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RIG-I–Mediated Antiviral Responses to Single-Stranded RNA Bearing 5′ Phosphates

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Double-stranded RNA (dsRNA) produced during viral replication is believed to be the critical trigger for activation of antiviral immunity mediated by the RNA helicase enzymes RIG-I and MDA5. Here, we show that influenza A virus infection does not generate dsRNA and that RIG-I is activated by single stranded (ss) viral genomic RNA bearing 5' phosphates. This is blocked by the influenza protein NS1, which is found in a complex with RIG-I in infected cells. These results identify RIG-I as a ssRNA sensor and potential target of viral immune evasion and suggest that its ability to sense uncapped RNA evolved as a means of self/nonself discrimination by the innate immune system.

The innate immune response to viral infection is characterized by the rapid production of a range of cytokines, most prominently type I interferons (IFN-α/β) (*1*). Specialised plasmacytoid dendritic cells (pDC) produce IFN-α/β upon triggering of toll-like receptors (TLRs) 7, 8 and 9 by RNA or DNA viral genomes in endosomes (*2*). Other cell types rely on cytoplasmic virus sensors such as the RNA helicases RIG-I and MDA5 (*3*–*7*), which are believed to be activated by double-stranded (ds) RNA produced during viral replication or convergent transcription of viral genes (*8*). Viral recognition pathways can be targeted as a means of immune escape (*9*). For example, influenza A virus NS1 protein suppresses IFN-α/β production in animal models, cell lines and primary cells (*10*), including conventional (non-plasmacytoid) dendritic cells (cDC), a critical cell type for the induction of adaptive immunity (*11*, *12*). NS1 possesses a RNA-binding domain at the N-terminus (*13*) and has been suggested to exert its suppressive effect by sequestering dsRNA (*10*).

To address whether this is the case, we first confirmed that influenza A virus (A/PR/8/34 strain) inhibits IFN-α/β production through the action of NS1. cDC derived from murine bone marrow progenitors (BM-DC; (*14*)) infected with a mutant virus lacking the protein (ΔNS1) produced 100-fold more IFN-α than cells infected with the parental wild type strain (Fig. 1A) (*11*, *12*). This was independent of the TLR7/8/9 adaptor MyD88 (Fig. 1A), suggesting an effect via the cytoplasmic pathway. We then assessed the extent to which dsRNA is generated during influenza replication. Consistent with recent data (*15*), no dsRNA was detected in BM-DC or in more permissive Vero cells (Fig. 1, B and C, and fig. S1) with either ΔNS1 or wild type virus despite the fact that the cells were uniformly infected (fig. S2). In contrast, dsRNA was detectable upon transfection with poly I:C, a synthetic dsRNA, or infection with encephalomyocarditis virus (EMCV), a picornavirus (Fig. 1, B and C, and fig. S1). We next examined the ability of NS1 to inhibit responses to Semliki Forest Virus (SFV), which, like EMCV, generates high levels of dsRNA (*16*). Cells infected with a recombinant SFV encoding NS1 (SFV-NS1) expressed NS1 protein (fig. S3) but produced comparable levels of IFN-α to cells infected with a control recombinant virus or wild type SFV (Fig. 1D). Similarly, transfection with NS1 had no effect on the induction of an IFN-β reporter in response to EMCV or SFV although it potently inhibited the response to ΔNS1 influenza or Sendai virus (SeV; Fig. 1E), as described (*13*). Notably, the latter two viruses generate minimal levels of dsRNA (Fig. 1, B and C) (*15*) but induce high levels of IFN-α (Fig. 1, A and E, and fig. S4) whereas EMCV and SFV induce high levels of dsRNA (Fig. 1, B and C, and fig. S3) (*16*) but lower levels of IFN-α (Fig. 1E and fig. S4). Collectively, these data indicate that neither IFN-α induction nor the inhibitory effect of NS1 correlate with the presence of dsRNA.

