JOURNAL OF VIROLOGY, Oct. 2010, p. 9760–9774 Vol. 84, No. 19
0022-538X/10/$12.00 doi:10.1128/JVI.01051-10
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

Inhibition of the Type I Interferon Response in Human Dendritic Cells by Dengue Virus Infection Requires a Catalytically Active NS2B3 Complex▽†§

Juan R. Rodriguez-Madoz, Alan Belicha-Villanueva, Dabeiba Bernal-Rubio, Joseph Ashour, Juan Ayllon, and Ana Fernandez-Sesma*

Department of Microbiology and the Emerging Pathogens Institute, Mount Sinai School of Medicine, New York, New York

Received 14 May 2010/Accepted 29 June 2010

Dengue virus (DENV) is the most prevalent arthropod-borne human virus, able to infect and replicate in human dendritic cells (DCs), inducing their activation and the production of proinflammatory cytokines. However, DENV can successfully evade the immune response in order to produce disease in humans. Several mechanisms of immune evasion have been suggested for DENV, most of them involving interference with type I interferon (IFN) signaling. We recently reported that DENV infection of human DCs does not induce type I IFN production by those infected DCs, impairing their ability to prime naive T cells toward Th1 immunity. In this article, we report that DENV also reduces the ability of DCs to produce type I IFN in response to several inducers, such as infection with other viruses or exposure to Toll-like receptor (TLR) ligands, indicating that DENV antagonizes the type I IFN production pathway in human DCs. DENV-infected human DCs showed a reduced type I IFN response to Newcastle disease virus (NDV), Sendai virus (SeV), and Semliki Forest virus (SFV) infection and to the TLR3 agonist poly(I:C). This inhibitory effect is DENV dose dependent, requires DENV replication, and takes place in DENV-infected DCs as early as 2 h after infection. Expressing individual proteins of DENV in the presence of an IFN-α/β production inducer reveals that a catalytically active viral protease complex is required to reduce type I IFN production significantly. These results provide a new mechanism by which DENV evades the immune system in humans.

Dengue virus (DENV), a member of the *Flaviviridae* family and grouped within the *Flavivirus* genus (27), is the most prevalent arthropod-borne human virus with significant medical and biodefense importance (8, 27). DENV is transmitted by mosquitoes, usually *Aedes aegypti*, with an estimated 100 million cases per year and 2.5 billion people at risk (53). Clinical manifestations include dengue fever, a febrile illness with rash, and dengue hemorrhagic fever, a severe and often lethal illness (53). There are four DENV serotypes (DENV1 to -4), and infection with one serotype confers life-long protection against that serotype at least by the induction of neutralizing antibodies (17). Currently there is no vaccine or effective antiviral treatment against this virus, and the most effective protective measures involve mosquito control.

The DENV genome is a positive-strand RNA molecule of about 11 kb in length, with one single open reading frame (ORF) flanked by 5′ and 3′ nontranslated regions (27). After viral infection and the release of the viral nucleocapsid into the cytosol, a 3,391-amino-acid (aa)-long polyprotein is translated from the viral RNA at the surface of the endoplasmic reticulum (ER) (27). This polyprotein is cleaved into three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins due to the combined and coordinated activities of host cell proteases in the ER and the viral protease complex (NS2B3) in the cytoplasm (27). The viral protease cleavages are mediated by the catalytic triad (His-51, Asp-75, and Ser-135) of the serine protease N-terminal domain of NS3 (56), with a hydrophilic segment of 40 residues from the transmembrane NS2B protein acting as a cofactor (55).

Dendritic cells (DCs) are the most potent antigen-presenting cells. They become activated after pathogen detection, and they migrate to the lymph nodes, where they activate $CD4^+$ and $CD8^+$ T lymphocytes, triggering adaptive immune responses (3). The phenotypic changes of DC activation include upregulation of major histocompatibility complex (MHC) class I and II molecules and costimulatory molecules, as well as the release of proinflammatory cytokines and chemokines that further potentiate their ability to stimulate T lymphocytes (3). Secretion of type I IFNs (alpha and beta interferon [IFN-α/β]) by DCs contributes to the generation of antiviral innate and adaptive immune responses (13, 23). DCs have been considered target cells for DENV infection (54), and several groups have identified membrane-bound molecules that enhance the infectivity of DCs and other cells by DENV, as is the case for dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) (36, 50). In a recent study, we showed that human monocyte-derived DCs are also productively infected by DENV, and they showed a distinct activated phenotype, with production of proinflammatory cytokines and chemokines, with the exception of type I IFNs (44). Interestingly, DC activation seems to be different in DENV-infected DCs from that in bystander DCs, with a preferential upregu-

* Corresponding author. Mailing address: Department of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1124, New York, NY 10029. Phone: (212) 241-5182. Fax: (212) 534-1684. E-mail: ana.sesma@mssm.edu.

† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 21 July 2010.

§ The authors have paid a fee to allow immediate free access to this article.

lation of costimulatory and HLA molecules on bystander cells (38). Additionally, a pattern of tumor necrosis factor alpha (TNF-α) and IFN-γ-inducible protein 10 (IP-10) production in bystander DCs that is different from that in DENV-infected DCs has been reported (37).

The important role played by IFN-α/β in antiviral host defense has been extensively reviewed elsewhere (1, 6, 15, 43). Recognition of viral components by membrane-associated and/or cytosolic pattern recognition receptors (PRRs) triggers type I IFN production in infected cells. Our current understanding indicates that TLR3 recognizes double-stranded RNA (dsRNA) in endocytic compartments, MDA5 recognizes long dsRNA in the cytoplasm, and RIG-I senses phosphate-containing dsRNA in the cytoplasm (6). In the case of DENV and other flaviviruses, like West Nile virus (WNV), both RIG-I- and MDA5-mediated detection has been described (28). After viral recognition, activation of IFN regulatory factor 3 (IRF-3), IRF-7, NF-κB, and activating transcription factor 2 (ATF-2)/c-Jun transcription factors is induced by different pathways, leading to type I IFN production. Binding of secreted IFN-α/β to the IFN receptor triggers a signal that is transduced by the JAK/STAT pathway, eventually leading to the expression of hundreds of interferon-stimulated genes (ISGs) with antiviral properties, effectively establishing the antiviral state in that cell (1). To counteract this potent antiviral response, viruses have evolved to develop a variety of mechanisms to overcome the antiviral state elicited by IFN-α/β (9, 14, 15). Often, viruses are able to express proteins that interfere with the type I IFN induction pathway, such as the influenza A virus NS1 protein (16), poxvirus E3L protein (24), or the VP35 protein of Ebola virus (5). Additionally, the type I IFN signaling pathway may also be targeted by viruses via the expression of IFN antagonist proteins acting at the level of STAT proteins, inducing STAT degradation or inhibiting the JAK kinases (9, 19, 26).

