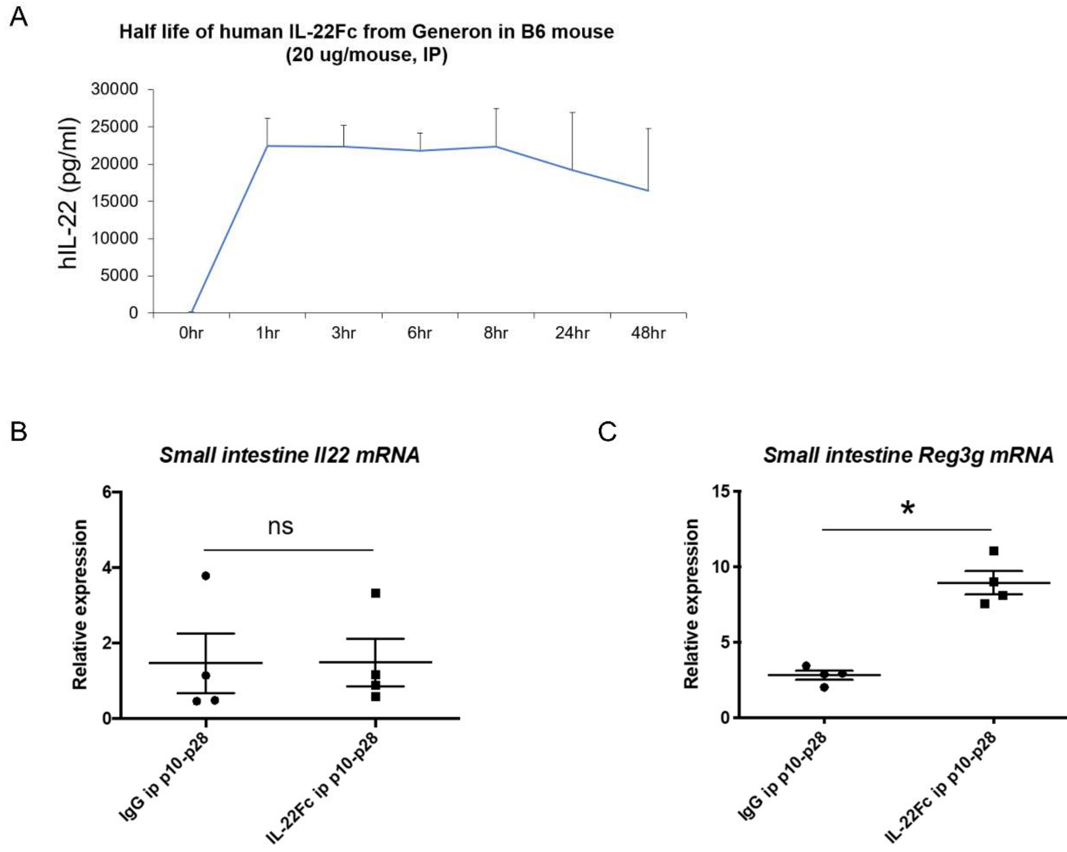

Figure S2. Properties and effects of human IL-22Fc in mice. Related to Figure 1.

(A) Half-life of human IL-22Fc was calculated to be approximately 107 hours. Data shown are mean ± SEM of 2 independent experiments.
(B-C) C57BL/6 (Jax) neonatal animals were injected with an equivalent amount of human IgG or IL-22Fc (1□g/gram body weight, Generon) intraperitoneally twice weekly from postnatal day 10-28. Small intestine mRNA for **(B)** *Il22* and **(C)** *Reg3g* was evaluated relative to the housekeeping gene *Rplo*. Representative data shown are mean ± SEM of 2 independent experiments and each mouse represents an individual symbol on the graph.
For statistical analysis, groups were compared using Mann Whitney test (A-B).

