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The RNA- and TRIM25-Binding Domains of Influenza Virus NS1 Protein Are Essential for Suppression of NLRP3 Inflammasome-Mediated Interleukin-1β Secretion

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ABSTRACT

Inflammasomes are cytosolic multimolecular protein complexes that stimulate the activation of caspase-1 and the release of mature forms of interleukin-1β (IL-1β) and IL-18. We previously demonstrated that the influenza A virus M2 protein stimulates IL-1β secretion following activation of the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome. The nonstructural protein 1 (NS1) of influenza virus inhibits caspase-1 activation and IL-1β secretion. However, the precise mechanism by which NS1 inhibits IL-1β secretion remains unknown. Here, we showed that J774A.1 macrophages stably expressing the NS1 protein inhibited IL-1β secretion after infection with recombinant influenza virus lacking the NS1 gene. Coimmunoprecipitation assay revealed that the NS1 protein interacts with NLRP3. Importantly, the NS1 protein inhibited the NLRP3/ASC-induced single-speck formation required for full activation of inflammasomes. The NS1 protein of other influenza virus strains, including a recent pandemic strain, also inhibited inflammasome-mediated IL-1β secretion. The NS1 RNA-binding domain (basic residues 38 and 41) and TRIM25-binding domain (acidic residues 96 and 97) were required for suppression of NLRP3 inflammasome-mediated IL-1β secretion. These results shed light on a mechanism by which the NS1 protein of influenza virus suppresses NLRP3 inflammasome-mediated IL-1β secretion.

IMPORTANCE

Innate immune sensing of influenza virus via pattern recognition receptors not only plays a key role in generating type I interferons but also triggers inflammatory responses. We previously demonstrated that the influenza A virus M2 protein activates the NLRP3 inflammasome, leading to the secretion of interleukin-1β (IL-1β) and IL-18 following the activation of caspase-1. Although the nonstructural protein 1 (NS1) of influenza virus inhibits IL-1β secretion, the precise mechanism by which it achieves this remains to be defined. Here, we demonstrate that the NS1 protein interacts with NLRP3 to suppress NLRP3 inflammasome activation. J774A.1 macrophages stably expressing the NS1 protein suppressed NLRP3-mediated IL-1β secretion. The NS1 RNA-binding domain (basic residues 38 and 41) and TRIM25-binding domain (acidic residues 96 and 97) are important for suppression of NLRP3 inflammasome-mediated IL-1β secretion. These results will facilitate the development of new anti-inflammatory drugs.

Influenza A virus, a member of the family *Orthomyxoviridae*, is an enveloped virus with an eight-segmented single-stranded negative-sense RNA genome. The virus causes a highly contagious disease of the human upper respiratory tract. The recognition of viruses (e.g., influenza virus) plays a key role not only in limiting virus replication and inflammatory responses at early stages of infection but also in initiating and orchestrating virus-specific adaptive immune responses (1–6). Infection by influenza virus is recognized by at least three classes of host pattern recognition receptors, including Toll-like receptor 7 (TLR7), retinoic acid-inducible gene-I (RIG-I), and NLRP3 (nucleotide-binding oligomerization domain [NOD]-like receptor family pyrin domain-containing 3) (7, 8). First, influenza virus genomic RNA is recognized by TLR7 within endosomal compartments (9, 10). Second, the cytosolic sensor RIG-I directly interacts with the panhandle structure of the viral nucleocapsid and detects the uncapped 5′-triphosphate RNA of the viral genome (11–14). Third, the influenza virus M2 protein, a proton-selective ion channel, stimulates ion flux from the *trans*-Golgi network and activates the NLRP3 inflammasome (15). Upon activation, NLRP3 is recruited to the mitochondria via mitochondrial antiviral signaling (MAVS) or mitofusin 2 (Mfn2) and forms the multimolecular protein complex termed the NLRP3 inflammasome (16, 17). This event activates the downstream molecule caspase-1, which cleaves the precursor forms of proinflammatory cytokines, such as interleukin-1β (IL-1β) and IL-18, and stimulates their secretion across

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the plasma membrane (18). These inflammasome-dependent cytokines play a key role in the induction of influenza virus-specific adaptive immune responses and the initiation of tissue repair following infectious damage (1, 3, 5, 6).

Recent reports indicate that several distinct inflammasomes, including the NLRP3 inflammasome, the NLRP1 inflammasome, the RIG-I inflammasome, the absent-in-melanoma 2 (AIM2) inflammasome, and the interferon gamma (IFN-γ)-inducible protein 16 (IFI16) inflammasome, are involved in viral recognition (7). NLRP3 senses cellular damage or distress induced by the viroporins of RNA viruses such as influenza virus (15), encephalomyocarditis virus (EMCV) (19), rhinovirus (20), or severe acute respiratory syndrome (SARS) coronavirus (21). In contrast, infection with vesicular stomatitis virus (VSV) or transfection with 5′-triphosphate RNA may activate the RIG-I inflammasome (22). Furthermore, the AIM2 inflammasome is activated by intracellular double-stranded DNA derived from DNA viruses (23–26). The IFI16 inflammasome in the nucleus is activated by the DNA genome of Kaposi's sarcoma-associated herpesvirus (KSHV) (27). Therefore, each of these viruses has developed strategies to evade host innate immune recognition systems.

The influenza virus PB1-F2 protein dissipates the mitochondrial membrane potential [$\Delta\Psi(m)$], which is required for full activation of the NLRP3 inflammasome and for MAVS-mediated antiviral signaling (16, 28, 29). In addition, the NS1 protein of influenza virus inhibits host interferon (IFN) responses either by sequestering viral RNA or by binding to RIG-I and other proteins required for RIG-I and IFN signaling pathways (30–34). Although the NS1 protein of influenza virus inhibits virus-induced IL-1β secretion following activation of caspase-1 (35), the precise mechanism by which it achieves this is unclear. Here, we examined the role of the NS1 protein in activating the NLRP3 inflammasome.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from The Jackson Laboratory. All animal experiments were approved by the Animal Committees of the Institute of Medical Science (The University of Tokyo).

Cells and viruses. Bone marrow-derived plasmacytoid dendritic cells (pDCs) were prepared as described previously (36). In brief, bone marrow was extracted from the tibia and femur by flushing with RPMI 1640 medium (Nacalai Tesque). Bone marrow cells were then cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and recombinant human Flt3 ligand (Flt3L; 100 μg/ml [PeproTech]) at 37°C for 7 days. J774A.1 and HeLa cells and the human embryonic kidney cell line, 293T (HEK293T), were maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque) supplemented with 10% FBS. Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential medium (E-MEM; Nacalai Tesque) supplemented with 10% FBS.

