Supporting Information

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SI Materials and Methods

Cell Culture and Biological Reagents. MRC5 human fibroblasts (ATCC CCL-171), human alveolar basal epithelial A549 cells (ATCC CCL-185), Madin–Darby canine kidney epithelial MDCK cells (ATCC CCL-34), African green monkey kidney epithelial Vero (ATCC CCL-81), mouse areolar/adipose fibroblast L929 cells (ATCC CCL-1), and human 293T cells (ATCC CRL-3216) that constitutively express the murine carcinoembyonic antigen-related cell adhesion molecule 1 (293T–mCEACAM1) were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 10% (vol/vol) FBS and 4.5 g·liter^{-1} glucose at 37 °C in a 5% (vol/vol) CO_2 atmosphere. Before HCMV infection, MRC5 cells were grown to confluence, resulting in ~3.2×10^4 cells per cm^2. Once confluent, medium was removed, and serum-free medium was added. Cells were maintained in serum-free medium for 24 h before infection. Human alveolar basal epithelial A549 (ATCC CCL-185), African green monkey kidney epithelial Vero (ATCC CCL-81), Madin–Darby canine kidney epithelial MDCK (ATCC CCL-34), and mouse areolar/adipose fibroblast L929 (ATCC CCL-1) cells were maintained in DMEM (Mediatech) supplemented with 10% (vol/vol) FBS, L-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in a 5% (vol/vol) CO_2 atmosphere. Human 293T cells constitutively expressing the murine carcinoembryonic antigen-related cell adhesion molecule 1 (mCEACAM1) were generated by transfecting a pCAGGS plasmid encoding mCEACAM1 and Hygromicin B resistance by using Lipofectamine 2000 as described (1, 2). At 48 hpi, cells were plated into 10-cm dishes at low density, and cell clones were selected in the presence of 500 μg/mL Hygromicin B (Cellgro). Drug-resistant clones were screened for mCEACAM1 expression by recombinant MHV-expressing GFP (rMHV–GFP) complementation. The established complementing clone was maintained in DMEM supplemented with 10% (vol/vol) FBS, penicillin/streptomycin, and Hygromicin B at a concentration of 500 μg/mL. HCMV (strain AD169) was used to infect cells at a MOI of 3 for all experiments, unless otherwise indicated.

Recombinant influenza A/Puerto Rico/8/34 H1N1 expressing the GFP fused to NS1 (NS1–GFP) (3), recombinant MHV expressing GFP (rMHV–GFP) (4), and recombinant VSV expressing GFP (rVSV–GFP) (4) were kindly provided by Adolfo Garcia-Sastre (Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York), Susan R. Weiss (Department of Microbiology, Perelman School of Medicine, Philadelphia), and John Hiscott (Vaccine and Gene Therapy Institute of Florida, Port St. Lucie, FL), respectively.

Adsorptions of influenza A (MOI = 0.005) and VSV (MOI = 0.0001) were carried out on A549 cells for 60 min at 37 °C, with the exception of influenza A, which was carried out at 25 °C. Subconfluent 293T cells constitutively expressing mCEACAM1 were infected with rMHV–GFP (MOI = 0.0005). After inoculation, for RNA viruses, viral titers were determined by fluorescent focus centers (FFU/mL) following standard viral infection and titration protocols.

Chemical Reagents. PALA (NCI Developmental Therapeutic Program's Open Compound Repository) and Leflunomide (Sigma-Aldrich) were maintained in DMSO at concentrations of 42 and 100 mM, respectively, at −20 °C. A3 (a gift of Megan L. Shaw, Icahn School of Medicine at Mount Sinai) was maintained in DMSO at a concentration of 100 mM. Uridine, UDP–glucose, and UDP–*N*-acetylglucosamine (Sigma Aldrich) were each maintained at 4 °C in serum-free DMEM at concentrations of 4, 82, and 77 mM, respectively. Orotate (Sigma Aldrich) was maintained in 0.1 M NaOH at a concentration of 50 mM. Live/dead assay reagents were obtained from Marker Gene Technologies, and the assay was performed according to the manufacturer's instructions. PNGase was obtained from New England BioLabs, and samples were treated after harvest according to the manufacturer's instructions.

