Rapid Communication

# NS1 Protein of Influenza A Virus Inhibits the Function of Intracytoplasmic Pathogen Sensor, RIG-I

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**Retinoic acid–inducible gene I (RIG-I) has recently been identified as one of the key intracellular sensors of virus infection. RIG-I binds to cytosolic double-stranded RNA and initiates a signaling cascade that leads to the activation of transcription factors required for expression of type I interferon (IFN-I). Previous evidence suggests that nonstructural protein 1 (NS1) encoded by influenza A virus (IAV) suppresses IFN-I secretion in virus-infected cells by an unknown mechanism. In the present study, we demonstrate that RIG-I is required for induction of IFN-I in an IAV-infected human lung epithelial cell line. Knockdown of RIG-I expression by RNA interference greatly impairs production of IFN-β in cells infected with different strains of wild-type IAV. Furthermore, co-expression of IAV NS1 down-regulates production of IFN-β induced by RIG-I agonists, and ectopic expression of RIG-I inhibits the replication of IAV. These results provide further information on the mechanism by which IAV NS1 antagonizes the host antiviral response.**

**CLINICAL RELEVANCE**

Our findings indicate the potential clinical utility of RIG-I–based therapeutic strategies to prevent and treat infections caused by seasonal and pandemic influenza viruses.

The innate immune system is the host's first line of defense against a variety of pathogens. One of the major mechanisms for rapid initiation of host innate immune responses is to recognize conserved motifs or pathogen-associated molecule patterns (PAMPs) unique to microorganisms by pattern recognition receptors, such as Toll-like receptors (TLRs; 1). Upon recognition of PAMPs, TLRs activate signaling pathways that lead to secretion of proinflammatory cytokines, such as type I interferon (IFN-I), that are essential in antiviral immunity. Viral infection is generally associated with double-stranded RNA (dsRNA) production. IFN-I is induced by ligation of extracellular dsRNA, lipopolysaccharide, single-stranded RNA (ssRNA), and unmethylated CpG DNA to TLR3, TLR4, TLR7, and TLR9, respectively (1). TLR3 and TLR4 act through a Myd88-independent pathway, while TLR7 and TLR9 appear to use Myd88 as an adaptor protein for IFN signaling. In addition to TLR-dependent pathways, IFN-I is also induced by retinoic acid–inducible gene I (RIG-I) through recognition of intracellular dsRNA, a component or replication product of many RNA viruses (2). RIG-I contains two caspase recruitment domains (CARDs) at its N-terminus and a DExD/H box RNA helicase domain at its C-terminus. RIG-I interacts with dsRNA through its helicase domain and initiates IFN production in an ATPase-dependent manner (2). RIG-I CARD-mediated signaling results in the activation of IRF-3 and NF-κB.

Several human viruses, including hepatitis C virus (HCV, 3), vaccinia virus (4), Ebola virus (5), and influenza virus (6), have evolved strategies to target and inhibit distinct steps in the early signaling events that lead to IFN-I induction, indicating the importance of IFN-I in the host's antiviral response. The viral protease NS3/4A encoded by HCV has recently been shown to block the activation of IRF-3 by targeting multiple signaling steps. NS3/4A inactivates TRIF and IPS-1, adaptor proteins in TLR3- and RIG-I–dependent pathway, respectively, to prevent IFN-I production (3, 7, 8). The role of nonstructural protein 1 (NS1) of influenza A virus (IAV) as an IFN antagonist is evidenced by the hyper-induction of IFN-I in response to IAV lacking the NS1 gene (delNS1 virus) as compared with wild-type virus infection (6, 9, 10). In addition, expression of NS1 inhibits activation of IRF-3 (6). It is suggested that sequestering of viral dsRNA by NS1 during virus replication prevents access of host dsRNA sensors (6). However, the mechanisms by which NS1 antagonizes IFN-I response to IAV infection are still not fully understood. We now report that RIG-I is required for induction of IFN-I in response to IAV, and that NS1 suppresses production of IFN-I by targeting and inactivating the RIG-I–dependent signaling pathway.

## MATERIALS AND METHODS

### Cell Lines and Viruses

A549 and 293T cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. Influenza viruses A/Puerto Rico/8/34 (PR8; H1N1) and A/Panama/2007/99 (H3N2) were grown in 10-d-old embryonated hen's eggs at 33.5°C for 48 h, while a highly pathogenic avian influenza (HPAI) virus A/Vietnam/1203/2004 (H5N1) was grown in eggs at 37°C for 24 h. All experiments with HPAI virus were performed in a biosafety level 3 laboratory with enhancement. Unless

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specified, infection of cells by virus was performed at a multiplicity of infection (MOI) of 1 plaque-forming unit (pfu) per cell in a 6-well plate without trypsin supplementation. Influenza viruses were quantified by plaque assay on MDCK cells.

