Influenza virus activates inflammasomes through intracellular M2 channel

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Supplementary Information

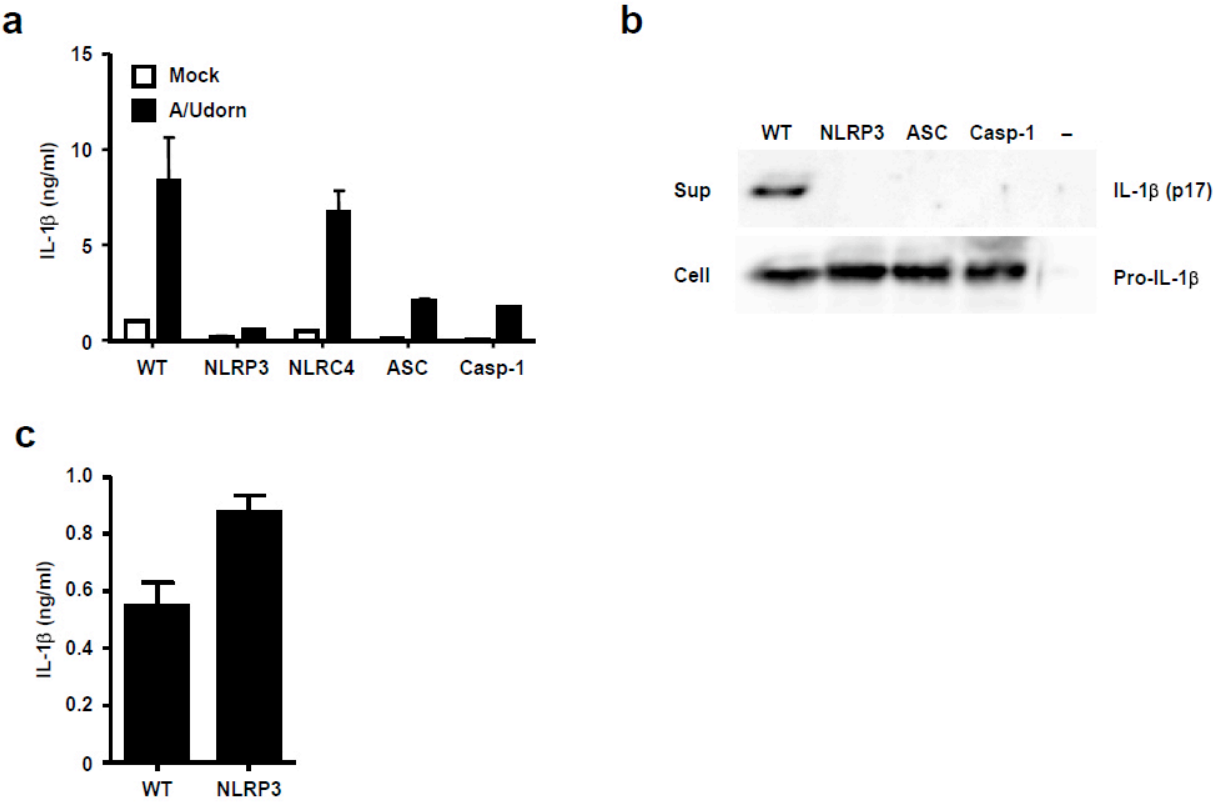

Figure S1. Influenza virus infection elicits NLRP3 inflammasomes in BM DCs. (**a**) BM DCs prepared from WT, NLRP3, NLRC4, ASC and caspase-1 knockout mice were infected with influenza virus (A/Udorn). Supernatant were collected at 24 hours post infection and analyzed for IL-1β by ELISA. (**b**) BM DCs prepared from WT, NLRP3, ASC and caspase-1 knockout mice were infected with influenza virus (A/Udorn). Supernatants were analyzed for the presence of mature IL-1β and cell extracts, for the presence of pro-IL-1β by Western blotting. (**c**) LPS-primed BM DCs prepared from WT and NLRP3 knockout mice were transfected with poly(dA:dT). Supernatants were collected at 20 hours post transfection and analyzed for IL-1β by ELISA.