Influenza virus A/Puerto Rico/8/34 (H1N1) (PR8) was grown in the allantoic cavities of 10-day-old fertile chicken eggs at 35°C for 2 days (3). Influenza A virus lacking the NS1 gene (37) was grown in MDCK cells stably expressing the influenza virus NS1 protein at 37°C for 2 days. Viruses were stored at −80°C, and the viral titer was quantified in a standard plaque assay using MDCK cells.

Plasmids. cDNAs encoding the influenza virus NS1 proteins of PR8 (H1N1) and A/Narita/1/2009 (H1N1) (Narita/2009) were obtained by reverse transcription and PCR of total RNA extracted from influenza virus-infected MDCK cells, followed by PCR using specific primers. Oligonucleotides corresponding to both strands of the full-length sequence of NS1 proteins from influenza virus strains A/Brevig Mission/1/18 (H1N1) (BM/1918), A/Hong Kong/483/1997 (H5N1) (HK/1997), and A/Anhui/1-BALF_RG1/2013 (H7N9) (Anhui/2013) and containing EcoRI and NotI sites at the 5′ and 3′ ends were synthesized (Eurofins Genomics) and cloned into the eukaryotic expression vectors pCA7-Flag (38) (a derivative of pCAGGS [39]) to produce Flag-tagged proteins. The R38A/K41A and E96A/E97A NS1 mutants were constructed by standard PCR-based methods. The integrity of the inserts was verified by sequencing. Plasmids encoding Flag-, myc-, or enhanced green fluorescent protein (EGFP)-tagged or untagged human NLRP3, RIG-I, ASC (apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain), procaspase-1, pro-IL-1β, uncoupling protein-2 (UCP-2), or influenza virus PB1-F2 protein were described previously (16, 19, 28, 29, 40).

Lentiviral vectors. To generate lentiviruses expressing the influenza virus NS1 protein, the full-length cDNA encoding the NS1 protein was cloned into the pLenti6.3/V5-TOPO vector (Invitrogen). 293FT cells cultured in a collagen-coated 10-cm dish were transfected with 3 μg of NS1 protein-expressing pLenti6.3/V5-TOPO vector together with ViraPower Packaging Mix (Invitrogen) using Lipofectamine 2000 (Invitrogen). The culture medium was replaced with fresh medium 24 h later. At 72 to 96 h posttransfection, the lentivirus-containing supernatants were harvested. A lentivirus encoding an irrelevant protein (EGFP) served as a control. The stock virus, containing Polybrene (10 μg/ml), was then inoculated into J774A.1 or MDCK cells. The culture medium was replaced with fresh medium 24 h later. Finally, the cells were cultured for 2 to 3 weeks in complete medium containing blasticidin (10 μg/ml) to kill nontransduced cells.

Infection. J774A.1 cells were infected with influenza virus at a multiplicity of infection (MOI) of 5 for 1 h at 37°C, washed with phosphate-buffered saline (PBS), and then cultured in complete DMEM for 18 to 24 h. Unless otherwise stated, all experiments were performed in lipopolysaccharide (LPS)-primed J774A.1 cells. Bone marrow-derived pDCs (5×10^5 cells in 96-well round-bottom plates) were stimulated with CpG-A (10 μg/ml; Invivogen) or influenza virus at an MOI of 0.5 for 24 h at 37°C.

ELISA. Cell-free supernatants were collected at 18 to 24 h postinfection or at 6 h after stimulation with LPS plus ATP. The supernatants were analyzed for the presence of IL-1β or IFN-α using an enzyme-linked immunosorbent assay (ELISA) utilizing paired antibodies (eBiosciences) (15, 41). To measure intracellular pro-IL-1β levels, cells were lysed by repeated cycles of freezing and thawing in PBS containing 2% FBS. The lysates were then analyzed by ELISA (16, 22, 40).

Coimmunoprecipitation and Western blot analysis. Subconfluent monolayers of HEK293T cells in 24-well cluster plates were cotransfected with 0.5 μg each of pCA7-EGFP, pCA7-Flag-NLRP3, pCA7-Flag-ASC, or pCA7-Flag-RIG-I together with 0.5 μg of pcDNA3.1-myc-NS1. At 24 h posttransfection, the cells were washed with PBS and lysed in 500 μl of 1× TNT buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol) containing protease inhibitors (Sigma). Lysates were centrifuged at 20,630 × *g* for 10 min at 4°C. A small amount (50 μl) of each supernatant was mixed with sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol [DTT], 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min. The rest of the supernatant was incubated for 60 min at 4°C with protein G-Sepharose (GE Healthcare AB), which had been pretreated with an anti-Flag (M2; Sigma) or normal mouse IgG1 (sc-3877; Santa Cruz) antibody overnight at 4°C. Complexes were obtained by centrifugation and washed three times with coimmunoprecipitation buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA). The polypeptides within the precipitated complexes were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) (10 to 15% gels) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). The membranes were incubated with rabbit anti-mouse IL-1β (Chemicon), mouse anti-influenza A virus M2 (14C2; Abcam), mouse anti-influenza A virus NS1 (NS1-23-1; Santa Cruz), mouse anti-tubulin (DM1A; Santa Cruz), mouse anti-NLRP3 (Cryo-2; AdipoGen), mouse anti-myc (9E10), or mouse anti-Flag (M2; Sigma) antibody, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (Jackson Immuno

Research Laboratories) or anti-rabbit IgG (Invitrogen). The PVDF membranes were then treated with Chemi-Lumi One Super (Nacalai Tesque) to elicit chemiluminescent signals, which were detected and visualized using an LAS-4000 Mini apparatus (GE Healthcare).

Flow cytometry analysis. The mitochondrial membrane potential [$\Delta\Psi$(m)] was measured by staining the cells with a cationic fluorescent dye, tetramethylrhodamine methyl ester (TMRM) (Molecular Probes/Invitrogen), according to the manufacturer's instructions. In brief, cells ($\sim 1 \times 10^6$ cells per ml) were treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma-Aldrich) or transfected with plasmids, washed once with PBS, and harvested into a centrifuge tube. The cells were then resuspended in 1 ml of PBS containing 2 μM TMRM and incubated at 37°C for 30 min. After three washes with PBS, flow cytometry analysis was performed in a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc.).