We and others have demonstrated that DENV is a weaker inducer of type I IFN responses after infection of human DCs, with minuscule production of IFN-α/β (20, 44, 49), especially compared to other viral infections capable of inducing significant levels of type I IFN, such as that of Newcastle disease virus (NDV) (13), Sendai virus (SeV) (30), or Semliki Forest virus (SFV) (18). This absence of type I IFN production by DENV-infected DCs resulted in an impaired ability of those DCs to prime T cells toward Th1 immunity, which was reversed by the addition of exogenous IFN-β (44). We have demonstrated in a primary human cell system that infection of human DCs with DENV did not induce IRF-3 phosphorylation, resulting in an inhibition of type I IFN production after DENV infection (44). Contrary to the knowledge gap regarding the pathway for IFN-α/β induction by DENV, several DENV proteins that inhibit type I IFN signaling have been identified (32). NS4B inhibits STAT-1 phosphorylation *in vitro* (33, 34), and STAT-2 degradation has been observed in DENV-infected cells (21), an action elicited by the DENV protein NS5 (2, 31). In human DCs, DENV infection antagonizes IFN-α/β but not IFN-γ signaling by inhibiting Tyk2-STAT (19).

In the current study, we explore the ability of DENV-infected DCs to respond to a variety of type I IFN-triggering signals. Our results demonstrate not only that DENV-infected DCs fail to produce type I IFN but also that they have reduced type I IFN production upon secondary infection or stimulation even when potent stimulators such as NDV, SeV, SFV, or TLR-3 ligand poly(I:C) are used. This effect is DENV dose dependent and takes place as early as 2 h after DENV infection. Also, we demonstrate that the inhibition of IFN production after NDV infection in DENV-infected DCs is not a bystander effect, suggesting a direct role of the DENV-infected DC population in the inhibition of IFN-α/β. Last, we show that the inhibition of IFN production in DCs by DENV is dependent on a catalytically active NS2B3 protein complex. These results provide new insight into the mechanism by which DENV evades immune recognition in human DCs.

MATERIALS AND METHODS

Cell lines. Vero, Madin Darby canine kidney (MDCK), 293T, and 293T cells stably transfected with a firefly luciferase reporter gene driven by the IFN-β promoter (293T-IFN-β-Luc cells; Juan Ayllon and Adolfo Garcia-Sastre, unpublished data) were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Baby hamster kidney cells (BHK) were grown in Glasgow minimal essential medium (MEM) supplemented with 5% FBS, 10% of tryptose phosphate broth, and 20 mM HEPES. Mosquito cells derived from *Aedes albopictus*, clone C6/36, were expanded at 33°C in RPMI medium with 10% FBS. All media were supplemented with 100 U/ml of L-glutamine and 100 μg/ml of penicillin-streptomycin. All tissue culture reagents were purchased from Invitrogen.

Generation of monocyte-derived dendritic cells. Human monocyte-derived DCs were obtained from healthy human blood donors (New York Blood Center), following a standard protocol as previously described (44). Briefly, after Ficoll-Hypaque gradient centrifugation, $CD14^+$ cells were isolated from the mononuclear fraction using a MACS CD14 isolation kit (Milteny Biotec) according to the manufacturer's instructions. $CD14^+$ cells were then differentiated to naive DCs by incubation during 5 to 6 days in DC medium (RPMI supplemented with 100 U/ml L-glutamine, 100 μg/ml penicillin-streptomycin, and 1 mM sodium pyruvate) with the presence of 500 U/ml human granulocyte-macrophage colony-stimulated factor (GM-CSF) (PeproTech), 1,000 U/ml human interleukin 4 (IL-4) (PeproTech), and 4% human serum serotype AB (Cambrex). The purity of the DCs was confirmed by flow analysis and at least 99% of DCs were $CD11c^+$, $CD86^{low}$, $CD83^-$, $HLA\text{-}DR^{low}$, and $CD14^-$.

Virus preparations. Dengue virus serotype 2 (DENV-2) strain 16681 was used in this study. DENV-2 virus was grown in C6/36 insect cells for 6 days as described elsewhere (11). Briefly, C6/36 cells were infected at a multiplicity of infection (MOI) of 0.01, and 6 days after infection, cell supernatants were collected, clarified, and stored at −80°C. DENV was titrated in Vero cells by immunofluorescence using the DENV E-protein-specific antibody 4G2 (ATCC). Briefly, monolayers of Vero cells were infected with serial dilutions of DENV-2 for 18 h. After washing, cells were fixed, permeabilized, and blocked. Cells were incubated with the DENV-2-specific monoclonal antibody for 1 h, and an anti-mouse IgG-fluorescein isothiocyanate (FITC) linked antibody was used as a secondary antibody (Invitrogen). Virus titers were determined by direct counting of FITC-positive cells. Also, the titers of DENV-2 stocks were determined by limiting-dilution plaque assay on BHK cells (10). Recombinant Newcastle disease viruses (NDV) B1 (Hitchner vaccine strain), recombinant NDV expressing green fluorescent protein (GFP) (NDV-GFP), influenza viruses A/PR8/34 lacking the NS1 gene (ΔNS1), Sendai virus (SeV), and Semliki Forest virus (SFV) expressing GFP have been previously described (16, 35, 39, 48). NDV and SeV viruses were grown in 9-day-old embryonated chicken eggs (Spafas Charles River). NDV and NDV-GFP viruses were titrated by immunofluorescence in Vero cells. SeV was titrated by hemagglutination assay (HA), and stocks with more than 12 HA wells were used for the experiments. Influenza A virus lacking the NS1 gene was grown in 6-day-old embryonated chicken eggs (Spafas Charles River) and titrated by immunofluorescence in MDCK cells. SFV-GFP was generated as described previously (48) and titrated in BHK cells by immunofluorescence (45).

Cloning of mammalian expression plasmids and NDV vectors coding for DENV proteins. Plasmid coding for TLR3 was previously described (47) and was kindly donated by Christopher F. Basler. Mammalian expression vectors coding for DENV proteins were generated using standard molecular biology methods. Some DENV proteins (prM, E, and NS1) were expressed fused with the transmembrane (TM) domain of the previous protein. The DENV NS2A protein was

fused in the N terminus to the last 22 amino acids of the E protein and the last 50 amino acids of the NS1 protein. Sequence coding for each protein was generated by reverse transcription using SuperScript One-Step reverse transcription-PCR (RT-PCR) with the Platinum *Taq* kit (Invitrogen) from RNA isolated with TRIzol reagent (Invitrogen) from DENV stocks. Primers used for PCR amplification are listed in Table S1 in the supplemental material. Reverse primers contained an HA tag sequence (ATGTACCCTTATGATGTCCCAGATTA TGCCTAA), and forward primers contained a Kozak sequence (CCGCCACC), in order to facilitate expression. Both forward and reverse primers contained specific restriction sites to facilitate cloning into pcDNA3.1 (Invitrogen). Mutations in the NS2B3 protein were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. NDV-based vectors were generated similarly from RNA isolated from DENV stocks using primers listed in Table S2 in the supplemental material. The NDV cDNA sequence was derived from the mesogenic Hitchner B1 strain, engineered to express the modified F cleavage site (F3aa), as previously described (40). PCR fragments were inserted as an additional transcription unit into the unique XbaI restriction site between the *P* and *M* genes in the full-length plasmid, pT7NDV/F3aa. Viruses were rescued using an established method of reverse genetics (35). NDV vectors expressing DENV proteins were grown and titrated as described above. All experiments with these recombinant NDV viruses were performed when these viruses were still classified as biosafety level 2 (BSL-2) agents.