Sendai virus and ΔNS1 influenza are recognized via RIG-I whereas EMCV is recognized by MDA5 (*6*, *7*). We therefore investigated whether the virus-specific effects of NS1 reflected its ability to interact with RIG-I. Consistent with this possibility, GFP- or HA-tagged RIG-I co-precipitated with anti-NS1 from post-nuclear lysates of influenza infected cells (Fig. 2, A and B) In addition, the cytoplasmic fraction of NS1 co-localised with GFP-RIG-I in infected cells (Fig. 2C). In contrast, MDA5 did not associate with NS1 in infected cells

(Fig. 2B). These results suggest that NS1 selectively targets RIG-I rather than dsRNA during influenza virus infection.

Given the lack of dsRNA in infected cells, we addressed whether RIG-I might be activated directly by the influenza single stranded (ss) RNA genome. Transfection with genomic RNA extracted from influenza virions (flu vRNA) induced potent activation of the IFN-β reporter (Fig. 3A) and production of IFN-α and IL-6 by BM-DC at levels comparable or superior to those obtained by transfection with poly I:C (Fig. 3C). This was not due to generation of progeny virus because flu vRNA transfection did not result in viral replication (Fig. 3B), consistent with the fact that vRNA from negative strand viruses is not infectious (*17*). The response to flu vRNA was RIG-I-dependent as it was inhibited by dominant negative RIG-I or by siRNA-mediated knockdown of RIG-I in either mouse or human cells (Fig. 3D and fig. S5). The specificity of RIG-I knockdown was confirmed by the fact that it blocked responses to Sendai virus but not EMCV (Fig. 3D), as expected (*6*). NS1 suppressed the response to flu vRNA and this was partly relieved by two point mutations previously reported to attenuate NS1 binding to RNA (*18*) (NS1 mut; Fig. 3A). Finally, addition of purified RIG-I to flu vRNA led to formation of high molecular weight complexes (Fig. 3E), demonstrating that RIG-I directly binds the influenza genome. Thus, RIG-I recognizes influenza ssRNA genomes and signals for cytokine production unless suppressed by NS1.

Mouse mRNA, total mammalian RNA (consisting of ~70% rRNA) and mammalian or bacterial tRNA did not elicit IFN responses (fig. S6), suggesting that RIG-I recognition is specific for viral RNA. Influenza vRNA is uncapped (*17*) and phosphorylated 5' termini present in siRNA and ssRNAs generated by *in vitro* transcription have been reported to induce IFN-α/β when transfected into cells (*19*). We confirmed the latter observation (fig. S7) and tested whether flu vRNA recognition similarly depends on the presence of 5' phosphates. Treatment with calf intestinal phosphatase (CIP) completely abrogated the stimulatory properties of flu vRNA (Fig. 4, A and B). This was due to phosphatase activity rather than non-specific effects of CIP as it could be blocked by inorganic phosphate or EDTA (fig. S8). Furthermore, CIP did not affect the ability of vRNA to stimulate TLR7-dependent IFN-α production from pDC-containing cell populations (fig. S8). Vesicular stomatitis virus (VSV) also has an uncapped genome (*17*) and is recognized via RIG-I (*6*). Similar to influenza, transfection with VSV vRNA induced an IFN response, which was completely abrogated by prior CIP treatment (Fig. 4, C and D). Consistent with the fact that EMCV is not recognized via RIG-I (*6, 7*), EMCV vRNA failed to induce a response when transfected into cells at amounts comparable to flu or VSV vRNA (Fig. 4C). These data suggest that cells use RIG-I to respond to the phosphorylated 5' termini of uncapped ssRNA viral genomes.

Finally, we assessed whether 5' phosphorylation contributes to RIG-I binding. *In vitro* transcribed RNA formed a complex with RIG-I in cell lysates, which was less resistant to salt extraction when the RNA was pre-treated with CIP (Fig. 4E). *In vitro* binding assays using purified RIG-I confirmed that complexes with CIP-treated RNA were less stable (fig. S9). Notably, addition of control but not CIP-treated RNA to cell lysates promoted the formation of a complex containing NS1 and RIG-I (Fig. 4F), mimicking the association seen in infected cells (Fig. 2). RIG-I associated only weakly with NS1 mut (Fig. 4F), which binds ssRNA poorly when compared with wild type protein (fig. S10). Thus, RIG-I preferentially forms stable complexes with RNA containing phosphorylated 5' ends and NS1 is recruited to such complexes via its RNA binding domain.