### Plasmids and Small Interfering RNA

The pCAGGS-myc-NS1 was constructed by cloning a full-length cDNA of segment 8 from influenza PR8 virus into expression vector pCAGGS with a fusion sequence encoding c-myc-tag located at the 5′ end of cloned cDNA. The splice acceptor sequence was mutated by overlap PCR. Constructs that express domains of NS1, pCAGGS-myc-NS1aa1–80 and pCAGGS-myc-NS1aa81–230, were derived from pCAGGS-myc-NS1. The pEF-FLAG-RIG-I, pEF-FLAG-N-RIG-I, and pEF-FLAG-C-RIG-I plasmids have been described (2). The (-110-IFN-β)-CAT, $(PRDIII\text{-}I)_3$-CAT, pEF-Bos-TRIF, and pCDNA3-IKKε were kindly provided by T. Maniatis, Harvard University, Cambridge, MA. The pUNO-hIPS1 was purchased from Invivogen (San Diego, CA). Predesigned small interfering RNA (siRNA) targeting human RIG-I (siRIG-I), human MDA5 (siMDA5), and control siRNA targeting luciferase (siLuc) were purchased from Dharmacon (Chicago, IL).

### Real-Time RT-PCR

Real-time RT-PCR was performed as described previously (11). Two sets of PCR assays were performed for each sample using primers specific for cDNA of the following genes: RIG-I, IFN-β, TNF-α, ISG15, MxA, and GAPDH. PCR product from above genes was cloned into PCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA) and the cloned constructs were used to create standard curves in real-time PCR. The cycle threshold of each sample was converted to copy number of cDNA per μg of RNA and was normalized to GAPDH quantity of the corresponding sample. Unless specified, all assays were performed at least three times from independent RNA preparations.

### Transient Transfection

Transient transfections of plasmid were performed using FuGENE 6 transfection reagent from Roche (Indianapolis, IN) according to the manufacturer's protocols. For transient transfection of dsRNA into 293T cells, 0.2 μg of poly (I:C) (Sigma-Aldrich, St. Louis, MO) was transfected with Lipofectamine 2,000 (Invitrogen). Transient transfections of siRNA into A549 cells were conducted using DharmaFECT 1 (Dharmacon) according to the manufacturer's protocols.

### Western Blot

Western blot was performed as described previously (11). Antibodies against FLAG-tag and β-actin were purchased from Sigma-Aldrich, and c-myc-tag from Invitrogen. Antibody against human RIG-I was purchased from IBL (Gunma, Japan). Antibody against human MDA5 was described previously (13).

## RESULTS

### RIG-I Is Required for IFN-β Response to IAV Infection in Lung Epithelial Cells

RIG-I has been identified as a receptor for dsRNA and is required for induction of type I IFN in response to infection by several RNA viruses (2). To determine whether RIG-I is required for IFN-I response to IAV infection, we knocked down the endogenous expression of RIG-I in the human lung epithelial cell line A549 by RNA interference (RNAi) technique, and infected cells with influenza virus 24 h later. Transfection of siRNA targeting RIG-I, but not a control siRNA targeting the luciferase gene, greatly reduced the level of IFN-β mRNA induced 16 h after infection with IAV, suggesting an essential role for RIG-I in IFN-I response to IAV infection in this human lung epithelial cell line (Figure 1A). Similarly, the induction of type I IFN-inducible genes, ISG15 and MxA, were greatly reduced in cells transfected with siRNA targeting RIG-I (Figures 1B and 1C). It has been shown that the RIG-I signaling pathway bifurcates to activate IRF-3 and NF-κB (2). To determine whether RIG-I plays a role in IAV-induced expression of NF-κB–responsive genes, we analyzed the expression level of TNF-α (12), in RIG-I knocked-down cells (Figure 1D). The induction level of TNF-α was also greatly reduced in cells transfected with siRNA targeting RIG-I, indicating that the signaling pathway leading to NF-κB activation by IAV infection might require RIG-I function. The importance of RIG-I in the IFN-I response to IAV infection was also demonstrated by IFN-β promoter and IRF-3-responsive promoter reporter assays. Consistent with the results from real-time RT-PCR, IFN-β promoter (IFN-β–CAT) (Figure 1E) or IRF-3–responsive promoter (PRDIII-I–CAT) (Figure 1F) reporter expression was decreased in RIG-I knocked-down cells as compared with controls. The specificity of RNAi was evidenced by the greatly reduced expression of RIG-I mRNA and protein only in cells transfected with siRNA targeting RIG-I (Figures 1G and 1H). Taken together, these data indicate that RIG-I is essential for induction of IFN-I and TNF-α in response to IAV infection, and that the induction activity involves activation of IRF-3 and NF-κB.