Protein Analysis. Protein accumulation was assayed by Western blotting. Cells were washed with PBS, solubilized, and scraped in disruption buffer containing 50 mM Tris (pH 7.0), 2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, and 2.75% (wt/vol) sucrose. Resulting extracts were sonicated, boiled for 5 min, and centrifuged briefly at 14,000 × *g* to pellet insoluble material. Extracts were then subjected to electrophoresis in an SDS–8% (wt/vol) polyacrylamide gel and transferred to a nitrocellulose sheet. Blots were then stained with Ponceau S to ensure equivalent loading and transfer, blocked by incubation in 5% (wt/vol) milk, and reacted with primary and subsequent secondary antibodies. Proteins were visualized by using an ECL system (Pierce) and imaged either by scientific film imaging (Kodak) or by CCD camera (BioRad Chemidoc XRS). Antibodies used were specific for CAD (Abcam), phospho-CAD (Thr456, Santa Cruz), α-tubulin (Epitomics), viral glycoprotein B (Virusys), viral protein IE-1 (5), viral protein UL44 (Virusys), viral protein UL26 (6), CD44S (R&D Systems), and GAPDH (Cell Signaling). Image densitometry of specific protein bands was performed with ImageLab software (BioRad), which includes controls that ensure signal intensities are linear.

LC-MS/MS Analysis. The accumulation of ^{13}C-labeled UTP and UDP–sugars as well as ^{12}C–UTP and UDP–sugars was monitored by using LC-MS/MS as described (7). After aspiration of cellular growth medium, metabolism was quenched by the addition of 4 mL of −80 °C 80% (vol/vol) methanol and subsequently incubated at −80 °C for 10 min. Cells were then scraped in methanol and centrifuged at 1,875 × *g* for 5 min at 4 °C, and the supernatant was collected. The pellet was extracted twice more in 500 μL of cold methanol, adding the resulting supernatants to the previously collected supernatant. After extraction, the supernatants were dried down under nitrogen gas and resuspended in 200 μL of 50% (vol/vol) methanol. Samples were subsequently spun down at full speed for 5 min at 4 °C, and the remaining supernatants were transferred to HPLC sample vials. LC-MS/MS was performed by using a LC-20AD HPLC system (Shimadzu) and a Synergi Hydro-RP column [150 × 2mm with a 5 μm-particle size; (Phenomenex)] coupled to a mass spectrometer. The LC parameters were as follows: autosampler temperature, 4 °C; injection volume, 10 μL; column temperature, 40 °C; and flow rate, 0.5 mL/min. The LC solvents were as follows: solvent A, 100% methanol; and solvent B, 10 mM tributylamine and 15 mM acetic acid in 97:3 (vol:vol) water:methanol. The gradient conditions were as follows (vol/vol): negative mode-t = 0, 85% B; t = 4, 3%B; t = 5, 3% B; t = 5.1, 85% B. Mass-spectrometric analyses were performed on a TSQ Quantum Ultra triple-quadrupole mass spectrometer running in multiple reaction monitoring mode (Thermo Fisher Scientific). Peak heights for metabolite chromatograms were analyzed by using Excalibur software (Thermo Fisher Scientific). For labeling experiments, labeled DMEM was prepared from glucose-free medium by adding 10 mM Hepes and either labeled (^{13}C) or

unlabeled (^{12}C) glucose to a final concentration of 4.5 g·L^{-1}. For flux analysis, samples were switched to fresh, unlabeled medium 24 and 1 h before final addition of ^{13}C-labeled medium. Samples were labeled until the indicated time, at which point the reaction was quenched by the addition of 4 mL of −80 °C 80% (vol/vol) methanol and processed for LC-MS/MS analysis as indicated above.