Infection of dendritic cells. Human DCs were obtained as described above, and at day 5 of culture, samples of 5×10^5 cells were resuspended in 50 μl of DC medium and were infected for 1 h at 37°C with the indicated MOI of DENV (diluted in DC media) or with DC medium (mock group) in a total volume of 100 μl. After the adsorption period, DC medium supplemented with 4% human serum (HS) was added up to a final volume of 500 μl, and cells were incubated for the appropriate time (between 0 and 24 h) at 37°C. NDV infection was done as follows. After centrifugation of the DENV- or mock-infected DCs at 3,500 rpm for 5 min, medium was removed and cells were infected with NDV-GFP virus at a MOI of 1 during 45 min in a total volume of 100 μl of DC medium. The control group received 100 μl of medium. After infection, 400 μl of DC medium with 4% HS was added, and cells were incubated for 18 h at 37°C. At the end of the incubation, cells were centrifuged at 3,500 rpm for 5 min, and cell supernatants were collected and stored until processed. Cell pellets were used for RNA extraction or fluorescence-activated cell sorter (FACS) analysis as described below. For some infections, DENV was inactivated by UV irradiation from a germicidal lamp (15 min at 6 in.). Virus inactivation was verified by immunofluorescence on Vero cells. All infections were performed in triplicate in at least three independent donors.

Infection of dendritic cells in transwell plates. Infection in the transwell plate experiments were performed as follows. Samples of 5×10^5 DCs were mock- or DENV-infected as described above (MOI of 1 for 1 h). After virus adsorption, 5×10^5 naive DCs were seeded in the lower compartment of the transwell plate, and 5×10^5 mock- or DENV-infected cells were seeded in the upper compartment in a total volume of 1 ml per well. Transwell plates were incubated for 12 h at 37°C, and then DCs from each independent compartment were collected and either infected as described above with NDV-GFP (MOI of 1) or mock infected. Following 18 h of incubation, the supernatant and cells were collected for posterior analysis. All infections were performed with at least three independent donors with three experimental replicates per infection.

Quantitative RT-PCR and analysis of cytokine production. RNA isolation was performed using the Absolutely RNA Microprep kit (Stratagene) according to the manufacturer's instructions. Viral and host RNA expression levels were quantified by quantitative RT-PCR (qRT-PCR) as described previously (44). Where indicated, qRT-PCRs were carried out using iQ SYBR green supermix (Bio-Rad) after retrotranscription of isolated RNA with the iScript cDNA synthesis kit (Bio-Rad) in a CFX96 real-time PCR detection system (Bio-Rad) according to the manufacturer's indications. Expression levels were calculated based on the $\Delta\Delta C_T$ values using three different housekeeping genes (rsp11, β-actin, and α-tubulin genes) to normalize the data. The presence of IFN-α in cell supernatants was measured using the panspecific human IFN-α enzyme-linked immunosorbent assay (ELISA) kit (Mabtech) according to the manufacturer's indications.

Flow cytometry. DENV-infected DCs were fixed and permeabilized with Cytofix and Cytoperm reagent (BD Pharmingen) according to the manufacturer's recommendations. Then, cells were stained with 4G2 (ATCC), a mouse monoclonal antibody specific for the E protein, as a primary antibody and an Alexa555-labeled anti-mouse antibody as a secondary antibody. NDV-GFP-infected DCs were analyzed either directly by GFP visualization or after fixing and permeabilization as described above. To measure cell surface expression markers, infected or noninfected DCs were stained with phycoerythrin (PE)- or FITC-conjugated monoclonal antibodies to CD86, CD83, HLA-ABC, HLA-DR, CD11c, and CD14 (Beckman Coulter). Mouse IgG1, IgG2a, and IgG2b were included as isotype controls. Staining for apoptotic cells was performed using PE annexin V (BD Pharmingen), and a positive control of induction of apoptosis with an anti-CD95 (FAS) antibody was included according to the manufacturer's instructions. Alexa555-, GFP-, PE-, and/or FITC-positive cells were analyzed using the Flowjo software program (Tree star) after sample acquisition on a FACScan flow cytometer (BD Pharmingen).

Transfections and type I IFN antagonist assay. 293T cells were transfected by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's specifications. A type I IFN production antagonist assay was performed as described previously (5). Each transfection of 5×10^5 cells contained 0.2 μg of the IFN-β promoter expressing a firefly luciferase reporter plasmid (34), 0.2 μg of the Renilla luciferase reporter plasmid pRL-Tk (Promega), and 1 μg of the indicated pcDNA expression plasmid coding for DENV proteins. To induce IFN-β promoter activity, cells were infected 24 h posttransfection with SeV at a MOI of 1. For TLR3-mediated IFN-β induction, 0.5 μg of pcDNA expressing a TLR3 plasmid (47) was included in the initial transfection, and 24 h later, cells were cultured in the presence of 25 μg/ml of poly(I:C) as described previously (47). In some experiments, the stably transfected 293T-IFN-β-Luc cell line was used. Cells were harvested and lysed in reporter lysis buffer (Promega) 24 h after the induction of the IFN-β promoter. Luciferase assays were performed by using the Promega luciferase assay system according to the manufacturer's directions.

Western blot analysis. Transfection of 293T cells and infection of human DCs was performed as described above. Cell lysates were obtained after incubation of cells with RIPA lysis buffer (Sigma Aldrich) supplemented with complete protease inhibitor (Roche) and were resuspended in a total of 50 μl of Laemmli sample buffer (Bio-Rad). Crude lysates were boiled for 10 min and then kept on ice. Each sample was loaded in a polyacrylamide-SDS gel, and the proteins were electrophoretically separated by conventional methods. Proteins were transferred to nitrocellulose, and blots were blocked in 5% fat-free milk and 0.5% Tween 20 in phosphate-buffered saline (PBS). Incubations with anti-HA, anti-β-actin (Sigma Aldrich), anti-IRF-3, and anti-IRF-3-P (Cell Signaling) were performed in blocking buffer at 4°C overnight on a rotating platform. Blots were washed three times for 10 min with PBS–0.05% Tween 20, incubated for 1 h with goat anti-rabbit or goat anti-mouse antibody (Amersham Bioscience), and washed again three times. Antibody-protein complexes were detected using a Western Lighting chemiluminescence system (Perkin Elmer).

Statistical analysis. Statistical analysis was performed using Student's *t* test or one-way analysis of variance (ANOVA), followed by Student's *t* tests between required samples. As indicated in figure legends, an asterisk represents a *P* values of <0.05, while double asterisks represent a *P* value of <0.01.