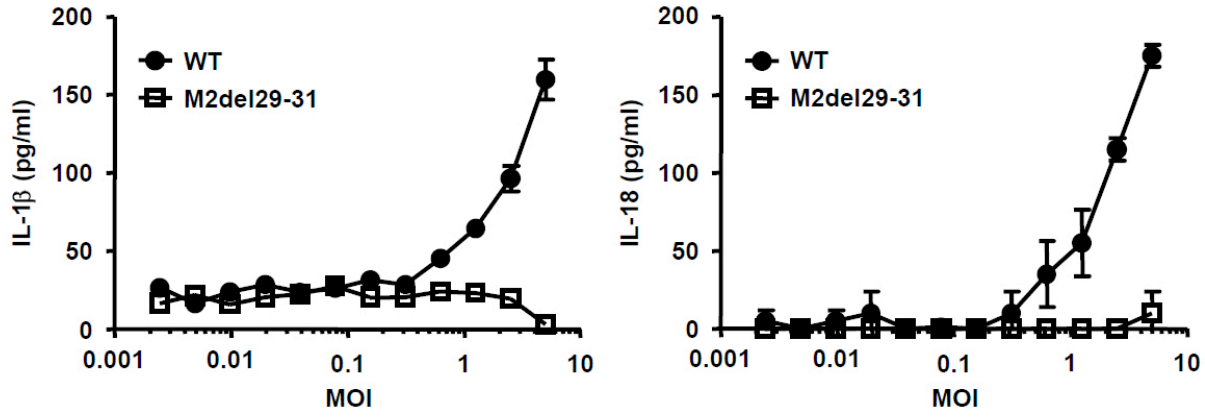

Figure S2. Inflammasome activation in BMM by influenza virus requires an intact M2 channel. BMM were infected with WT or M2del29-31 virus at indicated MOIs. Supernatants were collected 24 h after stimulation and analyzed for IL-1β and IL-18 by ELISA.

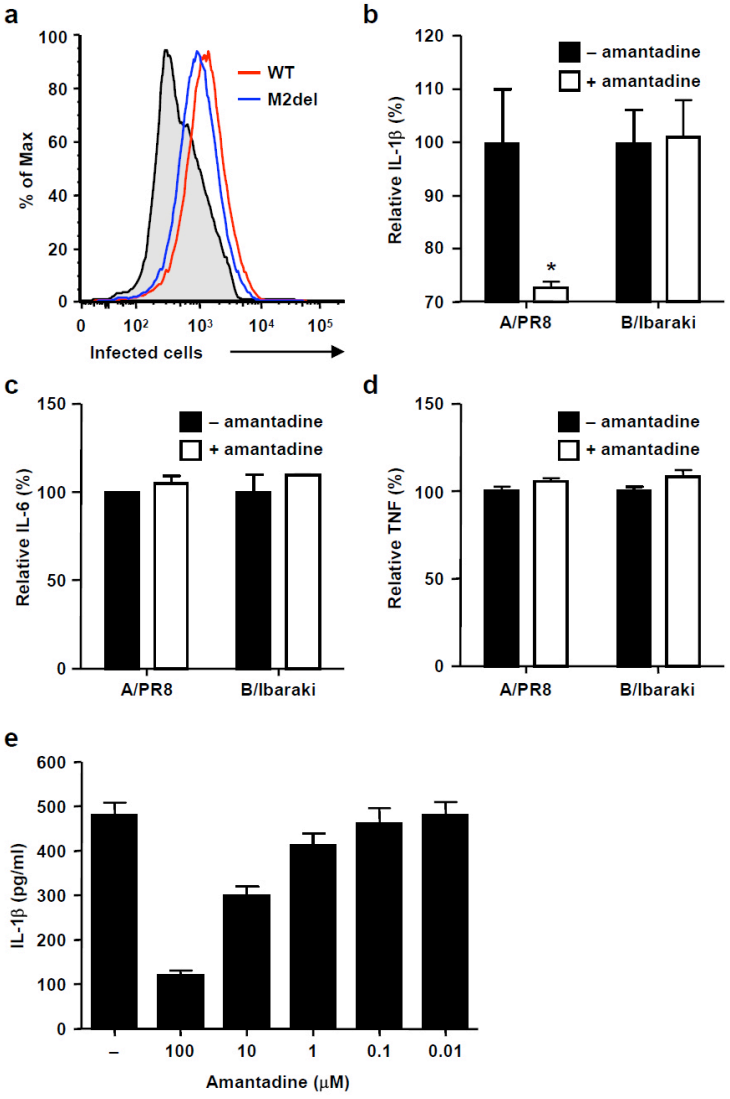

Figure S3. M2 is required for inflammasome activation but not infection. (**a**) BMM were infected with A/Udorn or M2del29-31 virus. Cells were collected at 24 hours post infection and stained with antiserum (diluted in 1:1000) from A/Udorn immunized mice followed by PE-anti-mouse IgG (diluted in 1:300). Histograms indicate infected cells within the $CD11b^{+}$ gate. Gray histogram show non-infected BMM control. (**b-d**) BMM were infected with A/PR8 or B/Ibaraki virus at MOI 2.5 and cultured in the presence (open bar) or absence (closed bar) of amantadine (6.25 μM) for 24 hours. IL-1β (**b**), IL-6 (**c**), and TNF-α (**d**) concentrations in the supernatants were determined by ELISA and depicted as percentage of controls. (**e**) BMM were infected with A/PR8 virus and cultured with the indicated doses of Amantadine. Supernatants were collected 24 h after infection and analyzed for IL-1β by ELISA. * $p<0.05$. These data are representative of 4 independent experiments.