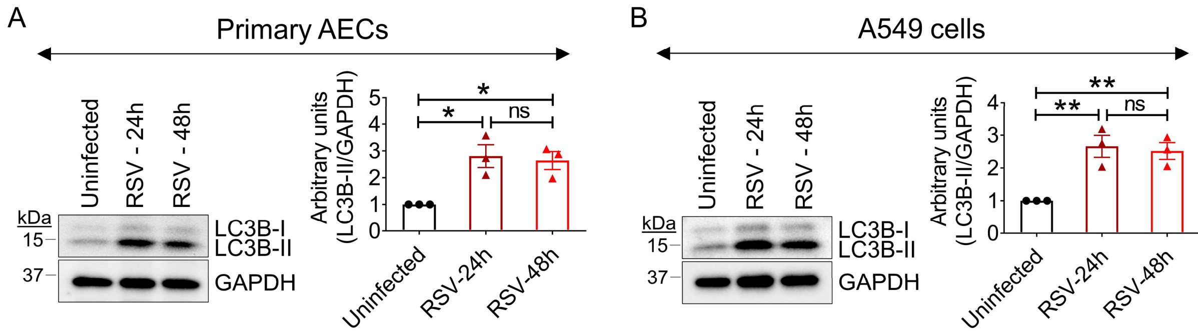

Figure S3. RSV-induced increase in LC3B expression in infected epithelial cells. Related to Figure 4.

(A-B) Representative immunoblot (left) and quantitation (right) of LC3B expression in **(A)** primary AECs and **(B)** A549 cells after RSV infection. Data shown are mean ± SEM of 3 independent experiments. *P < 0.05, **P < 0.01, ns - non-significant.

For statistical analysis, groups were compared using one-way ANOVA with Tukey post hoc test (A-B).

TRANSPARENT METHODS

EXPERIMENTAL MODEL AND RESEARCH DETAILS

Mice

C57BL/6J mice (Cat# 000664) were purchased from The Jackson Laboratory. IL-22$^{-/-}$ mice on C57BL/6 background (Zheng et al., 2007) were available from MMRRC. Mice were housed and bred under pathogen-free conditions in the Department of Laboratory Animal Resources at the University of Pittsburgh to generate neonatal mice. 5 day old neonatal mice were used for experiments irrespective of their gender. No gender bias in immune response was observed in our experiments. All protocols involving animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh.

Virus

Two different strains of virus, RSV Line 19 and RSV 2-20 were used for experiments. The viruses were kind gifts of Drs. Stokes Peebles and Martin L. Moore respectively. HEp-2 cells were used to amplify the viruses, as described previously (Moore et al., 2007). Virus titration after amplification was done by plaque assay using NY3.2 STAT1$^{-/-}$ fibroblast cells in 24-well tissue culture plates precoated with poly-L-lysine (Cat# P4707, Millipore Sigma).

Cell culture

Primary human airway epithelial cells (AEC) were harvested from lung airways of donor human subjects. Cells were then cultured and maintained in collagen (Cat# C7521, Millipore Sigma) coated flasks with Bronchialife basal medium containing supplements (1 µM epinephrine, 5 µg/mL transferrin, 10 nM T3, 0.1 µg/mL hydrocortisone, 5 ng/mL rh EGF, 5 µg/mL rh insulin) (Cat# LL-0023, Lifeline Cell Technology) and 5 µM ROCK inhibitor (Cat# ALX-270-333, Enzo Life Sciences) at 37°C and 5% CO_2. After attaining 80% confluency, cells were passaged, plated on collagen coated transwells and differentiated in air-liquid interface (ALI) using HBTEC ALI differentiation medium (Cat# LM-0050, Lifeline Cell Technology). A549 cells (ATCC CCL-185) were cultured and maintained in DMEM/F-12 medium (Cat# 10565-018, Gibco) containing 10% FBS (Cat# 100-106, Gemini Bio-Products) and 100 U penicillin with 100 µg/ml streptomycin (Cat# 15140-122, Gibco) at 37°C and 5% CO_2. HEp-2 (ATCC CCL-23) and Vero (ATCC CCL-81) cells were cultured and maintained in EMEM medium (Cat# ATCC 30-2003) containing 10% FBS and 100 U penicillin with 100 µg/ml streptomycin at 37°C and 5% CO2. NY3.2 STAT1-/-fibroblast cells (a kind gift of Dr. J.E. Durbin) were cultured and maintained in DMEM medium (Cat# ATCC 30-2002) containing 5% FBS and 100 U penicillin with 100 µg/ml streptomycin at 37°C and 5% CO2.