Melanoma differentiation–associated gene 5 (MDA5), an RNA helicase related to RIG-I, has been shown to share a common signaling cascade with RIG-I (13). To determine whether MDA5 plays a role similar to RIG-I in IFN-I response to IAV infection, we knocked down the endogenous expression of MDA5 in A549 cells by RNAi, and infected cells with IAV 24 h later. Expression of MDA5 was induced by IAV infection, and this induction was greatly reduced only in cells transfected with siRNA targeting MDA5 (Figure 2A). However, in comparison to RIG-I, transfection of siRNA targeting MDA5 only slightly affected the expression level of IFN-β, ISG15, MxA, and TNF-α induced by IAV infection (Figure 2B), suggesting that MDA5 might be inessential for IFN-I response to IAV infection in this human lung epithelial cell line.

An alternative approach to demonstrate the critical role of RIG-I in the IFN-I response to IAV infection relied on transient overexpression of FLAG-tagged RIG-I (Figure 3A). Transient transfection of a full-length RIG-I expression vector into 293T cells was sufficient to induce CAT expression from the IFN-β–CAT reporter in a dose-dependent manner. IAV infection further enhanced the level of induction, which might occur through enhanced expression of endogenous RIG-I after IAV infection. Similarly, endogenous expression of IFN-β, ISG15, MxA, and TNF-α (Figure 4B) was induced by transient overexpression of RIG-I in A549 cells, and their expression was also further induced by IAV infection (data not shown). The N-terminal domain of RIG-I (N-RIG-I) has been found to be constitutively active in inducing IFN-β, while the C-terminal domain (C-RIG-I) functions as a dominant negative inhibitor (2). To determine whether expression of C-RIG-I can block IAV-initiated IFN-β induction, 293T cells were co-transfected with a FLAG-tagged C-RIG-I expression vector and the IFN-β–CAT reporter construct, and infected with IAV 24 h later. The induction level of IFN-β reporter was inhibited by C-RIG-I in a dose-dependent manner (Figure 3A), confirming that C-RIG-I is a dominant-negative inhibitor for IFN-β induction by IAV infection and that RIG-I does play an important role in IFN-I response to IAV infection. The ectopic expression of RIG-I and C-RIG-I was confirmed by Western blot analysis (Figure 3B).

This is the first report demonstrating that RIG-I is required for IFN-I response to wild-type IAV infection in human epithelial