RESULTS

Infection of DCs with DENV does not induce type I IFN production. We recently showed that infection of human DCs with DENV resulted in upregulation of costimulatory molecules and production of proinflammatory cytokines without type I IFN production (44). To further determine the potential contribution of the viral load in the production of IFN-α/β by infected cells, DCs were infected with increasing MOIs of DENV, and the levels of infectivity and replication of DENV, as well as the induction of type I IFN, were analyzed on those DCs (Fig. 1). Flow cytometry analysis revealed a MOI-dependent infectivity of DCs by DENV reaching almost 80% 24 h after infection with a MOI of 25 (Fig. 1A). The number of infected cells with a MOI of 5 was slightly lower (73.77%), indicating that the infectivity is most likely saturated at a MOI of 5. In agreement with the flow cytometry analysis, we observed a MOI-dependent DENV replication by qRT-PCR (Fig. 1B) with increasing DENV RNA levels between 24 h and 48 h after infection. Again, the RNA levels 24 h after infection with a MOI of 5 were similar to those observed with a MOI of 25, confirming the likelihood of having reached a plateau at a MOI of 5 (Fig. 1A). On the other hand, the levels of IFN-α in the supernatants of

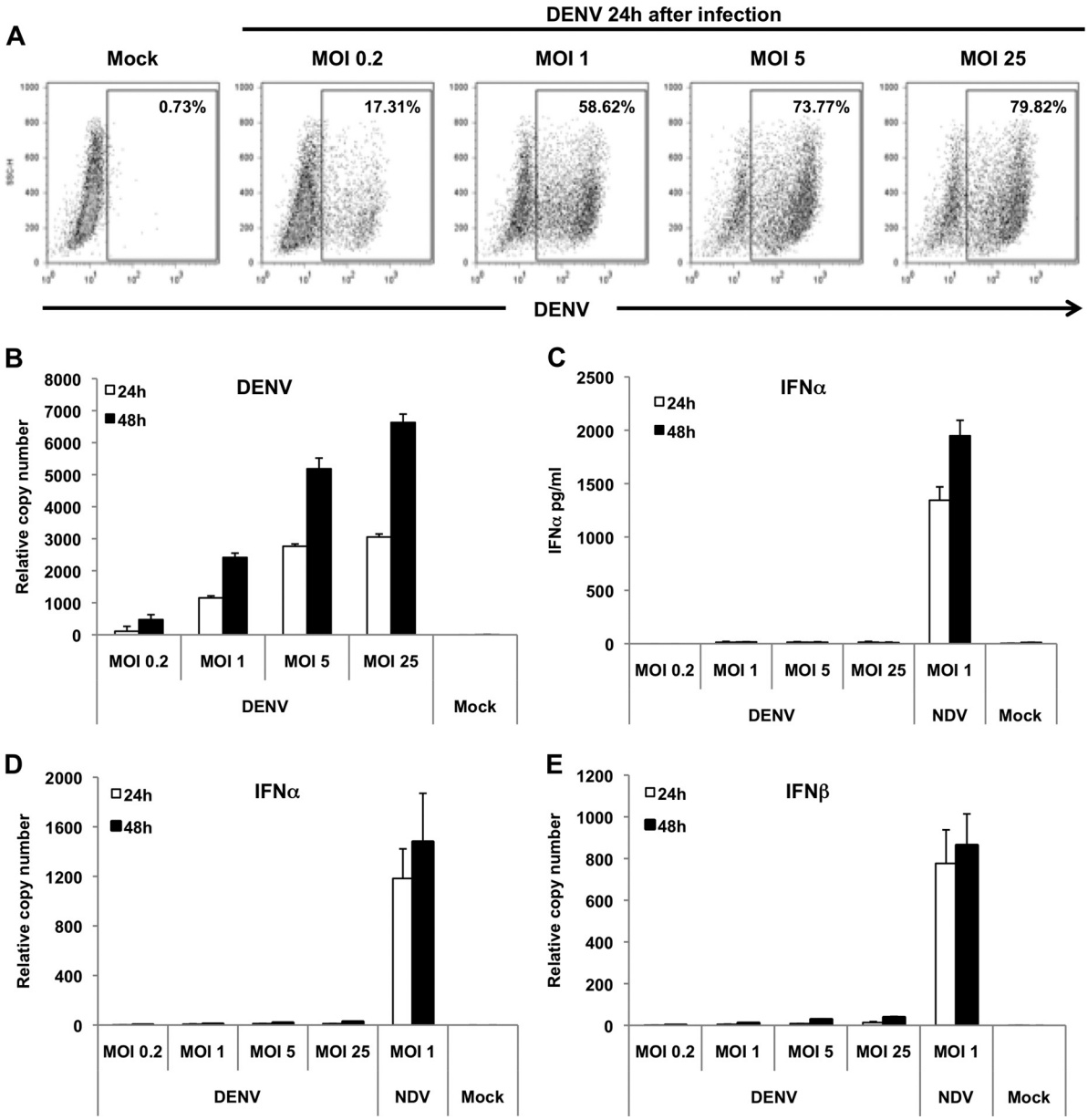

FIG. 1. Infection of human DCs by DENV at different MOIs does not induce type I IFN production. (A) FACS analysis of the infectivities of DCs with different DENV MOIs 24 h after infection using a specific antibody against the DENV E protein. The percentage of positive cells is represented for each condition. (B to E) Quantification of the RNA levels of DENV (B), the IFN-α protein (C), IFN-α RNA (D), or IFN-β RNA (E) in DCs infected with DENV and/or NDV at the indicated MOI 24 h (white bars) or 48 h (black bars) after infection. Error bars represent standard deviations for three sample replicates from a representative donor.

DENV-infected DCs were very low (<25 pg/ml) at any of the tested MOI or time points (24 h or 48 h). However, NDV-infected DCs produced high levels of IFN-α (2,000 pg/ml at 48 h), indicating that DCs were able to react to IFN-inducing stimulus (Fig. 1C). Additionally, we measured IFN-α and IFN-β RNA levels on those DENV-infected DCs 24 h and 48 h after infection (Fig. 1D and E), and the levels of expression were almost undetectable, even when a MOI of 25 was used. These data confirm that DENV is not able to induce the production of type I IFN after infection of human DCs even when high doses of virus were used.

DENV infection inhibits type I IFN production in DCs after NDV infection. In order to investigate if the inhibition of type I IFN production after DENV infection resulted in a general inhibition of the IFN pathway in those infected DCs, we performed a secondary infection with NDV expressing GFP (NDV-GFP) 12 h after infection with DENV, both at a MOI of 1. NDV was used since this virus is not only a great inducer of type I IFN production in DCs (13) but is also very sensitive to the antiviral effects of type I IFN (39). Mock-infected DCs or singly infected DCs with each virus were used as controls. RNA and protein levels of IFN-α/β produced in infected DCs