The ability to sense viral presence is critical for initiating innate and adaptive immunity to viral infection. Here, we find that virus recognition can be accomplished by RIG-I-mediated sensing of ssRNA viral genomes bearing 5' phosphates. This can be blocked by viral antagonists such as the NS1 protein of influenza A virus, which is found in a complex with RIG-I (SOM text S1). Our results demonstrate that the repertoire of antiviral defense strategies includes the detection of cytoplasmic ssRNA, explaining how some viruses that produce little or no dsRNA (*15*) can be efficiently recognised, even prior to viral replication (*20*). Consistent with the fact that RIG-I can bind to dsRNA *in vitro* (*4, 5*), our data do not exclude that the complex of RIG-I and ssRNA is stabilized by the presence of intramolecular double stranded regions, such as the panhandle structures that are found at the ends of the influenza genome (*17*). However, this is not sufficient to induce RIG-I activation unless the ssRNAs also contain phosphorylated 5' termini. Notably, many RNA viruses have uncapped RNAs bearing 5' tri-phosphates. A significant exception are picornaviruses, in which the vRNA is covalently linked to a small protein, VPg (*17*), perhaps explaining why EMCV cannot be recognised via RIG-I (*6, 7*). 5' phosphates are also absent from self mRNA due to the addition of a 7-methyl-guanosine cap and may be largely inaccessible in rRNA and tRNA, through association with ribosomal proteins or formation of cloverleaf structures containing 3' overhangs. Thus, the cytoplasmic presence of RNA containing accessible 5' tri-phosphates allows discrimination between self and viral RNA, indicating that, like dsRNA, 5' phosphate-bearing ssRNA constitutes a viral "pathogen-associated molecular pattern" (*21*). This finding, added to the recent discovery of innate sensing of cytoplasmic DNA (*22–24*), suggests a remarkable parallel between cytosolic and endosomal viral recognition, with MDA5, RIG-I and the cytosolic DNA receptor constituting

functional homologues of TLR3, 7/8 and 9. Like virologists, the innate immune system may therefore have learned to classify viruses by their genomes.

References and Notes

1. A. N. Theofilopoulos, R. Baccala, B. Beutler, D. H. Kono, *Annu Rev Immunol* **23**, 307 (2005).
2. T. Kawai, S. Akira, *Nat Immunol* **7**, 131 (2006).
3. M. Yoneyama *et al.*, *Nat Immunol* **5**, 730 (2004).
4. M. Yoneyama *et al.*, *J Immunol* **175**, 2851 (2005).
5. S. Rothenfusser *et al.*, *J Immunol* **175**, 5260 (2005).
6. H. Kato *et al.*, *Nature (London)* **441**, 101 (2006).
7. L. Gitlin *et al.*, *Proc Natl Acad Sci U S A* **103**, 8459 (2006).
8. B. L. Jacobs, J. O. Langland, *Virology* **219**, 339 (1996).
9. A. Garcia-Sastre, C. A. Biron, *Science* **312**, 879 (2006).
10. A. Garcia-Sastre, *Virology* **279**, 375 (2001).
11. S. S. Diebold *et al.*, *Nature (London)* **424**, 324 (2003).
12. A. Fernandez-Sesma *et al.*, *J Virol* **80**, 6295 (2006).
13. X. Wang *et al.*, *J Virol* **74**, 11566 (2000).
14. Materials and methods are available as supporting material on *Science* Online.
15. F. Weber, V. Wagner, S. B. Rasmussen, R. Hartmann, S. R. Paludan, *J Virol* **80**, 5059 (2006).
16. O. Schulz *et al.*, *Nature (London)* **433**, 887 (2005).
17. *Fields Virology*. D. Knipe, P. M. Howley, Eds. (Lippincott Williams & Wilkins, Philadelphia, ed. Fourth, 2001).
18. W. Wang *et al.*, *Rna* **5**, 195 (1999).
19. D. H. Kim *et al.*, *Nat Biotechnol* **22**, 321 (2004).
20. S. E. Collins, R. S. Noyce, K. L. Mossman, *J Virol* **78**, 1706 (2004).
21. C. A. Janeway Jr, *Cold Spring Harb Symp Quant Biol* **54**, 1 (1989).
22. Y. Okabe, K. Kawane, S. Akira, T. Taniguchi, S. Nagata, *J Exp Med* **202**, 1333 (2005).
23. K. J. Ishii *et al.*, *Nat Immunol* **7**, 40 (2006).
24. D. B. Stetson, R. Medzhitov, *Immunity* **24**, 93 (2006).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1132998/DC1
Materials and Methods
SOM Text
Figs S1 to S10
References