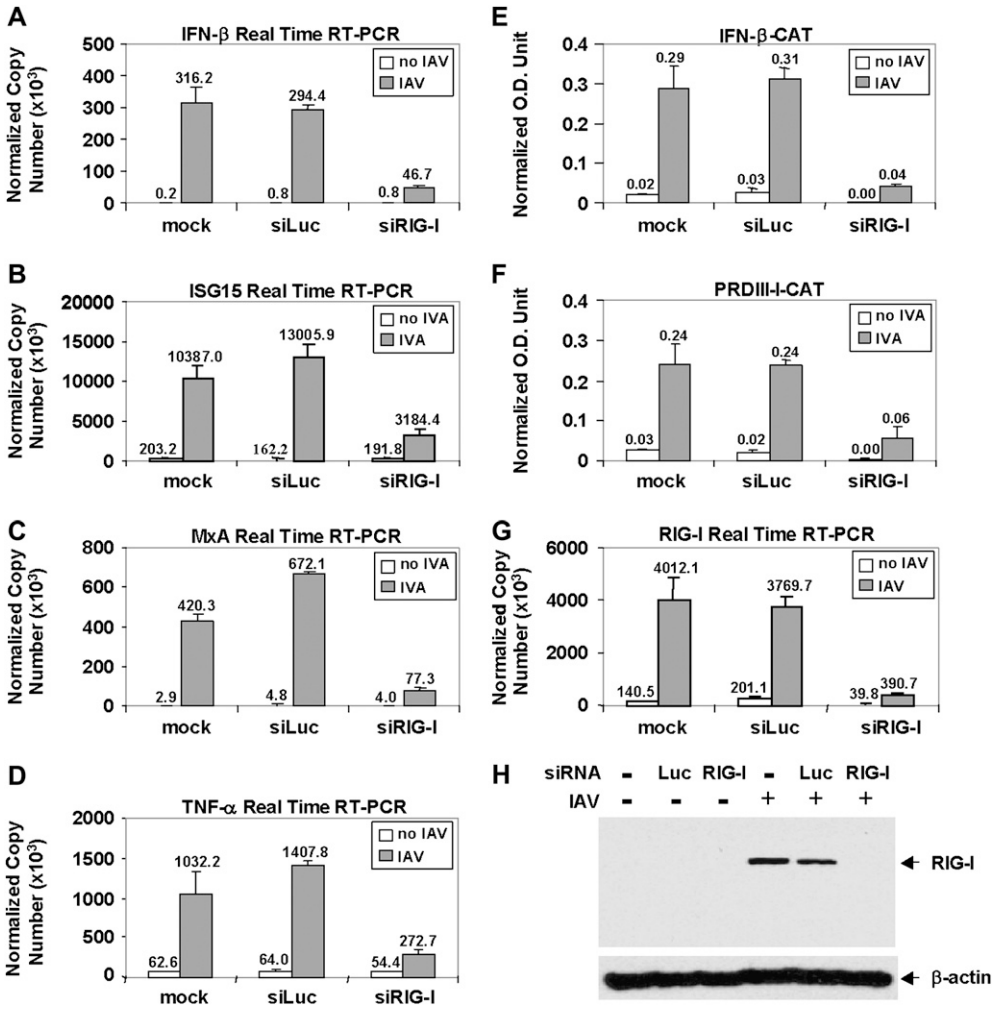


*Figure 1.* RIG-I is required for the induction of type I interferon against influenza A virus infection. A549 cells were transfected with siRNA targeting RIG-I (siRIG-I) or control siRNA targeting luciferase gene (siLuc). After 24 h incubation, transfected cells were infected with influenza virus A/Panama/2007/99 and incubated for 16 h. Total RNA was isolated, and real-time RT-PCR was performed to analyze IFN-β (*A*), ISG15 (*B*), MxA (*C*), TNF-α (*D*), and RIG-I (*G*) expression. For reporter assay and protein analysis, A549 cells were transiently co-transfected with siRNA and reporter plasmids as indicated, followed by infection with IAV PR8. Cell lysates were collected and analyzed by CAT ELISA (*E* and *F*), or by Western blot analysis using antibodies against RIG-I or β-actin (*H*). The average of three independent experiments is shown with SD.

cell lines. MDA5 is homologous to RIG-I and shares a common signaling cascade with RIG-I (13). Therefore, the function of MDA5 could compensate for the loss of RIG-I activity. However, our RNAi data demonstrate that knockdown of RIG-I expression greatly inhibits the IFN-I response to IAV infection (> 80% at mRNA level; Figure 1A). Furthermore, knockdown of MDA5 expression by RNAi only slightly represses the IFN-I response to IAV infection (Figure 2B). These results might indicate that MDA5 may not play a major role in the recognition of IAV, and the roles of RIG-I and MDA5 in innate response to virus infection may be differentiated in cells of lung epithelial cell lines (14).

### RIG-I–Initiated Induction of Type I IFN Is Inhibited by NS1 Encoded by IAV

Influenza virus lacking the NS1 gene is a potent inducer of IFN-I and NS1 has been shown to inhibit activation of IRF-3 (6). However, the precise mechanism by which NS1 antagonizes induction of IFN-I remains unknown. The critical role of RIG-I in the IFN-β response to IAV infection prompted us to hypothesize that NS1 might target the RIG-I signaling pathway and inhibit production of IFN-I. To test this hypothesis, RIG-I expression construct and IFN-β–CAT reporter were co-transfected with various amounts of NS1 expression vector into A549 cells, and the activity of IFN-β promoter was analyzed by CAT ELISA. Transfection of the RIG-I expression vector alone greatly induced CAT expression from the IFN-β–CAT reporter, and co-transfection of the NS1 expression vector inhibited the induction activity of RIG-I in a dose-dependent manner (Figure 4A). Similarly, the endogenous expression of IFN-β, ISG15, MxA, and TNF-α was greatly induced by overexpression of RIG-I, and co-transfection of the NS1 expression vector almost completely blocked the induction (Figure 4B). It should be noted that transfection of NS1 expression vector alone caused a slight reduction (< 2-fold) in the basal level of IFN-β expression (*see* Figure E1 in the online supplement). However, the inhibitory function of NS1 on RIG-I signaling was not due to altered expression of RIG-I, as comparable levels of RIG-I expression were found in cells transfected with RIG-I or RIG-I plus NS1 expression constructs (Figure 4C).