Reconstitution of the NLRP3 inflammasome in HEK293T cells. HEK293T cells, grown to ~90% confluence in 24-well cluster plates, were transfected with pCA7-NLRP3 (30 ng), pCA7-ASC (5 ng), pCA7-procaspase-1 (5 ng), and pCA7-pro-IL-1β (150 ng) and 500 ng of either pCA7-EGFP, pCA7-hUCP-2 (where hUCP-2 is human UCP-2), or pCA7-NS1 using polyethylenimine (PEI) Max. Cell-free supernatants were collected 24 h after transfection, and IL-1β levels were measured in an ELISA.

To analyze the activation of caspase-1, HEK293T cells in 24-well cluster plates were transfected with pCA7-NLRP3 (30 ng), pCA7-ASC (5 ng), pCA7-procaspase-1 (500 ng), and pCA7-pro-IL-1β (150 ng) and 500 ng of either pCA7-EGFP, pCA7-Flag-NS1, pCA7-Flag-R38A/K41A, or pCA7-Flag-E96A/E97A using PEI Max. Cell extracts were collected at 24 h posttransfection, and samples were analyzed by immunoblotting with rabbit monoclonal antibody against human caspase-1 (D7F10; Cell Signaling).

Confocal microscopy. HeLa cells were seeded on coverslips in 24-well cluster plates and transfected with 0.5 μg each of pCA7-myc-NS1, pCA7-Flag-NS1, pCA7-PB1-F2, or control plasmid (empty vector) together with 30 ng of pCA7-EGFP-NLRP3 and 5 ng of pCA7-Flag-ASC or pCA7-HA-ASC (where HA is hemagglutinin). At 24 h posttransfection, cells were fixed and permeabilized with PBS containing 4% formaldehyde and 1% Triton X-100. The cells were then washed with PBS and incubated with rabbit anti-Flag (F7425; Sigma), mouse anti-HA (F-7; Santa Cruz), or mouse anti-myc (9E10) antibody, followed by incubation with Alexa Fluor 647-conjugated goat anti-mouse IgG(H+L) and Alexa Fluor 568-conjugated goat anti-rabbit IgG(H+L) antibodies (Life Technologies).

To analyze the subcellular localization of influenza virus NS1 and PB1-F2 proteins, HeLa cells were seeded on coverslips in 24-well plates and transfected with 0.5 μg each of pCA7-myc-NS1 or pCA7-PB1-F2. At 24 h posttransfection, cells were fixed and permeabilized with PBS containing 4% formaldehyde and 1% Triton X-100. The cells were then washed with PBS and incubated with a rabbit anti-Tom20 (FL-145; Santa Cruz) antibody together with mouse anti-myc (9E10) or mouse anti-PB1-F2 antibody (29), followed by incubation with Alexa Fluor 647-conjugated goat anti-mouse IgG(H+L) and Alexa Fluor 568-conjugated goat anti-rabbit IgG(H+L) (Life Technologies). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). The stained cells were then observed under a confocal microscope (A1R$^+$; Nikon).

Statistical analysis. Statistical significance was tested using a two-tailed Student's *t* test. *P* values of <0.05 were considered statistically significant.

RESULTS

The influenza virus NS1 protein inhibits NLRP3 inflammasome-mediated IL-1β secretion. We used an NLRP3 reconstitution assay in HEK293T cells to test whether the NS1 protein of influenza virus inhibits NLRP3 inflammasome-mediated IL-1β secretion because HEK293T cells are deficient in endogenous NLRP3 inflammasomes (42); however, they can produce mature IL-1β upon transfection with plasmids encoding NLRP3, ASC, procaspase-1, and pro-IL-1β (40, 43). Indeed, reconstitution of the NLRP3 inflammasome resulted in IL-1β secretion by HEK293T cells, for which NLRP3 was absolutely required (Fig. 1A and B). Consistent with previous reports (16, 29), transfection of HEK293T cells with human uncoupling protein-2 (hUCP-2), which reduces $\Delta\Psi$(m) via proton leakage (44, 45), suppressed IL-1β secretion (Fig. 1A and B). Similarly, NS1 protein significantly inhibited IL-1β secretion (Fig. 1A and B) without affecting the expression of pro-IL-1β in the cytosol (Fig. 1C and D).

We next examined whether the suppression of NLRP3 inflammasome-mediated IL-1β secretion by NS1 protein in HEK293T cells can be reproduced by recombinant influenza virus lacking the NS1 gene (ΔNS1 virus). We first confirmed the deletion of the NS1 protein in HEK293T cells infected with ΔNS1 virus by immunoblotting (Fig. 2A). Consistent with a previous report (12), bone marrow-derived pDCs produced more IFN-α in response to mutant ΔNS1 virus than in response to the wild-type (WT) influenza virus (Fig. 2B). Since the mutant ΔNS1 virus has a lower replicative capacity than wild-type influenza virus *in vitro* (37), we decided to compare IL-1β secretion by EGFP-expressing and NS1 protein-expressing J774A.1 macrophages infected with ΔNS1 virus. To this end, we established J774A.1 macrophages stably expressing EGFP or the NS1 protein (Fig. 2C). J774A.1 macrophages stably expressing the NS1 protein secreted significantly less IL-1β in response to ΔNS1 virus or LPS plus ATP (NLRP3 agonist) than control cells (Fig. 2D and E), indicating that the NS1 protein inhibits NLRP3 inflammasome-mediated IL-1β secretion. Thus, the inhibitory activity of the NS1 protein appears to be specific for the NLRP3 inflammasome.

The mitochondrial membrane potential is not affected by the NS1 protein. We previously demonstrated that the PB1-F2 protein of influenza virus dissipated $\Delta\Psi$(m) and suppressed NLRP3 inflammasome-mediated IL-1β secretion (29). To gain insight into the mechanism by which the NS1 protein inhibits NLRP3 inflammasome activation, we first measured $\Delta\Psi$(m) (which is required for full activation of the NLRP3 inflammasome) (16). Consistent with previous reports (16, 29), the PB1-F2 protein localized to mitochondria and dissipated $\Delta\Psi$(m) (Fig. 3A and B). Similarly, $\Delta\Psi$(m) was dissipated in cells treated with the protonophore carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) and in cells transfected with hUCP-2 (Fig. 3B). In contrast, the NS1 protein of influenza virus did not localize to mitochondria and alter $\Delta\Psi$(m) (Fig. 3A and B), indicating that the mechanism by which the NS1 protein inhibits NLRP3 inflammasome-mediated IL-1β secretion is different from that of the PB1-F2 protein.