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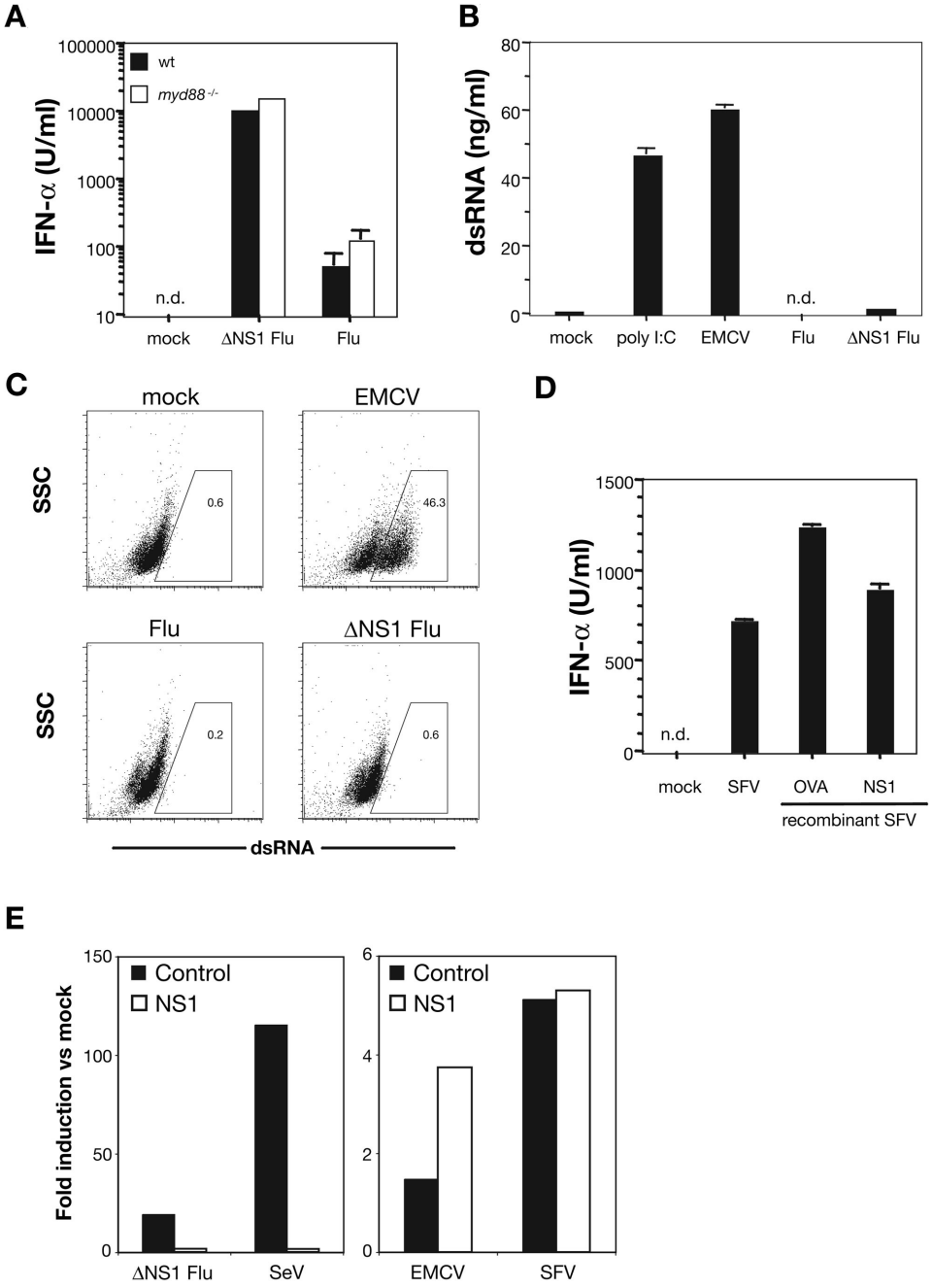
Fig. 1. NS1 inhibition or IFN-α/β induction do not correlate with viral dsRNA. (**A**) BM-DC from C57BL/6 (wt) or *myd88*$^{-/-}$ mice were cultured overnight in medium alone (mock) or with PR8 or ΔNS1 influenza virus. Data are average IFN-α levels of triplicate samples ± 1SD (**B** and **C**) DsRNA in Vero cells 6h after infection with influenza virus or EMCV or transfection with poly I:C measured by ELISA (B) or flow cytometry (C). Numbers in (C) indicate % cells in gate. (**D**) as for (A) but cells were infected with wild type SFV or recombinant SFV encoding NS1 or an irrelevant protein (ovalbumin; OVA). (**E**) Induction of luciferase activity in HEK293 cells co-transfected with IFN-β reporter plasmids together with NS1-encoding plasmid or control empty vector and subsequently infected with the indicated viruses. n.d., none detectable.