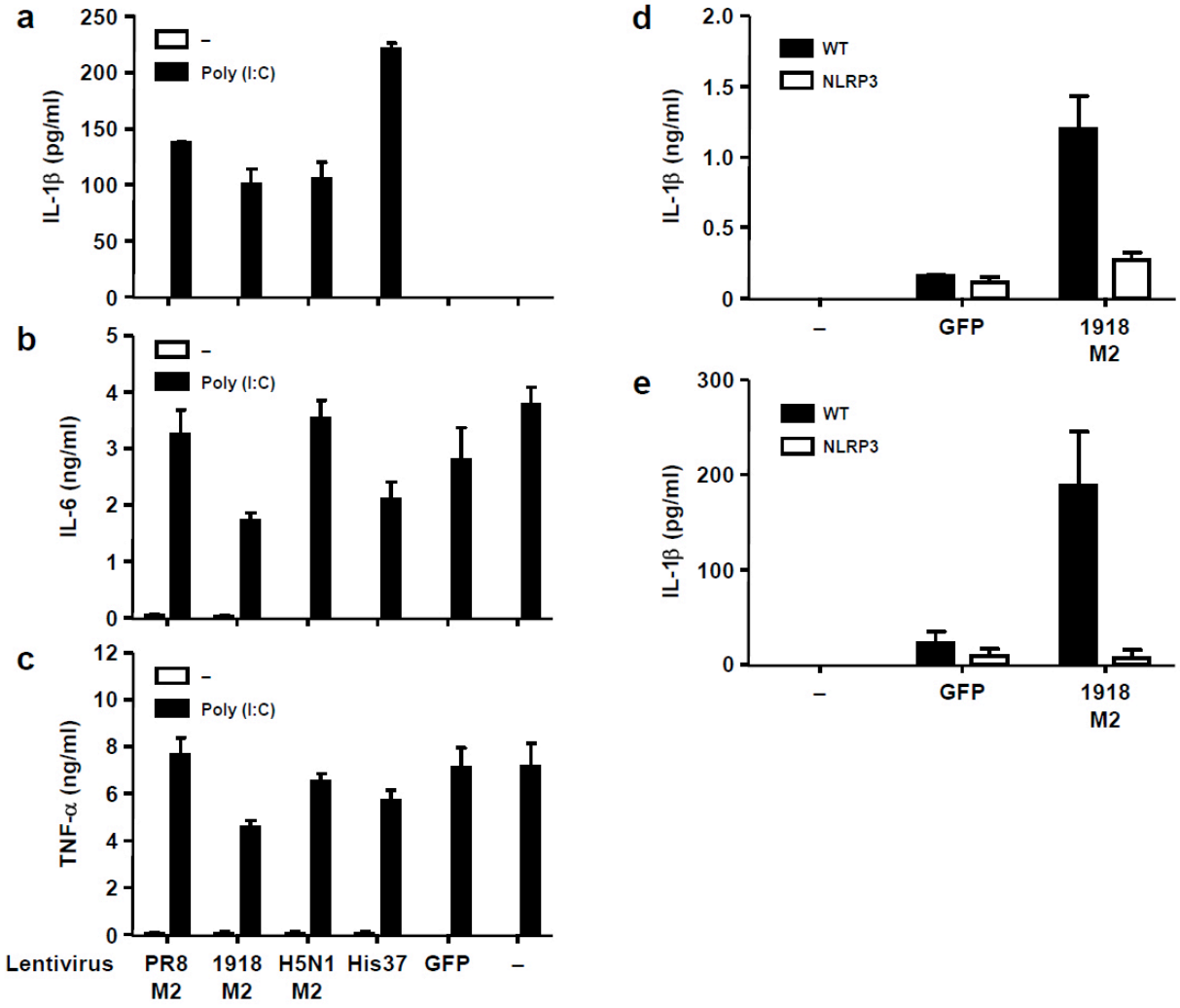

Figure S4. (**a-c**) Poly (I:C) primed BMM require M2 channel for IL-1βrelease but not for non-inflammasome dependent cytokine secretion. BMM were transduced with A/PR8 M2-expressing lentivirus. Twenty four hours later, cells were washed thoroughly and stimulated with poly(I:C). GFP-expressing lentivirus was used as negative control. Supernatants were collected at 18 hours post poly(I:C) stimulation and analyzed for IL-1β (**c**), IL-6 (**b**), and TNF-α (**c**) by ELISA. (**d-e**) BM DCs were infected with HSV-1 (**d**) or SeV (**e**) in combination with M2- or GFP-lentivirus. Supernatants were collected at 24 hours post infection and analyzed for IL-1β. These data are representative of 3 independent experiments.