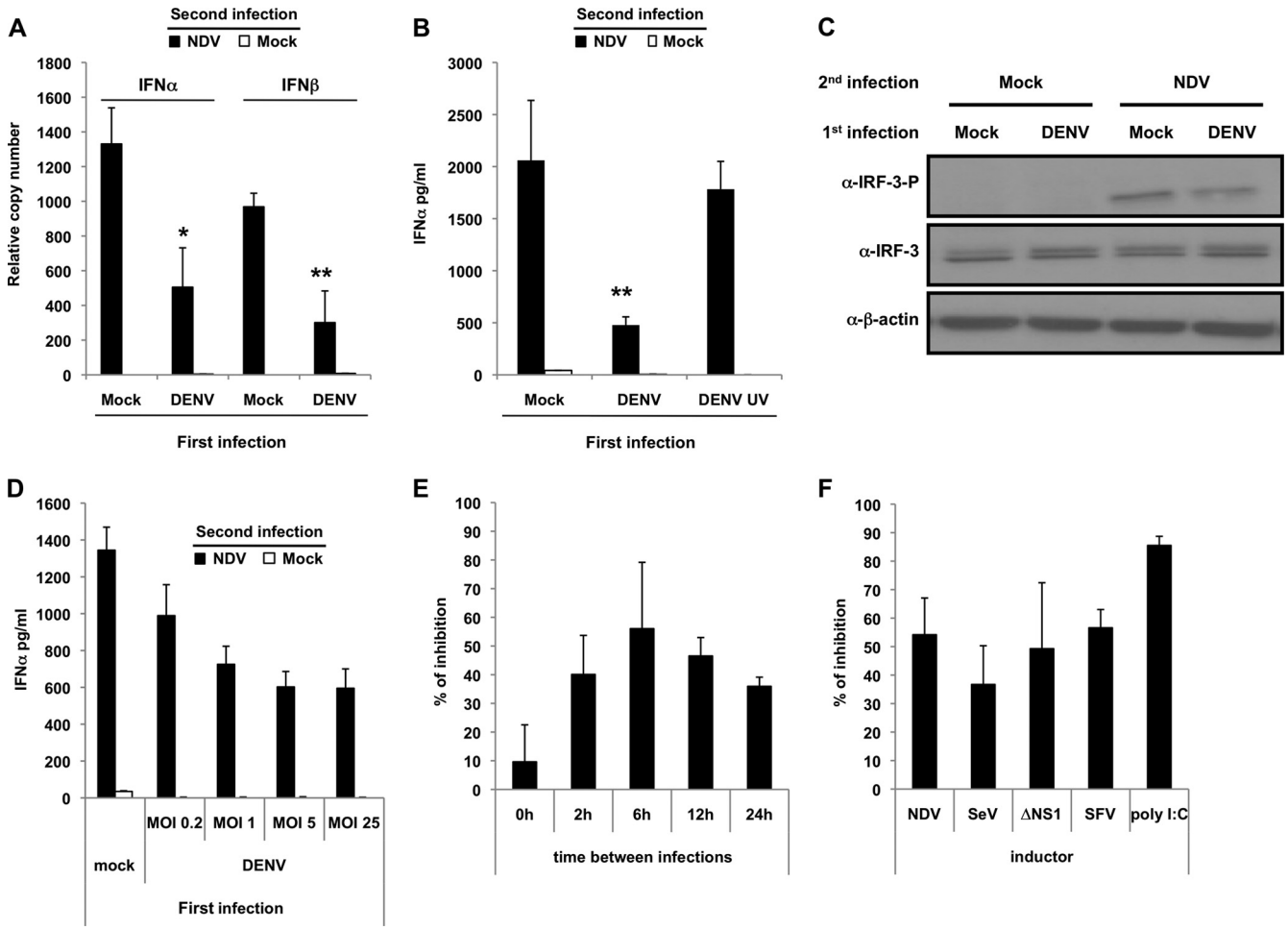

FIG. 2. Infection of DCs with DENV reduces type I IFN production after a secondary infection. DCs were infected with DENV at a MOI of 1, and 12 h later, we infected the same cells with NDV at the same MOI. Samples were collected and analyzed 18 h after secondary infection. (A) IFN-α and IFN-β RNA levels quantified by qRT-PCR. (B) IFN-α protein levels measured by specific ELISA in supernatants from infected DCs as indicated above. In this case, UV-inactivated DENV virus was included at the same MOI. (C) Analysis by Western blot of the phosphorylated and total levels of IRF-3 in DCs infected as described above. (D) Analysis of the contribution of the amount of DENV in the inhibition of type I IFN production after NDV infection. DCs were infected with DENV at the indicated MOIs, and 12 h later, DCs were infected with NDV at a MOI of 1. IFN-α protein levels were quantified in cell supernatants by ELISA 18 h after secondary infection. (E) Role that the time between infections had on the inhibition of type I IFN production. DCs were infected with DENV at a MOI of 1, and at the indicated times after DENV infection, a secondary infection with NDV was performed. Data represent the percentages of inhibition observed in IFN-α protein production after NDV infection in the group previously infected with DENV versus the mock one. (F) The ability of DENV to reduce type IFN production was not limited to a secondary infection by NDV. Twelve hours after DENV infection of DCs, a variety of IFN inducers recognized through RIG-I (NDV, SeV, and dNS1), MDA5 (SFV), or TLR3 [poly(I:C), 25 μg/ml] were used to trigger type I IFN production. Data represent the percentages of inhibition observed in IFN-α protein production after IFN triggering in the group previously infected with DENV versus the mock one. Error bars represent standard deviations for three sample replicates from a representative donor. *, $P < 0.05$; **, $P < 0.01$.

18 h after NDV infection were analyzed, and we observed that the DCs that were previously infected with DENV had on average 59% lower levels of IFN-β RNA than DCs singly infected with NDV (Fig. 2A). The IFN-α RNA levels were also reduced in the cells infected with both viruses, with an average of 58% inhibition (Fig. 2A). On the other hand, no significant IFN-α/β levels were observed after DENV infection or in the uninfected cells (Fig. 2A and B). These results were also confirmed at the protein level, where the presence of DENV reduced significantly the amount of IFN-α that was produced by the DCs in response to NDV (Fig. 2B). The average reduction of IFN-α at the protein level was 54%. Although the variability in gene expression and protein production of type I IFN within donors is high (Table 1), the relative values of inhibition of type I IFN production observed after a secondary infection with NDV in previously DENV-infected DCs were comparable (Table 1; see also Fig. S1 in the supplemental material). Since we have recently described that DENV infection of DCs does not induce IRF-3 phosphorylation (44), we analyzed the IRF-3 phosphorylation levels in each group. As expected, no IRF-3 phosphorylation was observed after DENV infection, and reduced levels of phosphorylated IRF-3 were observed after NDV infection of previously DENV-infected DCs compared to results for NDV-infected ones (Fig. 2C). The quantification of the bands by densitrometic analysis revealed a 37% reduction of the phosphorylated IRF-3 levels

TABLE 1. Variability within donors in IFN-α protein production after NDV infection in previously mock- or DENV-infected DCs

Donor no.	IFN-α level (pg/ml)		% IFN-α protein expression[a]	% inhibition[b]
	Mock-NDV	DENV-NDV		
1	933.02	415.93	44.58	55.42
2	1,117.41	652.99	58.44	41.56
3	477.16	140.71	29.49	70.51
4	1,344.44	817.22	60.79	39.21
5	1,301.94	698.44	53.65	46.35
6	6,182.29	1,433.54	23.19	76.81
7	1,451.90	554.62	38.20	61.80
8	4,847.31	2,872.38	59.26	40.74
All				
Mean value			45.95	54.05
SD			14.45	14.45

[a] Percentage of IFN-α protein production in DENV-NDV group relative to that in the mock-NDV group.
[b] Percentage of inhibition of IFN-α production in the DENV-NDV group relative to that in the mock-NDV group.