Reagents

Recombinant human Interleukin-22 (rh IL-22) (Cat# 8931), Interleukin-17A (rh IL-17A) (Cat# 8928) and cell lysis buffer (Cat# 9803) were purchased from Cell Signaling Technology. Chloroquine (Cat# H0915), fludarabine (Cat# F9813), sunitinib (Cat# PZ0012), poly-L-lysine (Cat#P4707), methylcellulose (Cat# 56340), and sodium orthovanadate (Cat# 6508) were obtained from Sigma. Halt protease inhibitors (Cat# 87785) were purchased from Thermo Scientific. Lipofectamine 2000 transfection reagent (Cat# 11668019) was ordered from Invitrogen. For plaque assay and Immunofluorescent staining, primary antibodies against RSV were purchased from EMD Millipore (Cat# AB112) and Meridian Life Science (Cat# B65860G), respectively. For western blotting, primary antibodies against LC3B (Cat# 2775), p62 (Cat# 39749), phospho-STAT1 (Y701) (Cat# 9167), total STAT1 (Cat# 14994), phospho-STAT3 (Y705) (Cat# 9131), total STAT3 (Cat# 4904),and GAPDH (Cat# 5174) were purchased from Cell Signaling. For immunofluorescent staining, primary antibody against LC3B (Cat# NB100-2220) was obtained from Novus Biologicals. Donkey serum (Cat# 017-000-121), HRP-conjugated donkey anti-goat IgG (Cat# 705-035-147), HRP-conjugated donkey anti-rabbit IgG (Cat# 711-035-152), HRP-conjugated goat anti-rabbit IgG (Cat# 111-035-144), Cy3-conjugated donkey anti-goat IgG (Cat#705-166-147), Cy5-conjugated donkey anti-rabbit IgG (Cat# 711-605-152) secondary antibodies for plaque assay, western blotting, and immunofluorescent staining were all purchased from Jackson Immunoresearch, while donkey anti-goat IgG AP secondary antibody (Cat# AP180A) and Hoechst (Cat# B2883) for nuclear staining were from

Millipore Sigma. F-actin counterstains Alexa Fluor 488 Phalloidin (Cat# 424201) and Alexa Fluor 647 Phalloidin (Cat# A22287) were from Biolegend.

METHOD DETAILS

RSV infection and treatment of mice

Neonatal mice (5 day old) were infected with 1 × 10^6 plaque-forming units (pfu) of RSV line 19 in 10 µl complete medium by the intranasal (i.n.) route. The infected pups were treated with 5 µg of human IL-22Fc fusion protein in 50 µl of saline by the intraperitoneal (i.p.) route on day 3 post infection (p.i). The lungs were harvested on days 1, 4, 6 and/or 8 p.i. to assay viral load or mRNA analysis depending on the experimental end point. Small intestines were harvested after IL-22Fc or hIgG (1µg/gram body weight) twice weekly treatment i.p. from postnatal day 10-28 to assay gene expression by RT-qPCR. The half-life of human IL-22Fc was examined in blood samples of mice following standard protocol (http://www-users.med.cornell.edu/~spon/picu/calc/halfcalc.htm). Using 1 hour level as peak and the measured level at 48 hours, the half-life estimate is approximately 107 hours.

RSV infection and treatment of epithelial cells

Differentiated AECs on transwell plates were infected with RSV line 19 (MOI-2) or RSV 2-20 (MOI-1) as indicated on the apical surface and allowed to adsorb for 2 hours at 37°C and 5% CO_2. After adsorption, unbound RSV was washed from the apical surface, and the cells were treated with rh IL-22 (50 ng/ml) on the basolateral surface. AECs were cultured in ALI condition for 24 hours to 48 hours p.i., and then samples were harvested to detect viral load, mRNA expression, and cytokines in culture media. A549 cells were infected with RSV line 19 (MOI-0.5) or RSV 2-20 (MOI-0.05) as indicated. At 2 hours after infection, the cells were treated with rh IL-22. At 24 hours and 48 hours after treatment, viral load, mRNA, and protein expression were assayed. For specific experiments, A549 cells were treated with rh IL-17A (50 ng/ml), chloroquine (40 µM), fludarabine (25 µM), or sunitinib (10 µM) for 24 hours and 48 hours.

Viral load detection in lungs and *in vitro* culture media

Viral load in lungs was detected by plaque assay on Vero cells as previously described (Schickli et al., 2015). Briefly, lung supernatant was collected after centrifugation of the lung homogenate and immediately processed for plaque assay. Two- and ten-fold dilutions of lung supernatants were made in Opti-MEM reduced serum medium (Cat# 31-985-070, Gibco) and applied to monolayers of Vero cells in 24-well tissue culture plates. After 1 hour of incubation at room temperature with constant shaking, the inoculum was replaced with methylcellulose-supplemented L-15 medium (2% methylcellulose mixed 1:1 with 2× L-15 [Cat# 12-669E, Lonza] supplemented with 2% FBS, 4 mM L-glutamine [Cat# 25030-081, Gibco], and 200 U penicillin with 200 µg/ml streptomycin) and incubated at 35°C and 5% CO_2 for 7 days. Overlay media was then aspirated, and cells were immune-stained with goat anti-RSV Abs (EMD Millipore) followed by HRP-conjugated donkey anti-goat IgG. Red-colored plaques were developed with 3-amino-9-ethylcarbazole (AEC) solution (Cat# K3464, Dako). Viral titer was recorded as PFU/g of lung tissue.