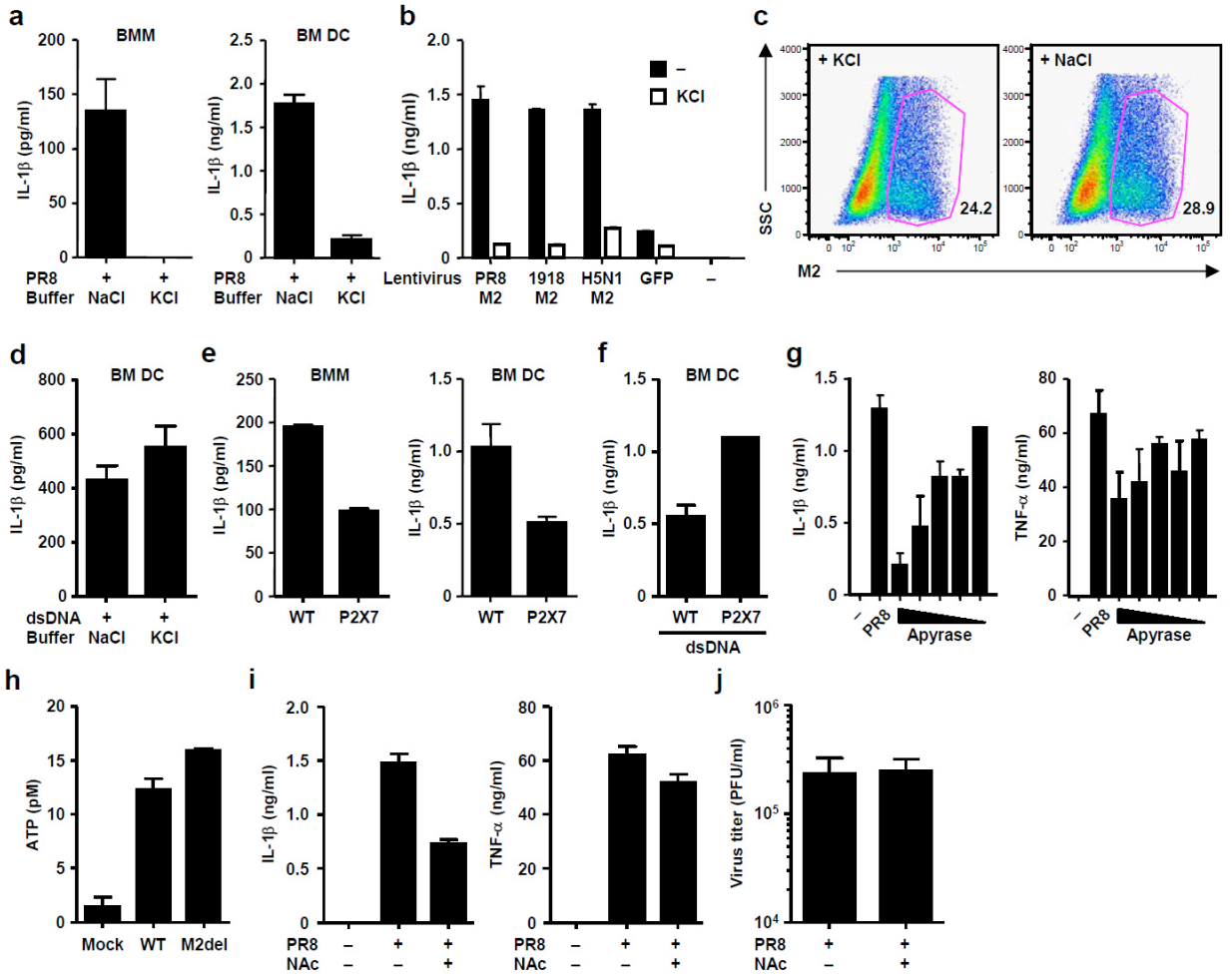

Figure S5 Analysis of pathways required for inflammasome activation by influenza virus infection. (**a**) BMM and BM DC were infected with A/PR8 virus and cultured in the presence or absence of KCl or NaCl (130 mM, each). (**b**) LPS-primed BMM and BM DC were transduced with M2-lentiviruses in the presence or absence of KCl. (**c**) MDCK cells were infected with A/PR8 virus in the presence of KCl or NaCl. Cells were collected at 6 h post infection, and intracellularly stained with M2-specific antibody. (**d**) LPS-primed BM DCs were transfected with poly(dA:dT) in the presence of KCl or NaCl. Supernatants were collected at 20 h post transfection and analyzed for IL-1β by ELISA. (**e**) BMM and BM DC from WT or P2X7$^{-/-}$ mice were infected with A/PR8 virus. (**f**) LPS-primed BM DCs prepared from WT and P2X7$^{-/-}$ mice were transfected with poly(dA:dT). Supernatants were collected at 20 h post transfection and analyzed for IL-1β by ELISA. (**g**) BM DC were infected with A/PR8 virus and cultured in the presence or absence of apyrase at different concentrations (twofold serial dilution from 2.5 U/ml). Supernatants