Flux Analysis. The rate of metabolite turnover via ^{13}C labeling was determined as described (8, 9). Briefly, to estimate rate constants of metabolic turnover, ^{12}C decay data were fit to the following equation:

$$V_U/V_T = (1-\alpha)\ \exp(-k_v t) + \alpha,$$

where V_U/V_T is the ratio of the unlabeled to the total sum of all metabolite isoforms for metabolite V, α is the ratio of unlabeled input of the metabolite resulting from macromolecular degradation, k_v is the rate constant of V turnover, and t is equal to time. This equation was used to calculate k_v and α from the ratio of unlabeled to the total sum of all metabolite isoforms over time after pulse labeling as measured by LC-MS/MS. Absolute abundance of UTP and UDP–sugars in pmol per 10^6 cells was determined by using dilution curves of pure standards as determined by LC- MS/MS and comparing them to the abundance of these metabolites at various times after infection in both mock- and HCMV-infected samples. Specific fluxes of given metabolites, e.g., V, were then estimated by the following equation:

$$\text{Flux}_V = V^{\text{total}} \times k_v$$

where V^{total} is the absolute concentration of V, and k_v is the aforementioned rate constant (8, 9).

RNA Interference Assay. CAD mRNA expression was targeted by using a CAD-specific Mission siRNA (Sigma; EHU130441). A nontargeting siRNA with the sequence 5′- CGUAAGCGACAUACUUACAUU-3′ in conjunction with its complementary sequence was used as a negative control. For siRNA transfections, MRC5 fibroblasts at 30% confluence were transfected with 37.5 pmol of RNA interference duplex using Oligofectamine (Invitrogen) as per the manufacturer's instructions.

Partial Purification of Secreted HCMV Virions. Supernatants of MRC5 human fibroblasts infected with AD169 (MOI = 1) were harvested at 144 hpi. The supernatant was centrifuged at 1,300 × *g* for 5 min to pellet debris. The supernatant was then underlaid with 7 mL of 20% (vol/vol) Sorbitol buffer and centrifuged for 1.25 h at 53,000 × g. pfus were measured by using a standard viral plaque assay.

Nucleic Acid Analysis. RNA and DNA accumulation was assayed by real-time qPCR. Viral DNA was harvested at various time points after infection in lysis buffer [100 mM NaCl, 100 mM Tris·HCl (pH 8.0), 25 mM EDTA, 0.5% SDS, 0.1 mg/mL proteinase K, and 40 μg/mL RNase A], and quantified by using the pp65-specific primer set: 5′-CAGGAAGATTTGCTGCCCGTTCAT-3′ (forward) and 5′-G-GCTTTACGGTGTTGTGTCCCAAA-3′ (reverse). The number of genomes in concentrated virions was measured by incubating 50 μL of virions in 50 μL of 2× lysis buffer [200 mM NaCl, 200 mM Tris·HCl (pH 8.0), 50 mM EDTA, 1.0% SDS, 0.2 mg/mL proteinase K, and 80 μg/mL RNase A] for 3 h and then quantified by using the pp65-specific primer set described above. The linearity of sample measurements was tested, and quantification of sample genomes was performed through comparison with a dilution series of a BAC-cloned AD169 reference genome. For RNA analysis, RNA was prepared by using TRIzol (Invitrogen) as recommended by the manufacturer. cDNA synthesis was performed by using SuperScript II First-Strand (Invitrogen) reagents with random hexamers as per the manufacturer's protocol. qPCR was performed by using Fast SYBR green PCR master mix, 7500 Fast real-time PCR system sequence detection, and 7500 software (Version 2.0.1; Applied Biosystems) following the manufacturer's instructions. Transcripts were quantified with specific primer pairs as follows: the gB transcript with 5′-CTGCAGAACCTCTTTCCCTATC-3′ (forward) and 5′- CTCTTCTTCGTCGGAGTCTTTC-3′ (reverse) and the GAPDH transcript, used for normalization, with 5′- CATGTTCGTCATGGGTGTGAACCA-3′ (forward) and 5′-ATGGCATGGACTGTGGTCATGAGT-3′ (reverse). qPCR was performed by using the Δ*CT* (threshold cycle) method, with GAPDH as a control.