RSV line 19 was cultured and amplified in HEp-2 cells, as described previously (Moore et al., 2007). Briefly, HEp-2 cells were infected with the virus and then maintained in culture for 3 days. Cells were lysed by sonication, and the cell supernatant containing virus was collected to titrate viral load. RSV-infected hAEC and A549 cells were lysed by three freeze-thaw cycles, and cell supernatant containing virus was used to determine viral load. Viral titration was then performed by plaque assay using NY3.2 $STAT1^{-/-}$ fibroblast cells in 24-well tissue culture plates pre-coated with poly-L-lysine. Culture medium containing virus stock was serially diluted in serum-free medium and was allowed to adsorb to the cell monolayer for 2 hours with constant shaking at 37°C. Adsorption of virus was stopped by adding 1% methylcellulose overlay medium and then incubated at 37°C and 5% CO_2 for 3 days. Viral plaques were then developed using goat anti-RSV all antigens primary antibody (Cat# B65860G, Meridian Life Science), donkey anti-goat IgG AP secondary antibody, and BCIP/NBT alkaline phosphatase substrate (Cat# B5655, Millipore Sigma).

RNA isolation and quantitative real-time PCR

Cell lysates were prepared in RLT-Plus buffer and RNA was isolated using RNeasy plus mini kit (Cat# 74136, Qiagen) according to manufacturer's instructions. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Cat# 4368813, Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time PCR was performed using validated TaqMan Gene expression assays. Primer and probe sets (Life Technologies) for the following human genes were used: *IFIT1* (Cat# Hs03027069_s1), *OASL* (Cat# Hs00984387_m1), *ISG15* (Cat# Hs01921425_s1), *IL22RA1* (Cat# Hs00222035_m1), *IL10RB* (Cat# Hs00175123_m1), *IFNα2* (Cat# Hs00265051_s1), *IFNβ1* (Cat# Hs01077958_s1), and *HPRT1* (Cat# Hs02800695_m1). *RSV-L polymerase* gene expression was detected using the following primers and probe: forward primer (5′-GAACTCAGTGTAGGTAGAATGTTTGCA-3′), reverse primer (5′-TTCAGCTATCATTTTCTCTGCCAAT-3′) and probe (5′-TTTGAACCTGTCTGAACATTCCCGGTT-3′). The primer sequences for the mouse genes were: *Rplo*, forward primer (5'-GGCGACCTGGAAGTCCAACT-3'), reverse primer (5'-CCATCAGCACCACAGCCTTC-3'); *Il22*, forward primer (5'-CGACCAGAACATCCAGAAGAA-3'), reverse primer (5'-GAGACATAAACAGCAGGTCCA-3'); *Reg3g*, forward primer (5'-GTACCCTGTCAAGAGCCTCA-3'), reverse primer (5'-TGTGGGGAGAATGTTCCCTT-3'). Gene expression was calculated using the $2^{-\Delta Ct}$ (normalized to *HPRT1* or *Rplo*). Results were analyzed using SDS 2.4 software.

ELISA

Levels of IFN-β (Type I) and IFN-λ (Type III) proteins were measured in debris-free cell culture supernatant from primary AECs and A549 cells using VeriKine Human Interferon Beta ELISA Kit (Cat# 41410-1, PBL Assay Science) and DIY Human IFN Lambda 1/2/3 (IL-29/28A/28B) ELISA (TCM) Kit (Cat# 61840-1, PBL Assay Science), respectively, according to the manufacturer's protocol.

Western Blotting

Cell lysates were prepared by harvesting cells in cell lysis buffer supplemented with halt protease inhibitors and sodium orthovanadate. After clearing the lysate by centrifugation, supernatants were collected, and total protein content was measured by DC protein assay kit II (Cat# 5000112, BioRad) according to manufacturer's instructions. Total protein (10 µg) was resolved on a 4-15% SDS–PAGE gel (Cat# 4568086, Biorad), and the proteins were transferred to an Immobilon-FL PVDF membrane (Cat# IPFL20200, Millipore Sigma). The blot was blocked using 5% milk and then probed with the specific primary antibody followed by HRP-conjugated secondary antibody and developed using SuperSignal West Femto Maximum Sensitivity Substrate (Cat# 34095, Thermo Fisher Scientific). ImageJ software was used to quantitate protein expression using GAPDH as an internal control.