NS1 protein interacts with NLRP3. We next examined the association between the NS1 protein and components of the NLRP3 inflammasome by performing coimmunoprecipitation experiments using HEK293T cells. In agreement with a previous report (12), the NS1 protein coimmunoprecipitated with RIG-I in HEK293T cells. The NS1 protein coimmunoprecipitated with NLRP3 to a lesser extent than in cells cotransfected with RIG-I (Fig. 4A). Recent reports indicate that both the NLRP3 and RIG-I inflammasomes are involved in recognition of influenza virus or cytosolic viral RNA (1, 3, 6, 15, 22). So, we next examined whether the NS1 protein of influenza virus inhibits NLRP3 and RIG-I inflammasome-mediated IL-1β secretion. Reconstitution of the NLRP3, but not RIG-I, inflammasome resulted in IL-1β secretion by HEK293T cells (Fig. 4B). Inclusion of the NS1 protein signifi-

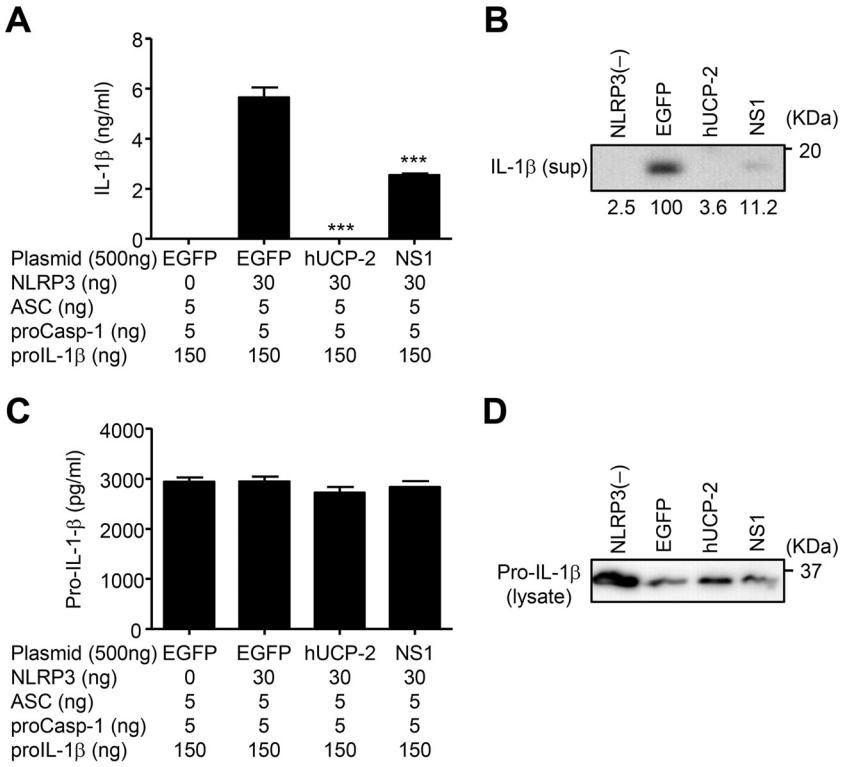

FIG 1 NS1 inhibits NLRP3 inflammasome-mediated IL-1β secretion. (A) HEK293T cells were transfected with expression plasmids encoding either NLRP3, ASC, procaspase-1, pro-IL-1β, and human UCP-2 (hUCP-2) or NS1. pCA7-EGFP was used as a control. (A and B) Cell-free supernatants were collected at 24 h posttransfection, and the mature (p17) form of IL-1β was analyzed by ELISA (A) or immunoblot analysis (B). sup, supernatant. (C and D) Cell extracts were collected at 24 h posttransfection, and the immature form of IL-1β (pro-IL-1β) was analyzed by ELISA (C) or immunoblot analysis (D). Data in panels A and C are representative of at least three independent experiments and are expressed as the means ± standard deviations. ***, $P < 0.001$.

cantly inhibited NLRP3 inflammasome-mediated IL-1β secretion (Fig. 4B). These results suggest that the NS1 protein interacts with NLRP3 and inhibits the NLRP3 inflammasome-mediated IL-1β secretion.

NS1 protein inhibits a single-speck formation of NLRP3. Upon activation of the inflammasomes, ASC forms speck-like structures, termed pyroptosomes (25, 46). It has been reported that ASC forms specks typically with one speck per cell when HEK293T cells are transfected with plasmids encoding ASC. The lack of a single-speck formation correlates with a loss of IL-1β processing in response to NLRP3 agonists in RAW264.7 cell (47). Thus, we next examined whether the NS1 protein of influenza virus inhibits a single-speck formation. To this end, we cotransfected HeLa cells with EGFP-tagged NLRP3 and Flag-tagged ASC to visualize speck formation. When HeLa cells were transfected with Flag-tagged ASC alone, most of the ASC protein localized to the nucleus and other cytoplasmic structures (Fig. 5). Transfection of HeLa cells with EGFP-tagged NLRP3 and Flag-tagged ASC led to a single-speck formation, which colocalized with NLRP3 and ASC (Fig. 5). In contrast, NLRP3 and ASC formed discrete specks when HeLa cells were cotransfected with EGFP-tagged NLRP3, Flag-tagged ASC, and myc-tagged NS1 or PB1-F2 (Fig. 5). Taken together, these results suggest that the NS1 protein of influenza virus inhibits a single-speck formation of NLRP3 and ASC.

The NS1 proteins from other influenza virus strains inhibit NLRP3 inflammasome-mediated IL-1β secretion. So far, we used the NS1 gene derived from the mouse-adapted A/Puerto Rico/8/34 (H1N1) (denoted PR8) strain. Next, we compared the inhibitory effect of NS1 proteins from other influenza viruses, including those from recent pandemic strains A/Narita/1/2009 (H1N1) (denoted Narita/2009), A/Brevig Mission/1/1918 (H1N1) (denoted BM/1918), A/Hong Kong/483/1997 (H5N1) (denoted HK/1997), and A/Anhui/1-BALF-RG1/2013 (H7N9) (denoted Anhui/2013), on NLRP3 inflammasome activation to determine whether viral pathogenesis could be explained (at least in part) by the ability of the respective NS1 proteins to inhibit host inflammatory responses. The NS1 protein from all five viral strains significantly inhibited NLRP3-mediated IL-1β secretion compared with the level in EGFP-transfected cells (Fig. 6A). Strikingly, IL-1β secretion was completely abrogated by transfecting cells with a plasmid encoding the BM/1918 NS1 protein. It has been reported that the NS1 protein of BM/1918 has an intact CPSF30-binding site that blocks host mRNA maturation (48, 49). Indeed, this inhibitory effect of IL-1β secretion was due to suppression of NLRP3 expression in BM/1918 NS1-transfected cells (Fig. 6B). Taken together, these data indicate that the NS1 protein from not only a mouse-adapted laboratory strain but also other human isolates inhibits NLRP3 inflammasome-mediated IL-1β secretion.