Fig. 2. The NS1 protein of influenza A virus interacts with RIG-I but not MDA5. (**A** and **B**) 293T cells were transfected with pGFP-RIG-I (A) or with pHA-RIG-I or pHA-MDA5 (B) and 12h later infected or not with influenza virus, as indicated. At 24h, cells were lysed and analysed by Western blot (WB) for the presence of NS1 and GFP (A) or NS1 and HA (B) in total cell lysates (lower panels) or after immunoprecipitation (IP) with anti-NS1 antibody (upper panels). (**C**) 293T cells were transfected with GFP-RIG-I, infected with influenza virus at 16h and stained for NS1 at 24h. Panels show GFP-RIG-I, NS1 and the merged image.

Fig. 3. Influenza vRNA triggers innate responses in a RIG-I-dependent manner. (**A**) HEK293 cells were co-transfected with IFN-β reporter plasmids and NS1 expression plasmid (NS1 or mutNS1) or empty vector (control). 24h later, cells were transfected with flu vRNA (0.2, 0.04 or 0.008 μg) and luciferase activity was measured at 38h. Western blots show the presence of NS1 or tubulin. (**B**) Lysates from cells transfected with flu vRNA or control mouse mRNA were probed with polyclonal antibody against influenza proteins. Influenza infected cells served as positive control. (**C**) IFN-α and IL-6 accumulation in overnight culture supernatants from BM-DC that were mock treated or transfected with flu vRNA (1 μg and 0.2 μg) or polyI:C (0.5 μg). n.d., none detectable. (**D**) Inhibition of responses to flu vRNA by RIG-I knockdown. Human HEK293 cells and mouse NIH3T3 were co-transfected with IFN-β reporter plasmids and siRNAs specific for mouse or human RIG-I. 72h later, cells were transfected with flu vRNA (0.2 or 0.04 μg) or were infected with SeV or EMCV. Luciferase activity was measured at 86h. (**E**) Cold EMSA analysis of RIG-I and flu vRNA interaction. Flu vRNA (1.08 nM) was incubated with the indicated

concentrations of purified FLAG-RIG-I or BSA and resolved by electrophoresis. RNA was visualized by SYBR Green staining.