(Fig. 2C). These results support that DENV infection interferes with IRF-3 phosphorylation in human DCs (44). Moreover, the inhibition of type I IFN observed in DENV-infected DCs after a secondary infection was DENV replication dependent, since infection of DCs with UV-inactivated DENV did not reduce the amount of IFN-α in response to NDV infection (Fig. 2B). These results suggest that infection of human DCs with DENV affects the type I IFN induction pathway by reducing the ability of infected cells to respond to a secondary infection.

Characterization of the type I IFN inhibition after NDV infection of DENV-infected DCs. To further characterize the inhibition of type I IFN production seen after NDV infection in human DCs previously infected with DENV, we analyzed the contribution of the DENV dose. Thus, DCs were infected with different MOIs of DENV (0.2, 1, 5, and 25), and 12 h later they were subsequently infected with NDV at a MOI of 1 as in previous experiments. IFN-α protein levels were measured in the cell supernatants 18 h after the secondary infection (Fig. 2D), and we observed that the ability of DENV infection to inhibit IFN-α production in DCs was directly correlated with the MOI of DENV used for the infection, as shown by the decrease in IFN-α production from 55% with a DENV MOI of 25 to 26% when a MOI of 0.2 was used. Mock- and DENV-infected DCs without subsequent infections did not produce significant amounts of IFN-α. A similar inhibition was observed when a MOI of 5 or 25 was used, indicating that a plateau was reached at a MOI of 5. Additionally, we studied the role that time between infections had on the inhibition of type I IFN production. DCs were infected with NDV at different times following the primary infection with DENV, from 0 to 24 h. With a time lapse as short as 2 h, a 40% inhibition of IFN-α protein production was observed (Fig. 2E), indicating that this inhibition starts at early times after DENV infection. Comparable levels of inhibition were observed when longer times of incubation between infections were performed (Fig. 2E), further supporting that the inhibitory effect on type I IFN production is an early event after DENV infection. To demonstrate that the ability of DENV to reduce IFN production after a secondary infection or stimulus is not limited to NDV, a variety of IFN-α/β inducers recognized through different PRRs by DCs were tested. As established in our prior experiments, DCs were infected with DENV at a MOI of 1, and 12 h later, DCs were stimulated via RIG-I by different viruses (NDV, SeV, and influenza A virus lacking the NS1 protein [ΔNS1]) (28), via MDA5 (Semliki Forest Virus [SFV-GFP]) (28, 46), and via TLR3 by its well-characterized ligand poly(I:C). Under all the conditions tested, DENV-infected DCs showed an impaired response against the secondary stimuli (Fig. 2F). Secondary infections with ΔNS1 or SFV resulted in inhibition similar to that observed against NDV, approximately 50%. After triggering IFN-α/β production with SeV, the inhibition was slightly lower (37%), but interestingly, after poly(I:C) treatment, the inhibition was increased up to 85%, indicating that DENV-infected DCs not only had an impaired response against RIG-I- and MDA-5-mediated induction but also inhibited TLR3-mediated type I IFN production. These data suggest that DENV-infected DCs have an impaired type I IFN response against secondary stimuli that are recognized by a variety of PRRs.

Enhanced NDV replication in doubly NDV- and DENV-infected DCs. Our data of reduced type I IFN production in DENV-infected DCs after NDV infection could reflect an inability of NDV to infect those cells. Thus, we tested the levels of NDV RNA in DCs previously infected with DENV. Samples were collected 18 h after secondary infection, and the levels of viral RNA were analyzed by qRT-PCR (Fig. 3A and B). Independently of the presence or absence of a secondary infection, the levels of DENV RNA were similar in the two groups (Fig. 3A). Interestingly, NDV RNA levels were about 4 times higher in the group that was previously infected with DENV than in DCs infected exclusively with NDV (Fig. 3B). These data are in accordance with the observation that the replication-dependent GFP intensity after NDV-GFP infection was higher when the DCs were previously infected with DENV than in the singly infected DCs with NDV (Fig. 3C and E). To test if the increased NDV RNA levels and the higher GFP intensity reflected a greater percentage of infection or more-efficient NDV replication, we quantified the percentage of positive cells for each virus in every group by flow cytometry (Fig. 3C and D). Our data show that the percentage of DCs infected only with one virus was 57.2% $DENV^+$ or 59.5% NDV^+, but when DENV-infected DCs were subsequently infected with NDV, the distribution was 20.6% $DENV^+$, 20.2% NDV^+, and 37.8% $DENV^+$ NDV^+. Since the frequency of DCs infected by NDV in the absence of DENV (59.5%) or in the presence of DENV infection (20.1% NDV^+ plus 37.8% $DENV^+$ NDV^+) was unchanged, these data indicate that NDV was replicating more efficiently in those DCs that were previously infected with DENV. Furthermore, the percentage of DENV-positive DCs was similar in both DENV-infected groups, independently of the secondary NDV infection, correlating with the similar RNA levels seen in Fig. 3A. Taking together, these data also support the lack of type I IFN production in DENV-infected DCs, since both DENV-infected and noninfected neighbor cells can be also infected with the very IFN-sensitive virus NDV (39), indicating that there is no induction of an antiviral state in those DCs after DENV infection.