R38A/K41A and E96A/E97A NS1 mutants are defective in blocking NLRP3 inflammasome-mediated IL-1β secretion. Finally, we examined the role of functional regions of the NS1 protein, including RNA-binding and TRIM25-binding domains, for the inhibition of NLRP3 inflammasome-mediated IL-1β secre-

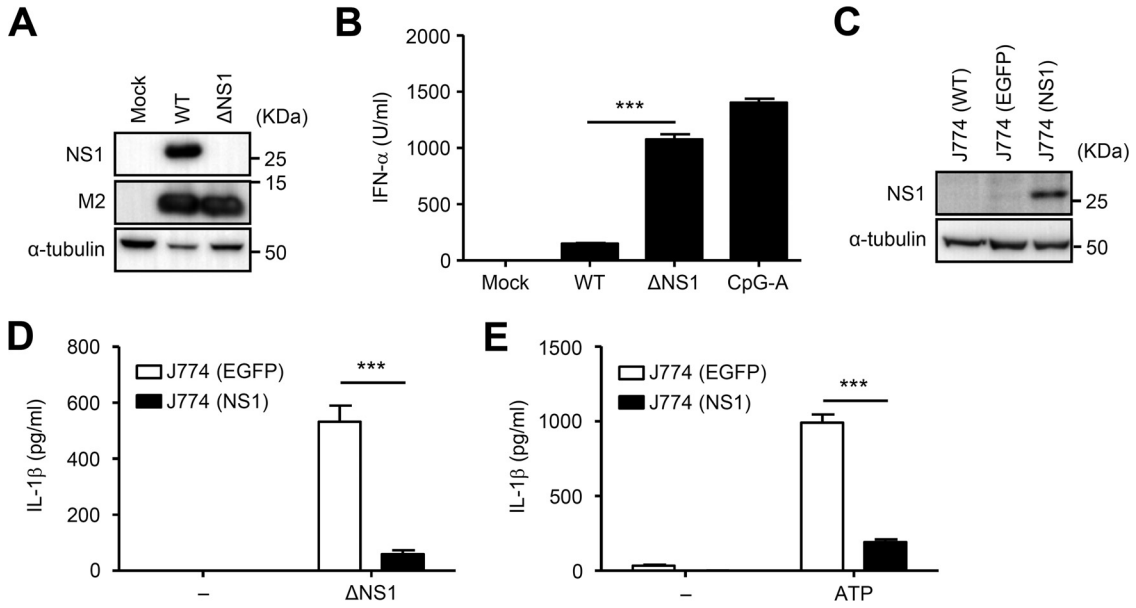

FIG 2 Influenza virus lacking the NS1 gene failed to stimulate IL-1β secretion from J774A.1 cells stably expressing the NS1 protein. (A) HEK293T cells were infected with wild-type (WT) or recombinant influenza virus lacking the NS1 gene (ΔNS1). Cell extracts were collected at 24 h postinfection, and NS1 and M2 protein expression was analyzed by immunoblotting. (B) Flt3L-cultured bone marrow pDCs were stimulated with CpG-A DNA (10 μg/ml) or WT or ΔNS1 influenza virus (MOI of 0.5) for 24 h. Cell-free supernatants were collected at 24 h poststimulation and analyzed for IFN-α by ELISA. (C) Samples from J774A.1 cells stably expressing EGFP or NS1 were analyzed by immunoblot analysis. (D and E) LPS-primed J774A.1 cells stably expressing EGFP or NS1 were stimulated with ΔNS1 or ATP. Cell-free supernatants were collected at 24 h poststimulation and analyzed for IL-1β by ELISA. Data are expressed as the means ± standard deviations. Similar results were obtained in three separate experiments. ***, $P < 0.001$.

tion. To this end, we first generated plasmids encoding Flag-tagged NS1 protein in which amino acids R38/K41 or E96/E97 were mutated to A residues and confirmed their expression in HEK293T cells by immunoblot analysis (Fig. 7A). To assess the role of the RNA-binding domain (R38A/K41A) and TRIM25-binding domain (E96A/E97A) of NS1 protein in NLRP3 inflammasome activation, we first transfected HEK293T cells with plasmids encoding NLRP3, ASC, procaspase-1, pro-IL-1β, and either EGFP, Flag-tagged WT, R38A/K41A, or E96A/E97A NS1. Reconstitution of the NLRP3 inflammasome resulted in IL-1β secretion by HEK293T cells, in which NLRP3 was absolutely required (Fig. 7B). Inclusion of Flag-tagged WT NS1 but not the R38A/K41A and E96A/E97A NS1 mutants inhibited NLRP3 inflammasome-mediated IL-1β secretion (Fig. 7B) and caspase-1 activation (Fig. 7C). These NS1 mutants failed to associate with NLRP3 (Fig. 7D). Furthermore, the WT but not mutant NS1 proteins inhibited a single-speck formation of NLRP3 and ASC (Fig. 7E). Taken together, these results suggest that the RNA- and TRIM25-binding domains of NS1 protein are important for NLRP3 inflammasome-mediated IL-1β secretion.

DISCUSSION

Here, we showed that the NS1 protein of influenza virus interacts with NLRP3 and inhibits the NLRP3/ASC-induced single-speck formation required for full activation of inflammasome, thereby inhibiting the NLRP3 inflammasome-mediated secretion of IL-1β.