Next, we determined whether NS1 could inhibit RIG-I activity in the presence of dsRNA. RIG-I expression vector and IFN-β promoter reporter plasmids were transfected with or without the NS1 expression vector into 293T cells. After 24 h of incubation, cells were transfected with dsRNA (poly [I:C]) and incubated for 8 h to induce IFN-I. The activity of IFN-β promoter was determined by CAT ELISA. Transfection of the RIG-I expression vector induced CAT expression driven by the IFN-β promoter, and the level of induction was further increased in cells transfected with poly (I:C), indicating that interaction of RIG-I with dsRNA enhanced the signaling activity of RIG-I (Figure 4D). Most importantly, the induction function of RIG-I was greatly inhibited by NS1 in the presence or absence of poly (I:C). CAT expression driven by IRF-3–responsive promoter

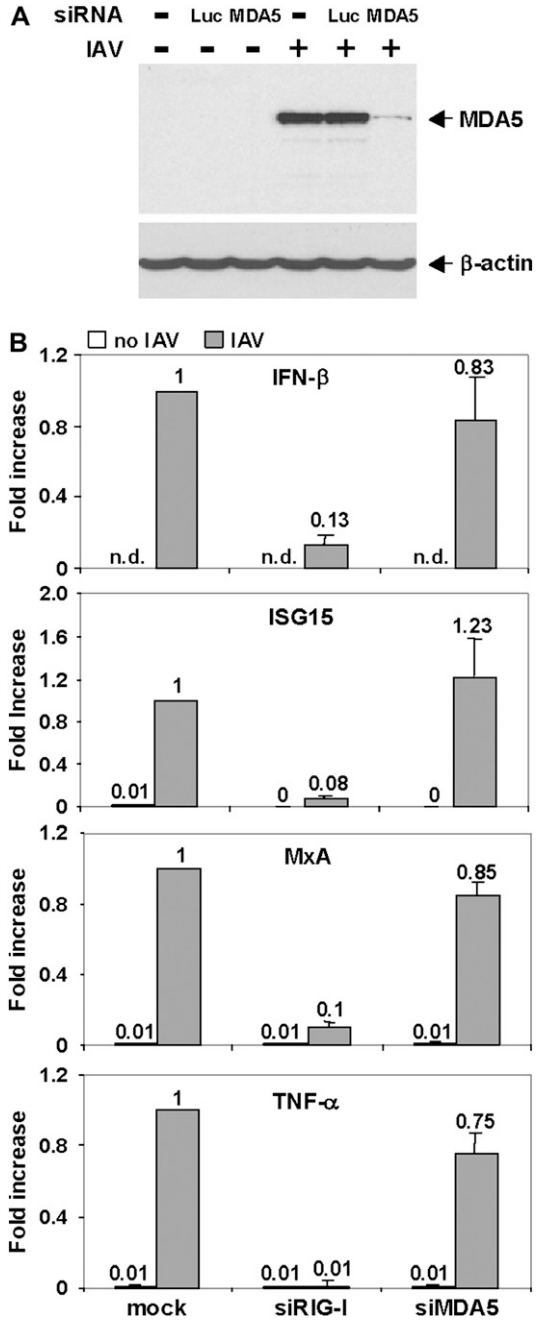


*Figure 2.* MDA5 is not essential for the induction of type I interferon against influenza A virus infection. A549 cells were transfected with siRNA targeting MDA5 (siMDA5), RIG-I (siRIG-I), or control siRNA targeting luciferase gene (siLuc). After 24 h incubation, transfected cells were infected with IAV PR8 and incubated for 16 h. Cell lysates were collected and analyzed by Western blot analysis using antibodies against MDA5 or β-actin (*A*). Total RNA was isolated, and real-time RT-PCR was performed to analyze the expression of IFN-β, ISG15, MxA, and TNF-α (*B*). The relative levels of mRNA expression were plotted as fold of increase with IAV-infected mock controls being set as 1-fold.