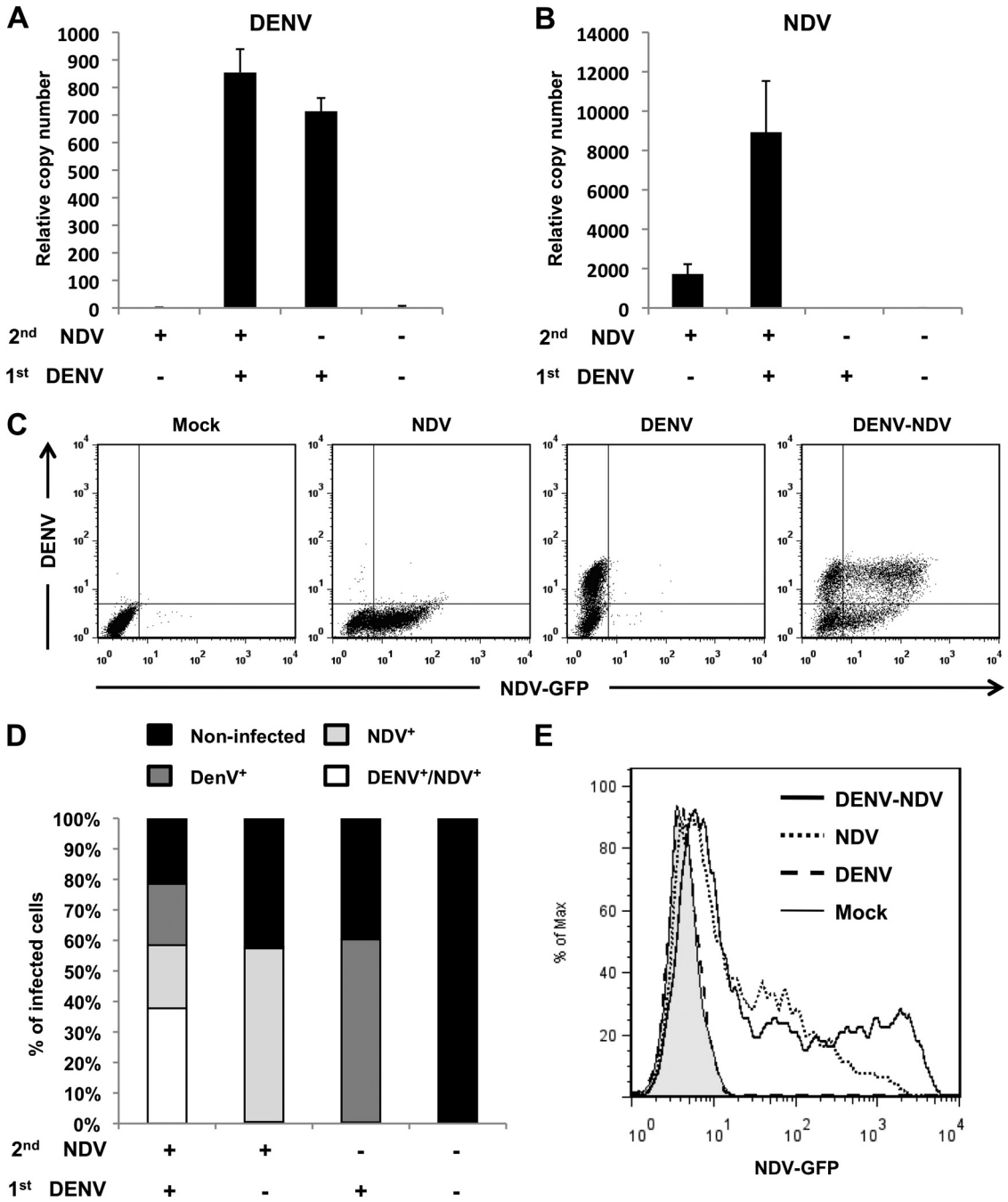

FIG. 3. Increased NDV replication after a secondary infection of DENV-infected DCs. DCs were infected with DENV at a MOI of 1, and 12 h later, we infected the same cells with NDV at the same MOI. Mock-infected DCs or singly infected DCs with each virus were used as controls. RNA levels for DENV NS5 (A) or NDV HN (B) were measured by qRT-PCR 18 h after secondary infection. The distribution of positive cells for each virus in every group of DCs 18 h after the secondary infection was analyzed by FACS (C), and the percentage of infected DCs for each virus was quantified (D). A histogram shows GFP intensity 18 h after NDV-GFP infection of DCs previously infected with DENV (E). Max, maximum. Error bars represent standard deviations for three sample replicates from a representative donor.

Inhibition of type I IFN production after NDV infection is not a bystander effect. Since several bystander effects have been described for DENV in DCs (37, 38), we tested if DENV-uninfected bystander cells also exhibited inhibition of type I IFN production upon a secondary infection. Thus, we performed experiments where DCs were seeded in the lower chamber of a transwell culture plate with a membrane between the two chambers that allows the diffusion of components in the culture medium but does not allow cell-to-cell contact (7). Other DCs were mock- or DENV-infected for 1 h in a sterile vessel and, following thorough washing to remove any excess DENV, were placed in the upper chamber of the transwell

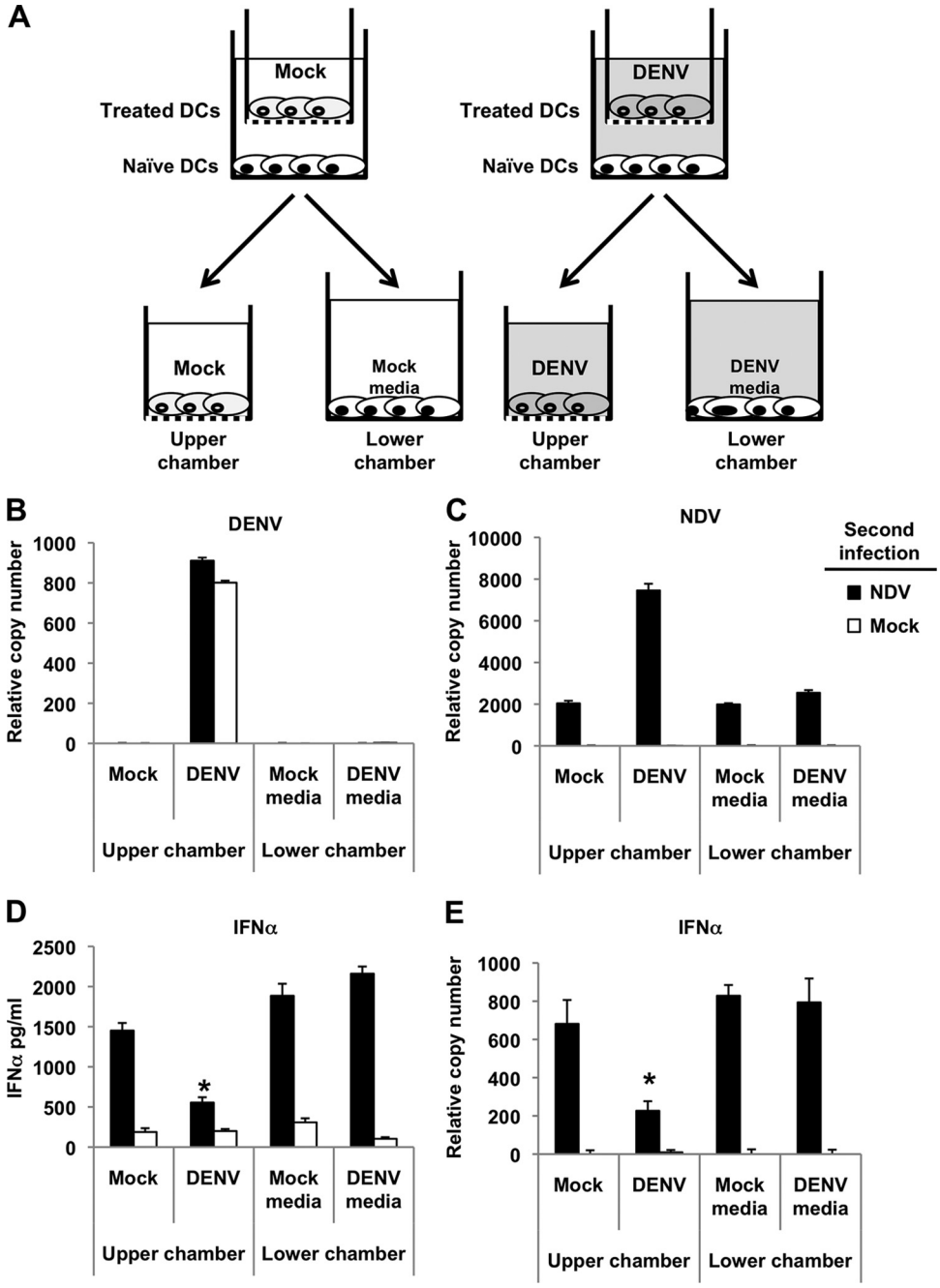

FIG. 4. Inhibition of type I IFN production by DENV after NDV infection is not a bystander effect. (A) Schematic representation of the experimental procedure in the transwell plates. Naive DCs were seeded in the lower chamber of a transwell culture plate with a membrane between the two chambers that allows the diffusion of components in the culture medium but does not allow cell-to-cell contact. Treated DCs were mock or DENV infected for 1 h and following thorough washing were placed in the upper chamber of the transwell plate. All transwell plates were cultured for 12 h, and each chamber was collected separately and subsequently mock (white bars) or NDV (black bars) infected. (B to E) Analysis of DENV NS5 RNA levels (B), NDV HN RNA levels (C), IFN-α protein levels (D), and IFN-α RNA levels (E) was done by measuring 18 h after mock or NDV infection in each chamber. Error bars represent standard deviations for three sample replicates from a representative donor. *, $P < 0.05$.