were collected at 24 h post infection and analyzed for IL-1β by ELISA. (**h**) BM DC were infected with A/Udorn or M2del29-31 virus at MOI 2.5. Supernatants were collected at 24 h post infection and analyzed for ATP. (**i**) BM DC were infected with A/PR8 virus and cultured with 2.5 mM N-acetyl-l-cysteine (NAc). Supernatants were collected at 24 h post infection and analyzed for IL-1β and TNF-α by ELISA. (**j**) MDCK cells were infected with A/PR8 virus and cultured in the presence or absence of 2.5 mM NAc. Supernatants were collected at 24 h post infection and analyzed for viral titer by plaque assay.

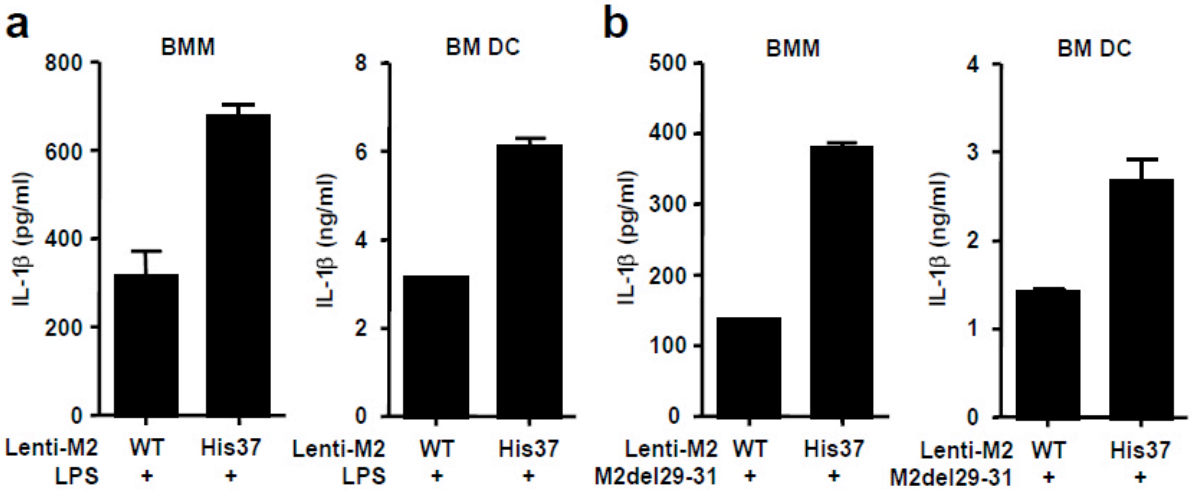

Figure S6. Proton selectivity of M2 is not required for inflammasome activation. (**a-b**) BMM or BM DC were with A/PR8 M2 or A/PR8 M2 mutant ($His_{37}Gly$)-expressing lentivirus. Six hours later, BMM or BM DC were stimulated with 50 ng/ml of LPS (**a**) or infected with M2del29-31 influenza virus (**b**) and cultured for additional 18 hours. Supernatants were collected at 24 hours post lentivirus infection and the levels of IL-1β were analyzed by ELISA. These data are representative of 3 independent experiments.