dsiRNA-mediated knockdown of gene expression

dsiRNAs specific to LC3B (dsiLC3B) (hs.Ri.MAP1LC3B.13.1) and negative control (dsiNC1) (Cat# 51-01-14-03) were purchased from IDT. Lipofectamine 2000 (Cat# 11668-019, Thermo Fisher Scientific) was used as a transfection reagent. A suspension of primary AECs was prepared at a density of $1–2 \times 10^5$ cells in 150 µl of BEBM media with supplements (Cat# CC-3170, Lonza) for each well of a 12-well transwell insert. Similarly, A549 cell suspension was prepared at a density of 2×10^5 cells in 400 µl of DMEM/F-12 complete medium for each well of a 24-well plate. For each transfection sample, dicer oligomer-Lipofectamine 2000 complex was prepared using 2 µl of Lipofectamine 2000 and 50 nM of dsiRNA oligomer. Briefly, 50 nM of each of dsiLC3 and dsiNC1 were prepared, and 2 µl of Lipofectamine 2000 was diluted in 50 µl Opti-MEM reduced serum medium in separate tubes, mixed gently, and incubated for 5 minutes at room temperature. After the 5-minute incubation, the diluted dsiRNA oligomer was combined with the diluted Lipofectamine 2000, mixed gently, and then incubated for 20 minutes at room temperature. Lipofectamine 2000 complex (100 µl) was combined with 150 µl of AEC and 400 µl of A549 cell suspension and then plated on the collagen-coated transwell inserts and 24-well plate, respectively. The cells were then incubated at 37°C and 5% CO_2 for 12–16 h, and then the medium was changed. Gene knockdown was assayed after 6 to 7 days and 48 to 72 hours of transfection for AEC and A549 cells, respectively. Transfected cells were then used for RSV infection.

Co-localization of RSV and LC3B in A549 cells

A549 cells were cultured on cover glasses, infected with RSV, and then treated with IL-22 as described before. After 24 hours of infection, cells were fixed with 2% PFA and then processed for immunofluorescent staining for RSV and LC3B. After rehydration and permeabilization, cells were blocked using 5% donkey serum and stained overnight at 4°C in the dark with goat anti-RSV all antigen antibody and rabbit anti-LC3B antibody. The cells were then stained with Cy3-conjugated donkey anti-goat IgG and Cy5-conjugated donkey anti-rabbit IgG secondary antibody respectively along with F-actin counterstain AF488 phalloidin for 1 hour at room temperature in the dark. Nuclei were stained with Hoechst and cells were mounted with gelvatol. Images were obtained under identical conditions at ×60 (1.43NA) magnification on Nikon A1 confocal microscope and quantitation was performed using NIS Elements software. Co-localization was assessed by determining the degree of overlap on a per pixel basis. RSV and LC3 emissions were first segmented based on intensity in order to generate a binary mask. Co-localization was determined using a binary "having" statement to identify LC3 positive puncta containing RSV.

Transduction using Tandem Sensor RFP-GFP-LC3B

The Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit (Cat# P36239, Thermo Fisher Scientific) combines the selectivity of an LC3B-fluorescent protein (FP) chimera with the transduction efficiency of the BacMam (insect Baculovirus with a Mammalian promoter) 2.0 technology and was used to discriminate between acidic and neutral LC3B-positive vesicles (i.e., autolysosomes and autophagosomes, respectively). A549 cells were plated at a density of 1×10^5 cells/well on 18 mm coverslips and then transduced with 50 particles per cell (ppc). The cells were then infected with RSV at 24 hours post-transduction and then treated with IL-22 as previously discussed. Cells were fixed using 2% PFA and stained with F-actin counterstain AF647 phalloidin for 1 hour at room temperature in the dark. Nuclei were stained with Hoechst 33342, and cells were mounted with gelvatol. Cells were then imaged on a Nikon A1 confocal microscope at 60× magnification. Quantitation was performed using spot detection to identify puncta that were positive for red and green, followed by a binary "and" statement to identify puncta that were positive for both red and green (yellow), with a binary in NIS Elements software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses

Mann Whitney test and one-way ANOVA with Tukey post hoc test were used as appropriate. Specific details with regard to statistical tests, as well as, sample sizes (n) and replicates can be found in the figure legends. Differences between groups were considered significant when $P < 0.05$. All statistical analyses were performed using GraphPad Prism 7 software.

SUPPLEMENTAL REFERENCES

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