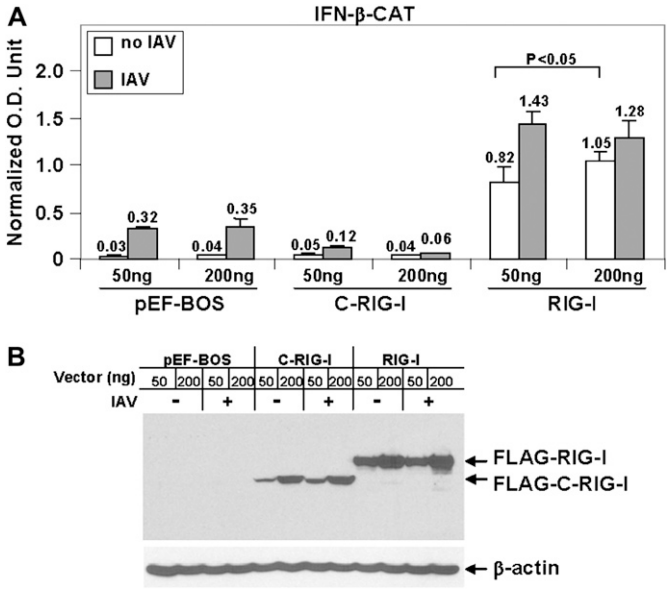


*Figure 3.* C-RIG-I functions as a dominant-negative inhibitor for IFN-β production induced by IAV infection. (*A*) 293T cells were transiently transfected with IFN-β promoter reporter plasmid DNA together with various amount of control vector pEF-BOS, or vectors that express FLAG-tagged C-terminal domain or full-length of human RIG-I. After 24 h incubation, cells were infected with IAV PR8 and incubated for another 24 h. Cell lysates were collected and a CAT ELISA was performed. The average of three independent experiments is shown with SD. (*B*) Samples tested by CAT ELISA from *A* were also analyzed by Western blot using antibodies against FLAG-tag or β-actin.

was also down-regulated by co-expression of NS1 (Figure 4E). Comparable levels of RIG-I expression were found in cells transfected with RIG-I or RIG-I plus NS1 expression constructs (Figure 4F). Also, transfection of NS1 expression vector had little effect on the expression level of β-galactosidase from pCDNA-LacZ, an RNA polymerase II–dependent reporter plasmid, suggesting that the inhibitory effect of NS1 on the expression of RNA polymerase II–dependent promoter reporter is minimal in our assay system (Figure E2). In addition, co-transfection of NS1 with IPS1, TRIF, or IKKε expression vectors failed to inhibit production of IFN-I that was induced by overexpression of these molecules, indicating the specificity of NS1 inhibitory activity on RIG-I pathway (Figure 4G).

To further determine the interaction between RIG-I and NS1, constructs that expressed domains of RIG-I or NS1 and IFN-β–CAT reporter plasmids were transfected with or without the full-length NS1 or RIG-I expression vectors into A549 cells (Figure 5A). Transfection of the N-RIG-I expression vector greatly induced CAT expression from the IFN-β promoter reporter, and co-transfection of the NS1 expression vector inhibited the induction activity of N-RIG-I. In addition, co-transfection of the constructs that expressed the N-terminus (amino acid 1–80), but not the C-terminus (amino acid 81–230) of NS1 with the RIG-I expression vector greatly repressed the induction of IFN-β–CAT reporter. Comparable levels of RIG-I expression were found in cells transfected with RIG-I or RIG-I plus NS1-domain expression vectors (Figure 5B).

NS1 of IAV is a multifunctional viral protein (15). Two cellular proteins that are required for the 3′-end processing of cellular pre-mRNAs, the 30-kD subunit of the cleavage and polyadenylation specificity factor (CPSF) and poly (A)-binding protein II (PABII), are bound and inactivated by IAV NS1, leading to decreased expression of the early type I IFN–independent antiviral genes (15). NS1 also inhibits the activation of another cellular antiviral gene, protein kinase R (PKR). Activation of PKR is known to phosphorylate the α-subunit of the translation initiation factor eIF2 to inhibit protein synthesis and therefore virus