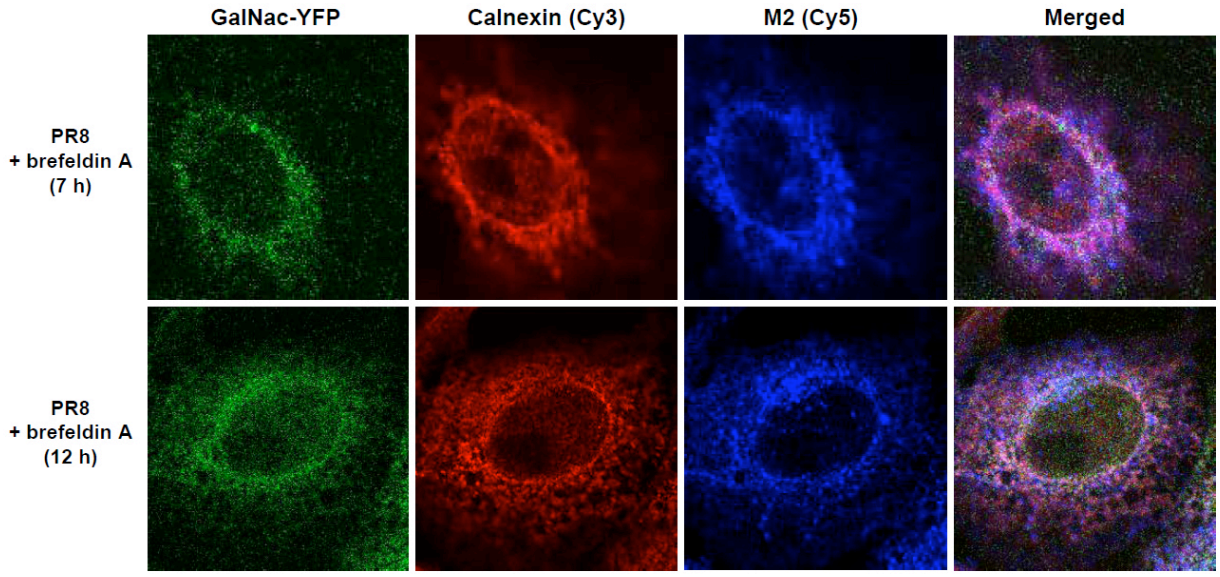

Figure S7. Brefeldin A treatment restricts M2 and Golgi markers to the ER. BSC-1 cells that stably produce the resident Golgi enzyme GalNAc fused to yellow fluorescent protein (GalNAc-T2-YFP) (green) were stimulated with A/PR8 virus in the presence of Brefeldin A (10 µg/ml, included during the first hour of infection). Cells were fixed at 7 and 12 h post infection and stained with anti-M2 (blue) and rabbit polyclonal anti-calnexin antibodies (red). Cells were imaged by confocal microscopy. These data are representative of 2 independent experiments.