Fig. 4. SsRNAs containing phosphorylated 5' ends bind RIG-I and activate antiviral responses. HEK293 transfected with IFN-β reporter and Renilla luciferase control plasmids (**A** and **C**) or BM-DC (**B** and **D**) were transfected with different amounts (0.6, 0.2, 0.06. 0.02 μg) of mouse spleen mRNA or flu vRNAs, or with vRNA from $8x10^7$ pfu VSV (C) or 5-fold serial dilutions thereof (D). RNAs were pre-treated (CIP) or not (NoCIP) with calf intestinal phosphatase. Luciferase activity (A, C) or IFN-α and IL-6 (B, D) were measured after overnight culture. n.d., none detectable. (**E**) In vitro transcribed biotinylated RNA was treated with or without CIP, bound to streptavidin-beads and incubated with lysates from 293T cells transfected with GFP-RIG-I. Data show protein eluted from the beads by washing with buffer containing the specified NaCl concentrations. (**F**) Lysates of HEK293 cells co-transfected with pFLAG-RIG-I and pCAAGS-NS1 or pCAAGS-mutNS1 were incubated with or without 7SK-as RNA that had been either mock or CIP-treated. Following immunoprecipitation with anti-NS1, the presence of RIG-I and NS1 was analyzed by Western blot.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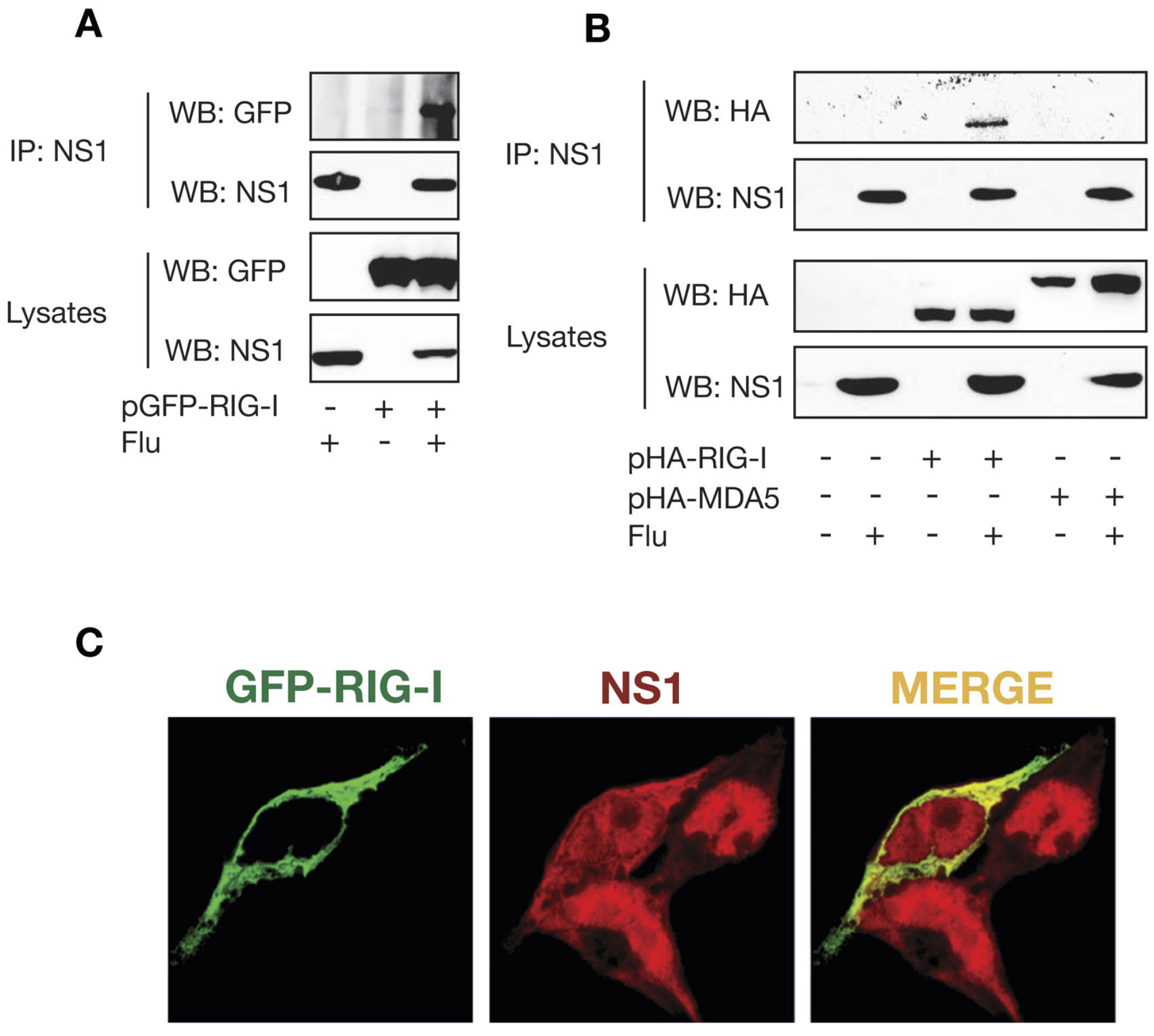
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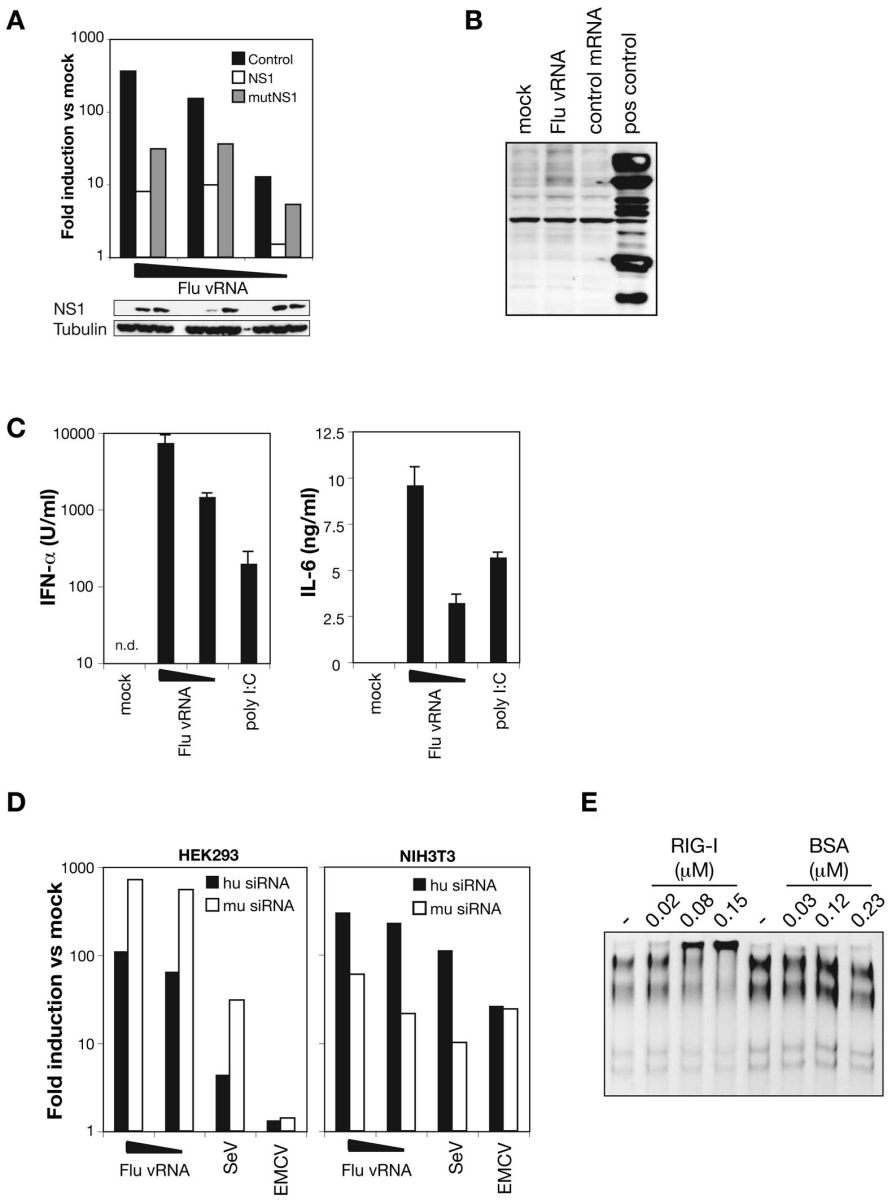
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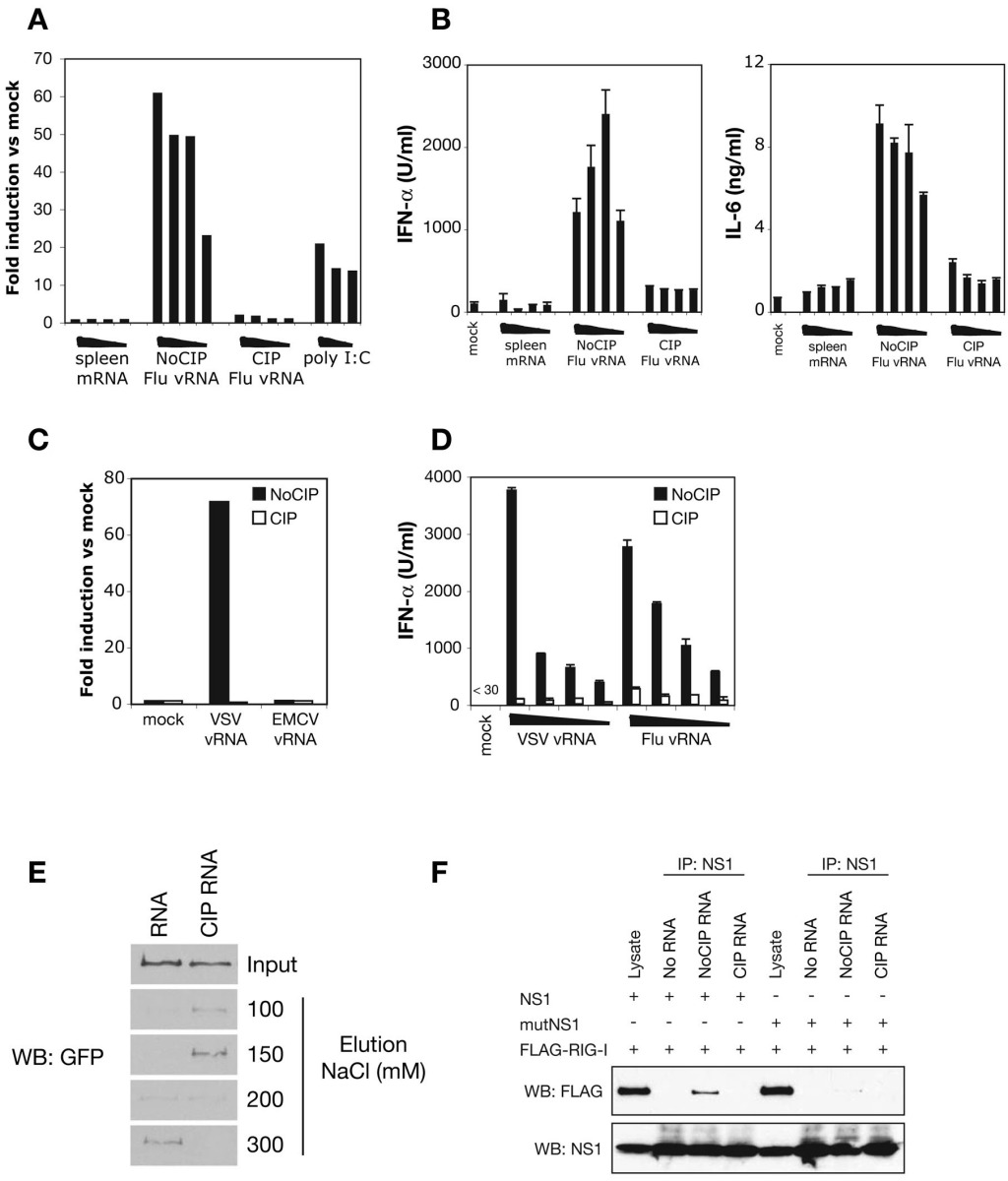
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