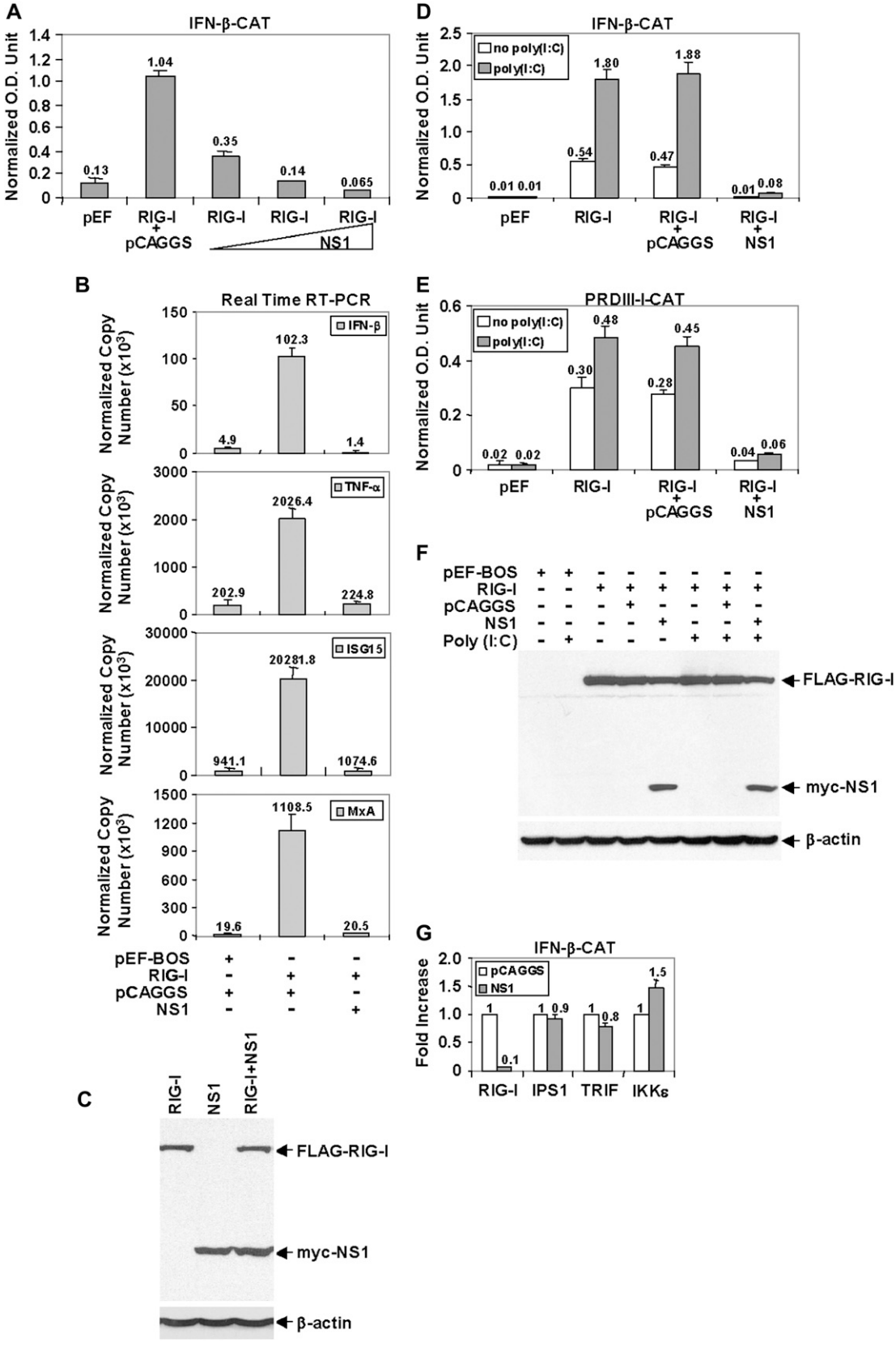


***Figure 4.*** NS1 from influenza A virus antagonizes production of IFN-β induced by RIG-I. (*A*) IFN-β–CAT reporter and FLAG-tagged RIG-I expression vectors were transiently transfected with increased amounts of the myc-tagged NS1 expression vector into A549 cells. Cell lysates were collected 24 h after transfection and analyzed by CAT ELISA. (*B*) A549 cells were transfected with vectors that express FLAG-tagged RIG-I or myc-tagged NS1, or their corresponding control vectors pEF-BOS or pCAGGS as indicated. After 24 h of incubation, cells were collected and total RNA was isolated, followed by real-time RT-PCR analysis for the expression of IFN-β, ISG15, MxA, and TNF-α. (*C*) Western blot was performed to confirm the ectopic expression of RIG-I and NS1 using antibodies against FLAG-tag or myc-tag. (*D–F*) 293T cells were transiently transfected with indicated promoter reporter plasmids together with vectors that express FLAG-tagged RIG-I or myc-tagged NS1. After 24 h of incubation, cells were transfected with poly (I:C) and incubated for another 24 h. Cell lysates were collected and analyzed by CAT ELISA to determine activities of the IFN-β promoter (*D*) and IRF-3–responsive promoter (*E*), or analyzed by Western blot analysis using antibodies against FLAG-tag or myc-tag (*F*). (*G*) IFN-β–CAT reporter plasmids and vectors that expressed RIG-I, IPS1, TRIF, or IKKε were co-transfected with or without the myc-tagged NS1 expression vectors into A549 cells. Cell lysates were collected 24 h after transfection and analyzed by CAT ELISA. The relative levels of CAT expression were plotted as fold of increase with samples transfected with pCAGGS and adaptor expression vectors being set as 1-fold. The average of three independent experiments is shown with SD.