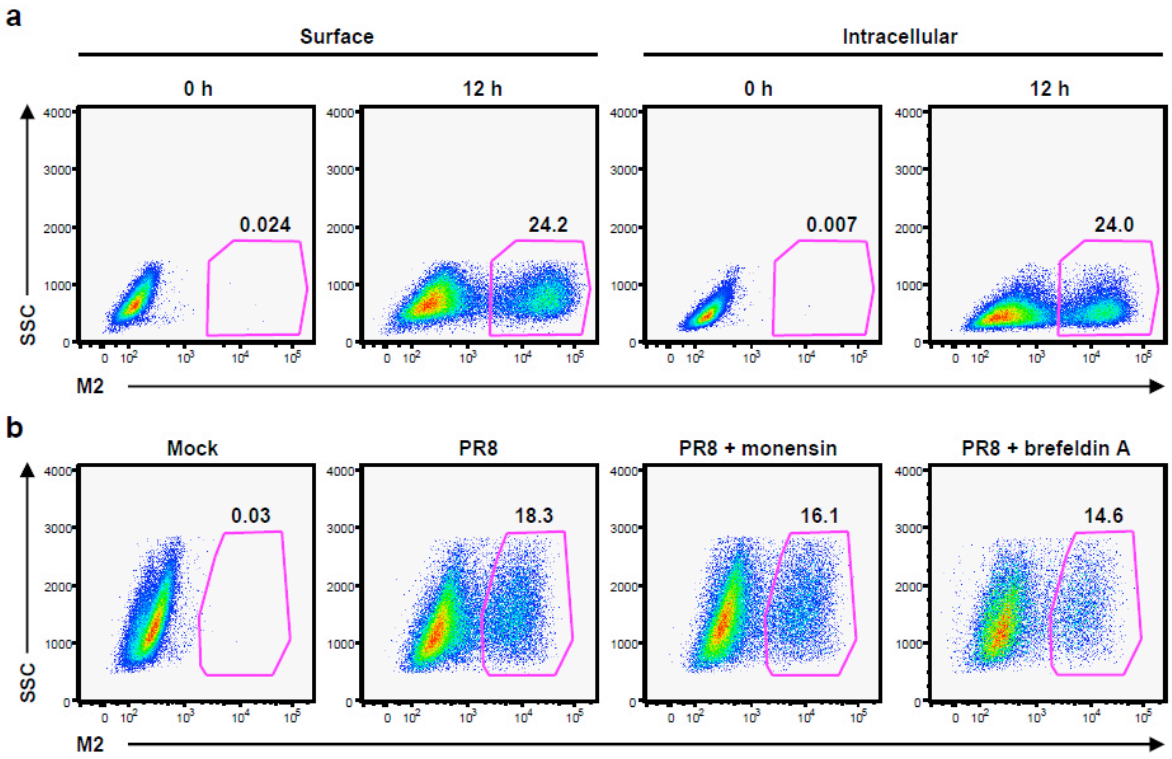

Figure S8. Surface and intracellular M2 expression levels in flu-infected cells. (**a**) HeLa cells were infected with A/PR8 virus for 12 h. Cell surface vs. intracellular expression of M2 were assessed by flow cytometry using M2-specific antibody. (**b**) HeLa cells were infected with A/PR8 virus in the presence or absence of Monensin (10 μM, 24 hours) or Brefeldin A (10 μg/ml, first 1 hour). Cells were collected at 24 h post infection. M2 expression was assessed by intracellular staining using M2-specific antibody. These data are representative of 3 independent experiments.

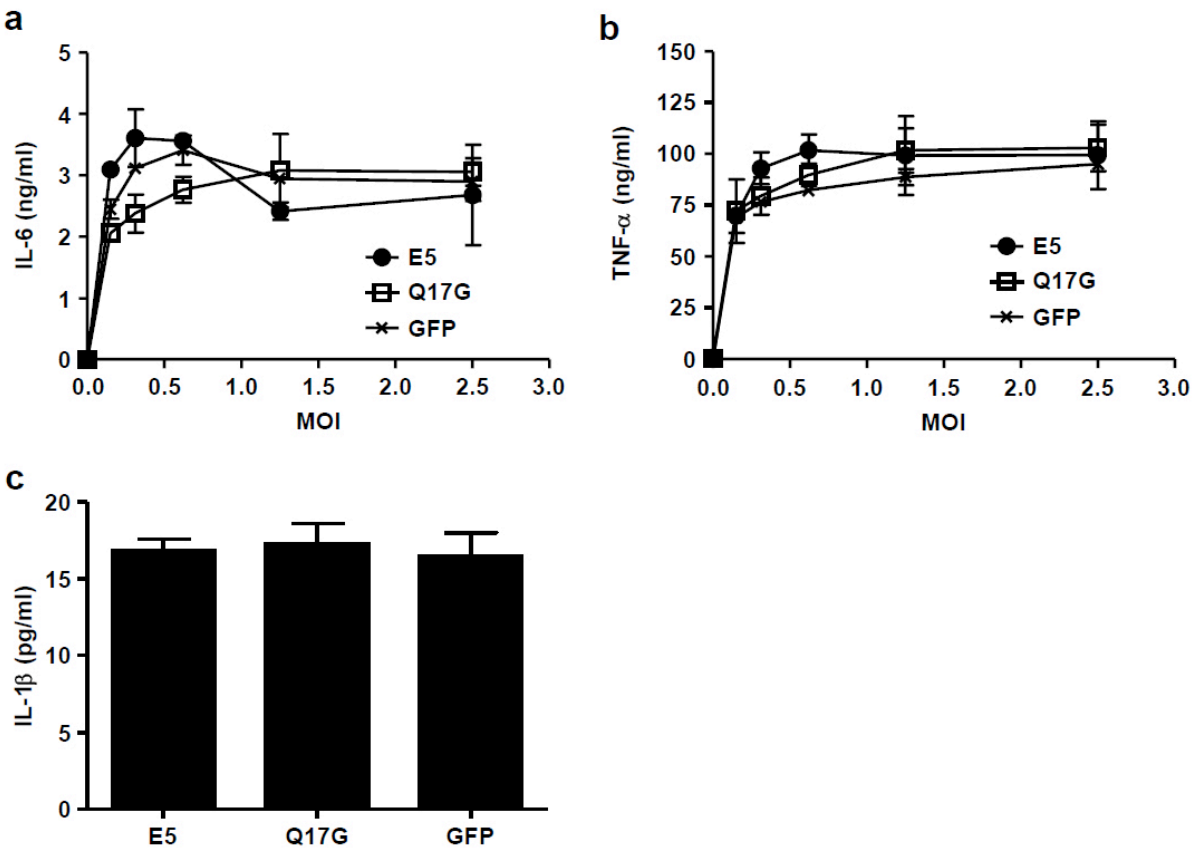

Figure S9. Golgi alkalinization does not affect non-inflammasome dependent cytokine secretion or non-influenza induced inflammasome activation. RAW264.7 cells were infected with WT E5-, E5 Q17G-, or GFP-expressing retrovirus. Cells were incubated in hygromycin-containing media between day 2-4. Selected cells were further cultured for 3 more days in the absence of hygromycin. (**a-b**) E5-, Q17G- and GFP-RAW cells were infected with A/PR8 virus at indicated MOIs. (**c**) E5-, Q17G- and GFP-RAW cells were stimulated with LPS for 6 hours and pulsed with ATP for 30 min. Supernatants were collected at 24 hours post infection and analyzed for IL-1β and TNF-α by ELISA. These data are representative of 3 independent experiments.