Both DNA and RNA viruses induce the secretion of IL-1β from infected cells. Recent reports indicate that the viroporins of RNA viruses, such as influenza virus M2 protein (15), EMCV 2B protein (19), rhinovirus 2B protein (20), or SARS coronavirus E protein (21), induce IL-1β secretion. In contrast, other studies suggested that viral RNA is involved in NLRP3- or RIG-I-mediated inflammasome activation (1, 22, 50, 51). In addition, Chakrabarti et al. showed that RNA cleavage products generated by RNase L bind to the DEXD/H-box helicase, DHX33, and activate the NLRP3 inflammasome (52). These results indicate that while influenza virus M2 protein triggers NLRP3 inflammasome activation in macrophages and dendritic cells (15), other cell types, such as lung epithelial cells, employ partially redundant recognition mechanisms and that virus-induced type I interferon through the RIG-I/TRIM25-dependent pathway stimulates RIG-I and NLRP3 expression and their assembly to form the RIG-I and NLRP3 inflammasome (51). Given that viral RNA triggers RIG-I and NLRP3 inflammasome activation, the NS1 protein may inhibit RIG-I and NLRP3 inflammasome-mediated IL-1β secretion by sequestering viral RNA or by binding to RIG-I in influenza virus-infected cells. Recently, Cheong et al. demonstrated that NS1 proteins derived from both highly pathogenic (A/Hong Kong/483/1997 [H5N1]) and low-pathogenic (A/WSN/1933 [H1N1]) strains suppressed IL-1β secretion from THP-1 cells after stimulation with LPS plus ATP (NLRP3 agonist) (53). In the present study, we demonstrated that NS1 protein derived from different virus strains suppressed NLRP3 inflammasome-mediated IL-1β secretion by different mechanisms. The BM/1918 NS1 protein, which has an intact CPSF30-binding site that blocks host mRNA maturation, suppressed expression of NLRP3, thereby abrogating NLRP3 inflammasome-mediated IL-1β secretion (Fig. 6). In contrast, other NS1 proteins did not change the expression levels of NLRP3 or $\Delta\Psi$(m). Instead, the RNA-binding domain (basic residues 38 and 41) and TRIM25-binding domain (acidic residues 96 and 97) of NS1 protein are required for suppression of

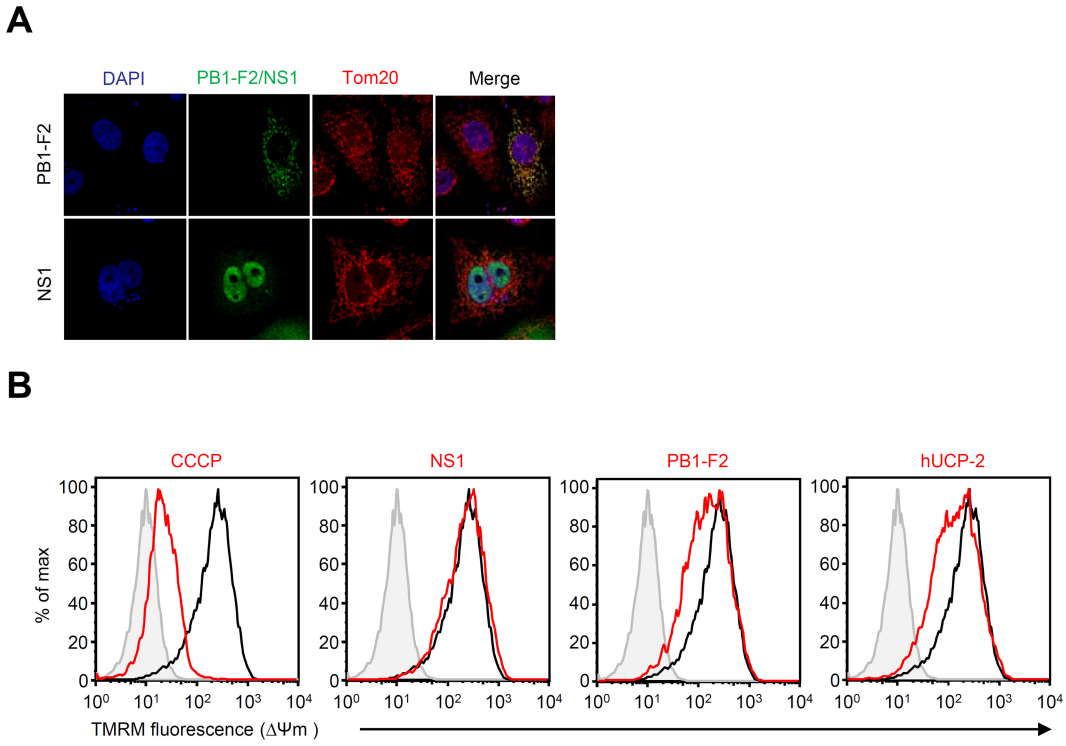

FIG 3 NS1 does not reduce mitochondrial membrane potential. (A) HeLa cells were transfected with an expression plasmid encoding influenza virus PB1-F2 or myc-tagged NS1. Twenty-four hours later, cells were stained with anti-myc, anti-PB1-F2, and anti-Tom20 (mitochondrion marker) antibodies and analyzed by confocal microscopy. Nuclei were visualized by staining with DAPI. (B) HEK293T cells were treated with CCCP (40 μM) or transfected with an expression plasmid encoding influenza virus PB1-F2 or NS1. Twenty-four hours later, the cells were stained with TMRM and analyzed by flow cytometry. Similar results were obtained in three separate experiments. max, maximum.

NLRP3 inflammasome-mediated IL-1β secretion. Since type I IFN signals enhance IL-1β secretion in response to influenza virus infection (3, 51), the NS1 protein appears to play a key role in inhibition of RIG-I and NLRP3 inflammasome-mediated IL-1β secretion from nonimmune cells such as lung epithelia.

Viruses utilize many strategies to inhibit inflammasome activation. We previously demonstrated that the PB1-F2 protein of influenza virus translocates to the mitochondrial inner membrane space and dissipates $\Delta\Psi(m)$, thereby suppressing NLRP3 inflammasome-mediated IL-1β secretion (29). In contrast, the NS1 protein did not dissipate $\Delta\Psi(m)$. Instead, the NS1 protein interacted with NLRP3 and inhibited a single-speck formation, which is required for full activation of inflammasomes (47). Similarly, the nonstructural V protein of measles virus interacted with NLRP3

