ONLINE METHODS

Animals

The generation of Nlrp3$^{-/-}$, Pycard$^{-/-}$, Nlrc4$^{-/-}$, casp1$^{-/-}$, Tlr7$^{-/-}$ and Myd88$^{-/-}$ mice has been reported previously[17, 36-39]. Nlrp3$^{-/-}$, Pycard$^{-/-}$ and Nlrc4$^{-/-}$ mice were provided by Lexicon Genetics Incorporated. All KO mice have been backcrossed at least nine generations onto the C57BL/6 background. Age- and sex-matched C57BL/6 mice from National Cancer Institute (Frederick, MD) were used as WT control. MAVS KO mice were a gift of Dr. Zhijian J. Chen (University of Texas Southwestern Medical Center, Dallas, TX). All procedures used in this study complied with federal guidelines and were approved by the Yale Animal Care and Use Committee.

Viruses

Influenza virus A/Puerto Rico/8/34 (A/PR8; H1N1), A/Yamagata/120/86 (A/Yamagata; H1N1), A/Beijing/262/95 (A/Beijing; H1N1), A/Aichi/2/68 (A/Aichi; H3N2), A/Sydney/5/97 (A/Sydney; H3N2), A/Guizhou/54/89 x A/PR/8/34 (A/Guizhou-X) (H3N2) and B/Ibaraki/2/85 (B/Ibaraki) were gift from Dr. Hideki Hasegawa (National

Institute of Infectious Diseases, Tokyo, Japan) and were grown in allantoic cavities from 10- to 11-day-old fertile chicken eggs for 2 days at 35˚C. Influenza A/Udorn/307/72 (A/Udorn; H3N2) and the mutant virus (M2del29-31; H3N2)[21] were provided by Dr. Robert Lamb (Northwestern University, Evanston, IL) and were propagated and titrated in Mardin-Darby canine kidney (MDCK) cells. HSV-2 strain (186TKΔKpn)[40] was propagated and assayed on Vero cells. Sendai virus (Cantell stain) was purchased from American Type Culture Collection (Manassas, VA). These viruses were stored at -80˚C.

Lentiviral vectors

Oligodeoxynucleotides corresponding to both strands of influenza virus M2 full-length sequences (A/PR8 (A/PR/8/34 (H1N1), Genbank EF190985), 1918 flu (A/Brevig Mission/1/1918 (H1N1), Genbank AY130766) and A/Vietnam/1194 (A/Vietnam/1194/2004 (H5N1), Genbank EF541452)) containing BamHI-NotI sites at the 5' and 3'ends were synthesized (Integrated DNA technologies, Inc), and cloned into the lentiviral vector pHRSIN-CSGW (expressing GFP) (gift of Dr. Mary K. Collins (University College London, London, UK)[41]. Lentivirus encoding an irrelevant gene (GFP) was used as a control. His_{37}→Gly M2 mutant was constructed using a standard

PCR-based method. The integrity of the inserts was verified by sequencing. Recombinant lentivirus was produced by co-transfection of the vector plasmid with pCMVR8.91 and pMDG as described previously[42].

Generation of E5-expressing retroviruses

pT2H-HA-E5 or pT2H-HA-Q17G were gifts from Dr. Daniel DiMaio (Yale University)[43]. The Packaging cell line GP2-293 (CLONTECH) in 10-cm dishes were transfected with 10 μg of pT2H-HA-E5 or pT2H-HA-Q17G by Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The supernatant was collected at 48 h. RAW264.7 cells were infected with E5-, Q17G-, or GFP-retroviruses and incubated in the presence of polybrene (10 μg/ml) for 24 h. Then, cells were cultured in the presence of hygromycin B (150 μg/ml) to kill non-transduced cells (day 2-4). On day 7, cells were infected with A/PR8 virus at indicated MOIs. Supernatants were collected at 24 h post A/PR8 virus infection.

Influenza virus infection

BMM and BM DCs prepared as described previously[1] were incubated with 2.5 MOI of

influenza virus, HSV-2, or SeV in 100 μl/well of 0.1% BSA PBS at 8 x 10^5 cells/24-well plate for 1 h, washed thoroughly, and incubated in complete media for an additional 24 h. Cell-free supernatant was collected and analyzed for IL-1β by ELISA (eBioscience). In some cases, supernatants and cell extracts were collected for Western blot analysis.