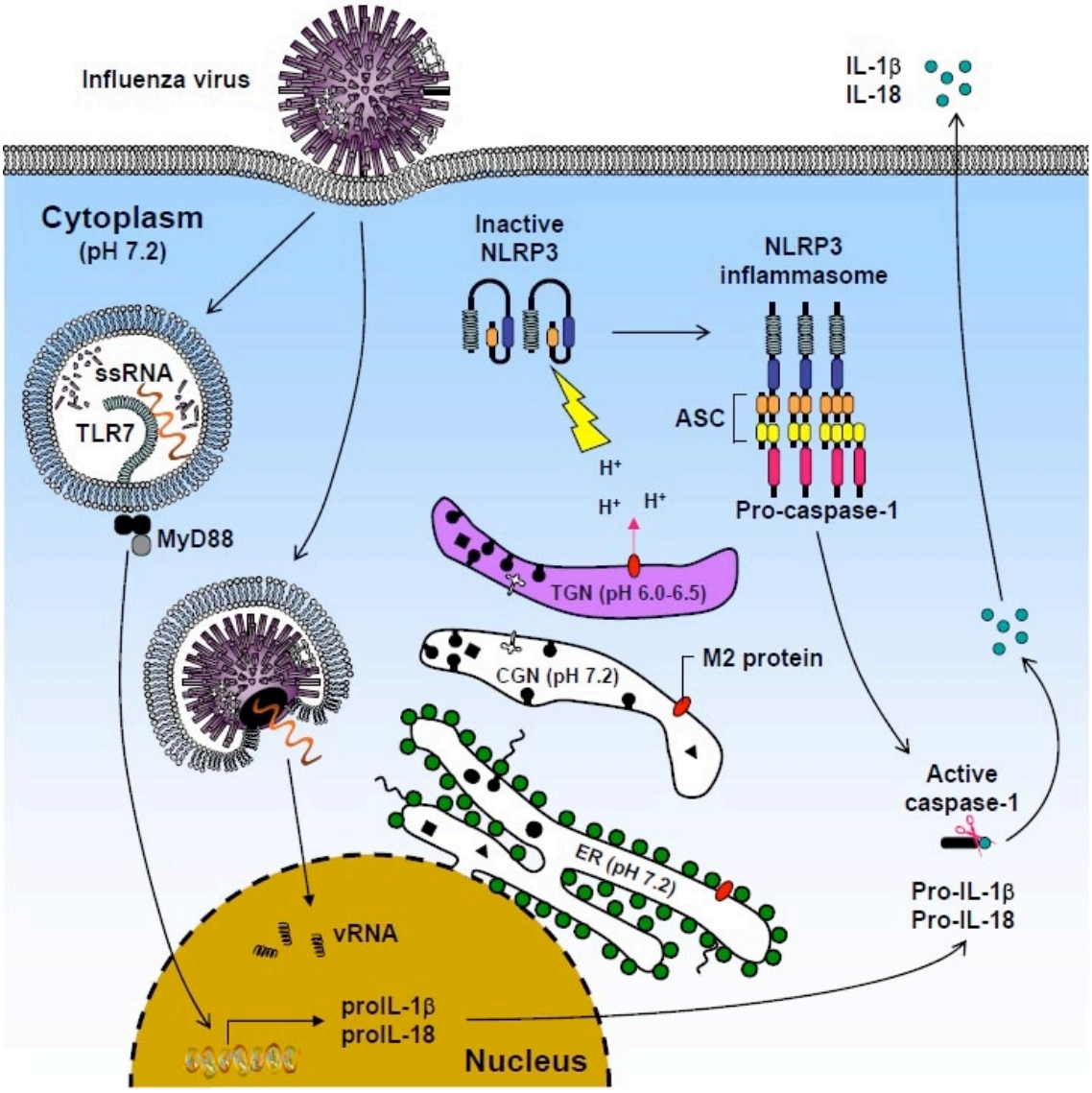

Figure S10. Proposed mechanism of M2-induced inflammasome activation. Influenza virus M2 protein (red) stimulates the NLRP3 inflammasome pathway. Within the acidic trans-Golgi network (TGN), M2 channel opens and transport H^+ ions out of the lumen, leading to neutralization of TGN pH. NLRP3 senses disturbances in ionic concentrations by M2 protein in TGN, leading to inflammasome complex formation and secretion of IL-1β and IL-18.