Statistics. Data normality and variance homogeneity were tested through the Shapiro Wilk and the Brown–Forsythe tests, respectively, at 95% confidence. For parametric datasets, Student's *t* tests (two-sample comparisons) or ANOVA (more than two sample comparisons) were used to compare means. For those judged to be significantly different by ANOVA ($P < 0.05$), individual sample comparisons were made by using a Tukey test, and marked with an asterisk.

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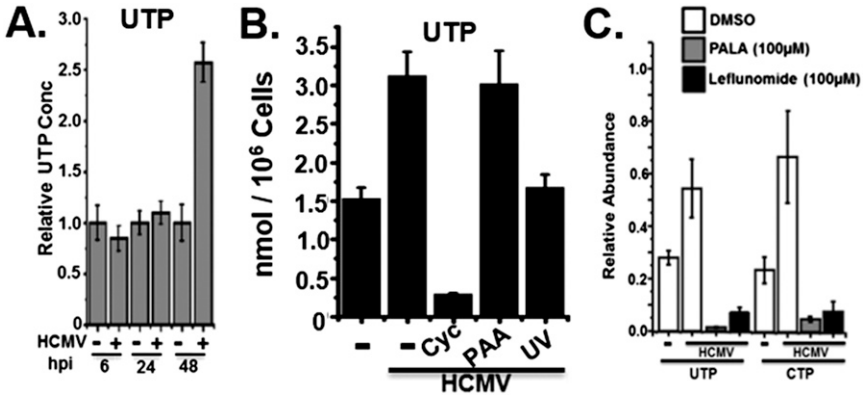

Fig. S1. (*A*) MRC5 cells were mock- or HCMV-infected (MOI = 3.0) and harvested at 6, 24, and 48 hpi for LC-MS/MS–based measurement of relative UTP concentrations (mean ± SEM, *n* = 6). (*B*) MRC5 cells were mock-infected or infected with UV-inactivated HCMV or nonirradiated HCMV (MOI = 3.0) and treated with PAA (50 μg/mL) or Cycloheximide (Cyc; 10 ng/mL) as indicated. Samples were harvested at 48 hpi for LC-MS/MS–based measurement of UTP concentrations (mean ± SEM, n = 6). (*C*) MRC5 cells were mock- or HCMV-infected (MOI = 3.0) and treated with DMSO, PALA, or Leflunomide as indicated. At 48 hpi, cells were harvested and processed for LC-MS/MS analysis. The relative abundance of metabolites was normalized to maximum metabolite levels (mean ± SEM; two biological replicates were analyzed in duplicate).

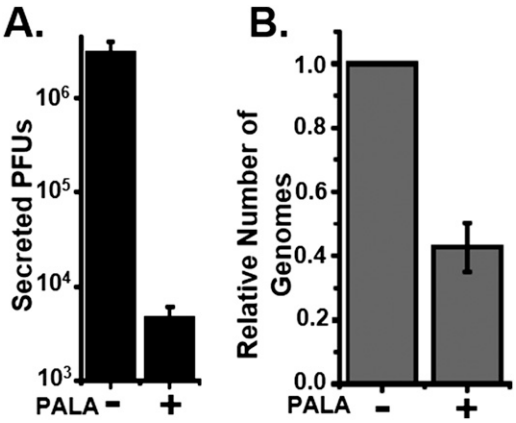

Fig. S2. Cells were HCMV-infected (MOI = 1.0) and treated with DMSO or 100 μM PALA. Supernatants were harvested at 144 hpi and partially purified through a sorbitol cushion via centrifugation at 53,000 × *g* for 1.25 h. (*A*) Samples were analyzed for the production of infectious viral progeny (total production from equivalent amounts of cells). (*B*) Genomes were measured by extracting viral DNA from the virion preparations in *A*. Viral DNA abundance was analyzed by qPCR, compared with a linear dilution of AD169 BAC, and plotted relative to the untreated sample (mean ± SEM; two biological replicates were analyzed in duplicate).