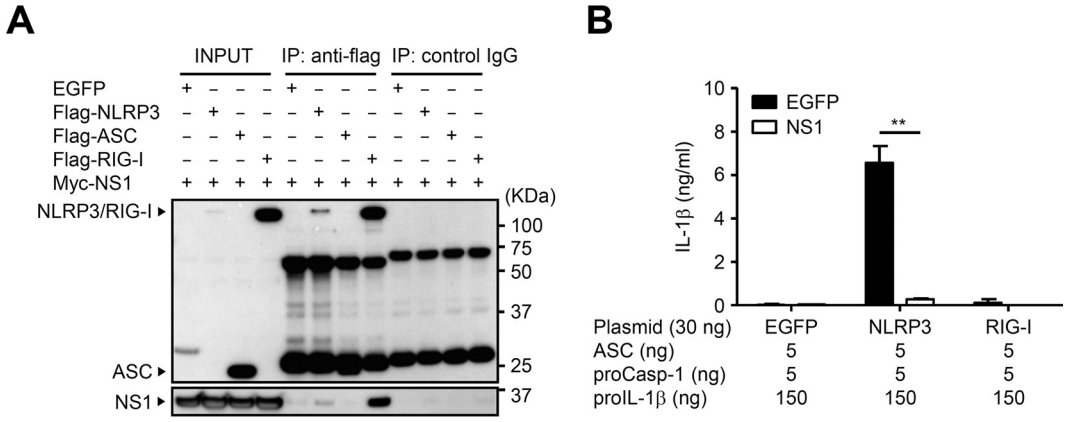

FIG 4 NS1 protein interacts with NLRP3. (A) HEK293T cells were cotransfected with expression plasmids encoding Flag-tagged NLRP3, ASC, or RIG-I together with myc-tagged NS1. pCA7-EGFP was used as a control. At 24 h posttransfection, proteins were immunoprecipitated with an anti-Flag antibody, followed by immunoblotting of total lysate (Input) and immunoprecipitates (IP) with anti-Flag or anti-myc antibodies. (B) HEK293T cells were transfected with expression plasmids as indicated. pCA7-EGFP was used as a control. Cell-free supernatants were collected at 24 h posttransfection and analyzed for IL-1β by ELISA. Data in panel B are representative of at least three independent experiments and are expressed as the means ± standard deviations. **, $P < 0.01$.

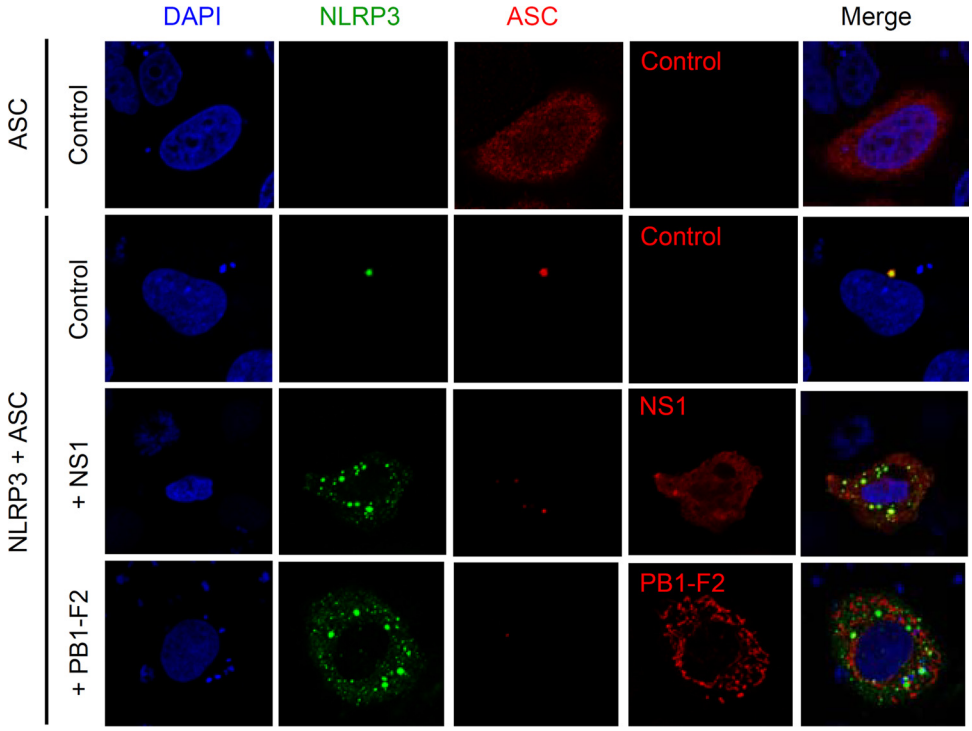

FIG 5 Inhibition of a single-speck formation by influenza virus NS1 protein. HeLa cells were cotransfected with expression plasmids encoding Flag-tagged ASC, EGFP-NLRP3, and myc-tagged NS1. PB1-F2 was used as a positive control. At 24 h after transfection, cells were stained with anti-Flag, anti-myc, and anti-PB1-F2 antibodies and analyzed by confocal microscopy. Nuclei were visualized by staining with DAPI. Data are representative of at least three independent experiments.

to specifically inhibit NLRP3 inflammasome activity (40). KHSV Orf63, a viral homolog of human NLRP1, inhibits inflammasome activation by interacting with NLRP1 or NLRP3. In addition, the pyrin domain (PYD)-containing M13L protein of myxoma virus, a rabbit-specific poxvirus, interacts with ASC and inhibits inflammasome activation. The Basic Local Alignment Search Tool for proteins, blastp, and ClustalW2 alignment confirmed that the NS1 protein of influenza virus does not contain any regions homologous with NLRP3, indicating that the interaction is not dependent on homologous conserved sequences.

It was reported that IL-1 receptor (IL-1R) signaling enhanced the ability of DCs to stimulate T cells (5, 54). In addition, inflammasome-dependent cytokine production plays a key role in the induction of virus-specific adaptive immunity and in the initiation of host repair responses to lung tissue damage after influenza virus infection (1, 3, 55). These observations suggest that inhibi-