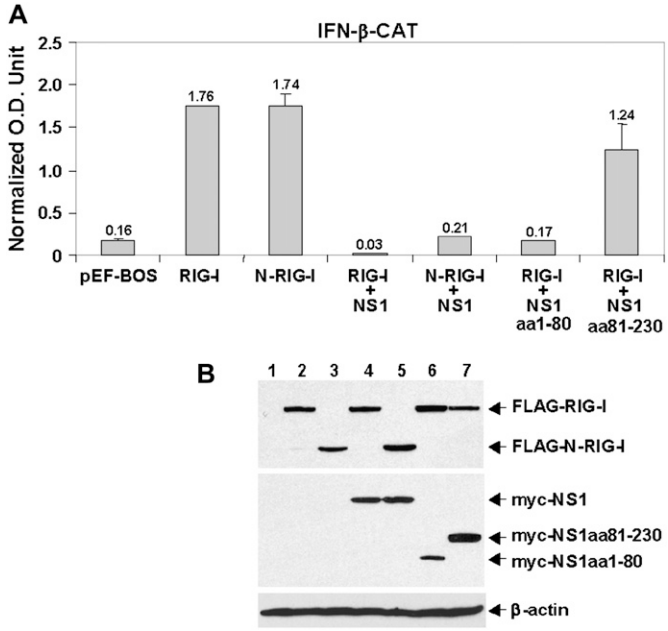


*Figure 5.* NS1 from IAV antagonizes RIG-I signaling through its N-terminal domain. A549 cells were transiently transfected with IFN-β–CAT reporter plasmids together with vectors that expressed FLAG-tagged RIG-I domains or myc-tagged NS1 domains. After 24 h of incubation, cell lysates were collected and analyzed by CAT ELISA (*A*), or analyzed by Western blot analysis using antibodies against FLAG-tag or myc-tag (*B*).

replication (15). Our data present further evidence that NS1 antagonizes the host antiviral response by targeting and inhibiting RIG-I signaling to block IRF-3 activation. It should be noted that NS1 inhibits the activity of RIG-I in the presence and absence of poly (I:C). The anti-IFN properties of IAV NS1 have been mapped to its N-terminal dsRNA-binding domain (10). Our data are consistent with the observation and indicate that the N-terminal domain of NS1 is sufficient to counteract RIG-I activity (Figure 5A).

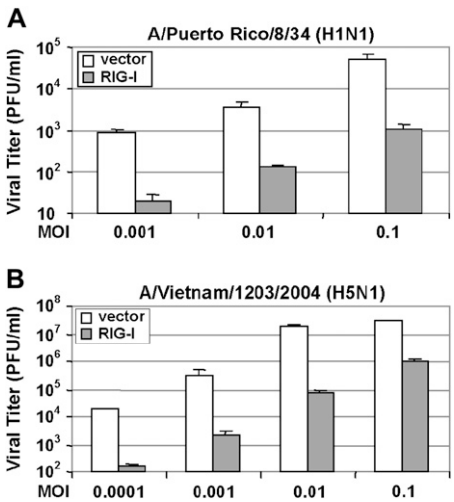


*Figure 6.* RIG-I inhibits replication of highly pathogenic avian influenza A virus. A549 cells were transiently transfected with control vector pEF-BOS or the vector that expresses full-length RIG-I. After 24 h of incubation, cells were infected with IAV PR8 (H1N1; *A*) or highly pathogenic avian IAV A/Vietnam/1203/2004 (H5N1; *B*) at various MOIs and incubated for another 24 h. Culture supernatants were collected and viral titers were determined by plaque assay on MDCK cells. The average of three independent experiments is shown with SD.

### RIG-I Inhibits IAV Replication

Increased expression of RIG-I has been shown to reduce the yield of vesicular stomatitis virus and encephalomyocarditis virus (2). To test whether RIG-I can inhibit replication of influenza virus, A549 cells were transiently transfected with the construct that expressed full-length RIG-I or its null expression control vector, and 24 h later were infected with IAV PR8 or highly pathogenic avian influenza virus A/Vietnam/1203/2004 (H5N1) at various MOI in the absence of trypsin. Compared with cells transfected with control vector, the yields for PR8 and H5N1 virus were reduced by 1 to 2 log of control in cells transfected with RIG-I expression vector (Figures 6A and 6B). Our results of inhibition of H1N1 and H5N1 IAV replication by RIG-I suggest the general capacity of RIG-I in anti-influenza function. Understanding the mechanism of how ectopic expression of human RIG-I induces type I IFN would provide insights for development of intervention strategy for viral infections.

***Conflict of Interest Statement***: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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***Note added in proof*:** After the submission of this manuscript, three additional groups reported NS1-mediated inhibition of RIG-I function.

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