Viral RNA isolation and transfection

To obtain influenza genomic RNA, QIAamp Viral RNA Mini Kit (QIAGEN) was used to isolate viral RNA from A/PR8 virus. The concentration of the resultant purified genomic RNA was determined by NanoDrop (Thermo scientific). Purified RNA was subjected to mock or dephosphorylation using calf intestinal phosphatase (CIP, New England Biolabs). The enzyme was removed by using the RNeasy kit (QIAGEN). BMM were transfected with 1 μg of poly (I:C), CIP-treated or non-treated PR8 genomic RNA with DOTAP (Roche) for 5 hours. Then, cells were washed 3 times and cultured in new media for 24 h. For dsDNA transfection, BMM were primed with LPS (100 ng/ml). After 6-12 hours of stimulation, cells were washed thoroughly and incubated in fresh culture medium containing 1-5 μg/ml poly(dA:dT) complexed with the cationic lipid LyoVec (InvivoGen).

RT Quantitative PCR

RNA isolation was performed using the RNeasy kit (Qiagen) according to manufacturer's instructions. Isolated RNA was used to synthesize cDNA using SuperScript II Reverse Transcriptase (Invitrogen) and qPCR was performed on a Stratagene Mx3000P unit using SyberGreen (Qiagen). Primers used for quantitative PCR were as follows: mpro-IL-1β forward, GCAACTGTTCCTGAACTCAACT; mpro-IL-1β reverse, ATCTTTTGGGGTCCGTCAACT; mIFN-β forward, GCACTGGGTGGAATGAGACTATTG; mIFN-β reverse, TTCTGAGGCATCAACTGACAGGTC; mGAPDH forward, ACCACAGTCCATGCCATCA; mGAPDH reverse, TCCACCACCCTGTTGCTGT.

Immunoblotting

For immunoblotting cells were lysed in buffer containing 50 mM Tris pH 7.5, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1mM EDTA, and protease inhibitor cocktail. For immunoprecipitation of IL-1β, samples were pre-cleared with protein G sepharose (GE Healthcare) for 1 hour at 4ºC and incubated with goat polyclonal anti-mouse IL-1β

antibody (R&D System, Minneapolis, MN) at 4ºC overnight. Immunoprecipitates were washed three times in lysis buffer and bound proteins were eluted by boiling in SDS sample buffer. Proteins were separated on a NuPAGE gel and were transferred onto poly vinylidine difluoride membrane. The membranes were blocked with 5% milk proteins in 1 x PBS and 0.5% Tween-20 for 1 h, then blocked with 5% BSA in TBST overnight. To detect caspase-1 and IL-1β, rabbit polyclonal anti-mouse caspase-1 p10 antibody (clone M-20; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-mouse IL-1β antibody (Millipore, Temecula, CA), or 3ZD anti-IL-1β monoclonal antibody were used. Anti-rabbit IgG HRP (ECL) or anti-mouse IgG (Jackson) secondary antibodies were used and proteins were detected using ECL Western Blotting Detection Reagents (Amersham/GE Healthcare).

Immunofluorescence microscopy

GalNAc-T2-YFP BSC-1 cells[44] which stably produce the resident Golgi enzyme N-acetylgalactosaminyltransferase II fused to yellow fluorescent protein (gift of Dr. Craig Roy, Yale University) were plated on alcian blue-coated coverslips and stimulated with A/PR8 virus (MOI 2.5) in the presence or absence of Monensin (10 μM) or Brefeldin A

(10 μg/ml, included during the first hour of infection). Cells were washed after 1 h and cultured in complete media with or without Monensin (10 μM). Cells were fixed in 4% PFA for 30 min and blocked and permeabilized in PBS containing 10% goat serum, 0.05% saponin, and 1% BSA (IF buffer) for 15 min at room temperature at 7 and 12 h post infection. Cells were incubated with primary and secondary antibodies diluted in IF buffer for 1 h and 30 min, respectively. Antibodies used for fluorescence microscopy were mouse monoclonal anti-influenza A virus M2 protein (abcam; diluted in 1:500) and rabbit polyclonal anti-calnexin (Stressgen; duluted to 1:200). Images of the cells were acquired at room temperature using an LSM 510 confocal microscope (Carl Zeiss MicroImaging, Inc.).

Statistics

All values are expressed as the mean ± s.d. of individual samples. We performed statistical analysis using the ANOVA test (GraphPad INSTAT). $p < 0.05$ was considered statistically significant.