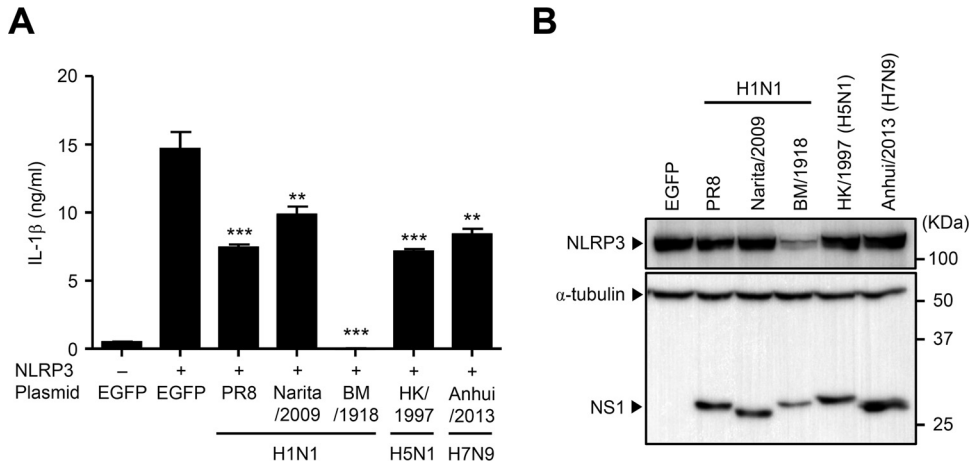

FIG 6 The NS1 protein from different strains suppresses NLRP3 inflammasome-mediated IL-1β secretion. (A) HEK293T cells were transfected with expression plasmids encoding NLRP3, ASC, procaspase-1, pro-IL-1β, and NS1 from different virus strains as indicated. pCA7-EGFP was used as a control. Cell-free supernatants were collected at 24 h posttransfection, and IL-1β was analyzed by ELISA. (B) HEK293T cells were transfected as described for panel A. Cell extracts were collected at 24 h posttransfection, and NLRP3 expression was analyzed by immunoblotting. Data in panel A are representative of at least three independent experiments and are expressed as the means ± standard deviations. **, $P < 0.01$; ***, $P < 0.001$.

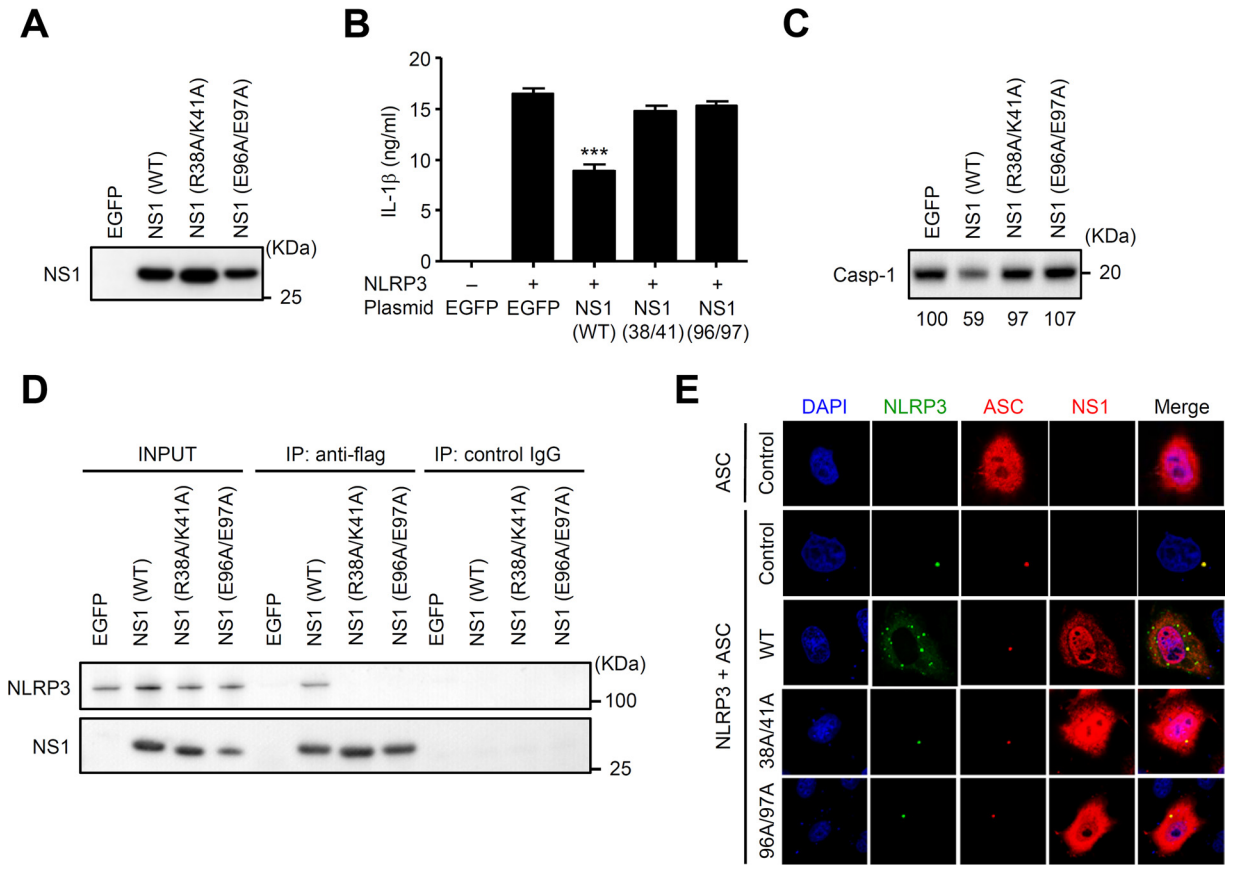

FIG 7 The RNA- and TRIM25-binding domains of NS1 protein are essential for inhibition of NLRP3 inflammasome activation. (A) HEK293T cells were transfected with plasmids encoding Flag-tagged WT or mutant NS1. Cell extracts were collected at 24 h posttransfection, and NS1 protein expression was analyzed by immunoblotting. (B) HEK293T cells were transfected with expression plasmids encoding NLRP3, ASC, procaspase-1, and pro-IL-1β together with WT or mutant NS1. pCA7-EGFP was used as a control. Cell-free supernatants were collected at 24 h posttransfection, and IL-1β was analyzed by ELISA. (C) HEK293T cells were transfected as described for panel B. Cell extracts were collected at 24 h posttransfection, and active caspase-1 (p20) was analyzed by immunoblotting. (D) HEK293T cells were cotransfected with expression plasmids encoding NLRP3 together with Flag-tagged wild-type or mutant NS1. pCA7-EGFP was used as a control. At 24 h posttransfection, proteins were immunoprecipitated with an anti-Flag antibody, followed by immunoblotting of total lysate (Input) and immunoprecipitates (IP) with anti-Flag or anti-NLRP3 antibodies. (E) HeLa cells were cotransfected with expression plasmids encoding HA-tagged ASC, EGFP-NLRP3, and Flag-tagged NS1. Twenty-four hours after transfection, cells were stained with anti-Flag and anti-HA antibodies and analyzed by confocal microscopy. Nuclei were visualized by staining with DAPI. Data in panel B are representative of at least three independent experiments and are expressed as the means ± standard deviations. ***, $P < 0.001$.

tion of inflammasome activation by the NS1 protein may be associated with influenza virus pathogenesis.

In summary, the findings of the present study reveal a mechanism by which NS1 protein inhibits inflammasome activation. These results will aid the development of new anti-inflammatory drugs. Also, inhibiting NS1 activity might be a potential novel therapeutic approach.

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