plates (Fig. 4A). Thus, after 12 h of culture, DCs from each chamber were collected separately and subsequently mock or NDV infected. Supernatants and RNA samples were analyzed 18 h after NDV infection. Analysis of viral RNA showed that DENV potentially budding from the upper compartment was not diffusing through the membrane and infecting DCs located in the lower chamber of the transwell plate when DENV-infected DCs were seeded in the upper compartment (Fig. 4B), indicating that any effect or change observed in the DCs in the lower chamber is not due to DENV infection of those cells.

NDV RNA was detected in both the upper and lower compartments that were directly infected with this virus (Fig. 4C). However, the previously seen enhancement in Fig. 2B when DCs were previously infected with DENV was not observed in those DCs that were incubated in the lower chamber and were exposed to any released factors by the DENV-infected DCs in the upper chamber (Fig. 4C). These data indicate that the enhancement of NDV RNA levels in DENV-preinfected DCs is not due to a soluble factor released from DENV-infected DCs that increases NDV replication. Moreover, inhibition of type I IFN production was observed only in those groups of cells directly infected with DENV and subsequently with NDV and not in those DCs sharing the culture medium with DENV-infected DCs (Fig. 4D and E). These results show that the inhibition of type I IFN production in DENV-infected DCs after a secondary infection takes place only on those DCs that are directly infected with DENV, suggesting that DENV by itself and/or some DENV-induced factor on DCs is inhibiting type I IFN production upon secondary infections/stimuli.

DENV NS2B3 protease complex reduces type I IFN production on DCs. Since we demonstrated that the inhibition of type I IFN production in DENV-infected DCs after a secondary infection takes place only on those DCs that are doubly infected, we further investigated the IFN antagonist properties of individual DENV proteins. Thus, we used a strategy based on NDV vectors, similar to the one previously used for the influenza A virus NS1 protein (13). We generated NDV vectors (35) coding for the nonstructural DENV proteins NS1, NS2A, NS2B3, NS4A, and NS4B. Due to the complex topology of the DENV polyprotein and the requirement of a proper localization in the ER and the proper cleavage by host and viral proteases for its function (27), some of the DENV proteins were expressed fused with the transmembrane (TM) domain of the previous protein, as explained in Methods. PCR fragments were inserted using the unique XbaI restriction site (Fig. 5A), and the ability of those recombinant viruses to induce type I IFN production after infection of human DCs was tested. The NDV vector expressing the DENV NS5 protein could not be rescued after several attempts, probably due to the insert size limitation in our NDV vector. As a positive control, an NDV vector coding for the influenza A NS1 protein (NDV-NS1flu) was included (13). DCs were infected with each virus at a MOI of 1, and expression of the different DENV proteins was confirmed 18 h after infection by Western blotting (Fig. 5B). Also, NDV RNA levels were quantified by qRT-PCR and showed comparable levels of replication for NDV-GFP and NDV vectors expressing DENV proteins (Fig. 5C). NDV-NS1flu showed higher replication levels, most likely due to its strong inhibition of type I IFN production and the ability of influenza virus NS1 to preferentially enhance viral mRNA processing (12). IFN-α protein levels were measured in supernatants from those NDV-infected DCs, and we observed a 35% significant reduction in IFN-α production when the NDV-NS2B3 vector was used (Fig. 5D), indicating an IFN antagonist activity of the NS2B3 protein complex of DENV, which also correlated with the slightly higher NDV RNA levels observed in DCs after infection with NDV-NS2B3 (Fig. 5C). Moreover, this reduction in the IFN-α protein levels was not a consequence of a higher induction of apoptosis in those NDV-infected DCs, since the levels of apoptotic cells (annexin V-positive cells) were similar after NDV-GFP and NDV-NS2B3 infection (Fig. 5E).

DENV NS2B3 protease complex mediates type I IFN inhibition on 293T cells. We moved to an *in vitro* system to further investigate the role of the DENV NS2B3 protease as well as those of other DENV proteins. This approach also allowed us to test additional DENV proteins that could not be successfully incorporated into NDV vectors using our reverse genetics system. Thus, we generated mammalian expression vectors coding for different DENV proteins that could be tested in a type I IFN production antagonist assay in 293T cells (5). First, we tested if DENV by itself was able to block type I IFN production in 293T cells containing the firefly luciferase reporter gene under the control of the IFN-β promoter (293T-IFN-β-Luc). Thus, 293T-IFN-β-Luc cells were infected with DENV at a MOI of 5, and DENV replication, as well as luciferase production, was measured. We observed that DENV was able to replicate in these 293T-infected cells (Fig. 6A), with more than 80% of the cells infected (data not shown). However, no IFN-β promoter activity was induced by DENV during the first 24 h after infection (Fig. 6B) as measured by luciferase expression. Minimal increased levels of luciferase were observed at later times after DENV infection compared to results for mock-infected cells (Fig. 6B), which was irrelevant compared with the induction produced by SeV infection (Fig. 6C). Also, we tested the ability of DENV to reduce type I IFN induction in 293T cells when DENV-infected cells were subsequently infected with SeV. We observed a reduction in the levels of luciferase of almost 60% (Fig. 6D), correlating with strongly decreased levels of IRF-3 phosphorylation when DENV-infected 293T-IFNb-Luc cells were challenged with SeV (Fig. 6E). In this case, when SeV was added to previously DENV-infected cells, SeV was present in DENV-infected cells and in noninfected cells, with a percentage of infected cells similar to that in the group infected with SeV that was previously mock infected (data not shown). These results indicate that the reduction observed in the luciferase and in the IRF-3-phosphorylated levels was not a consequence of a lack of SeV infectivity. Together these data indicate that DENV is able to block SeV-induced IFN-β promoter activation in 293T cells. In order to test the mammalian expression vectors coding for different DENV proteins, 293T cells were cotransfected with each DENV protein-expressing plasmid together with a reporter plasmid expressing the firefly luciferase gene under the control of the IFN-β promoter. After transfection, protein expression was corroborated (Fig. 6G), and IFN-β production was triggered by SeV infection. For TLR3-mediated induction, a TLR3 plasmid was included in the initial transfection mix, and IFN-β production was triggered with poly(I:C). The results shown in Fig. 6F indicate that expression of the viral protease complex (NS2B3) significantly reduced the IFN-β promoter activation induced by both SeV and poly(I:C) to levels in a range similar to the inhibition observed in DENV-infected DCs and 293T cells after a secondary infection with SeV (Fig. 2F and 6D). Similar results were observed when the 293T-IFN-β-Luc cell line was used for the transfections (data not shown).

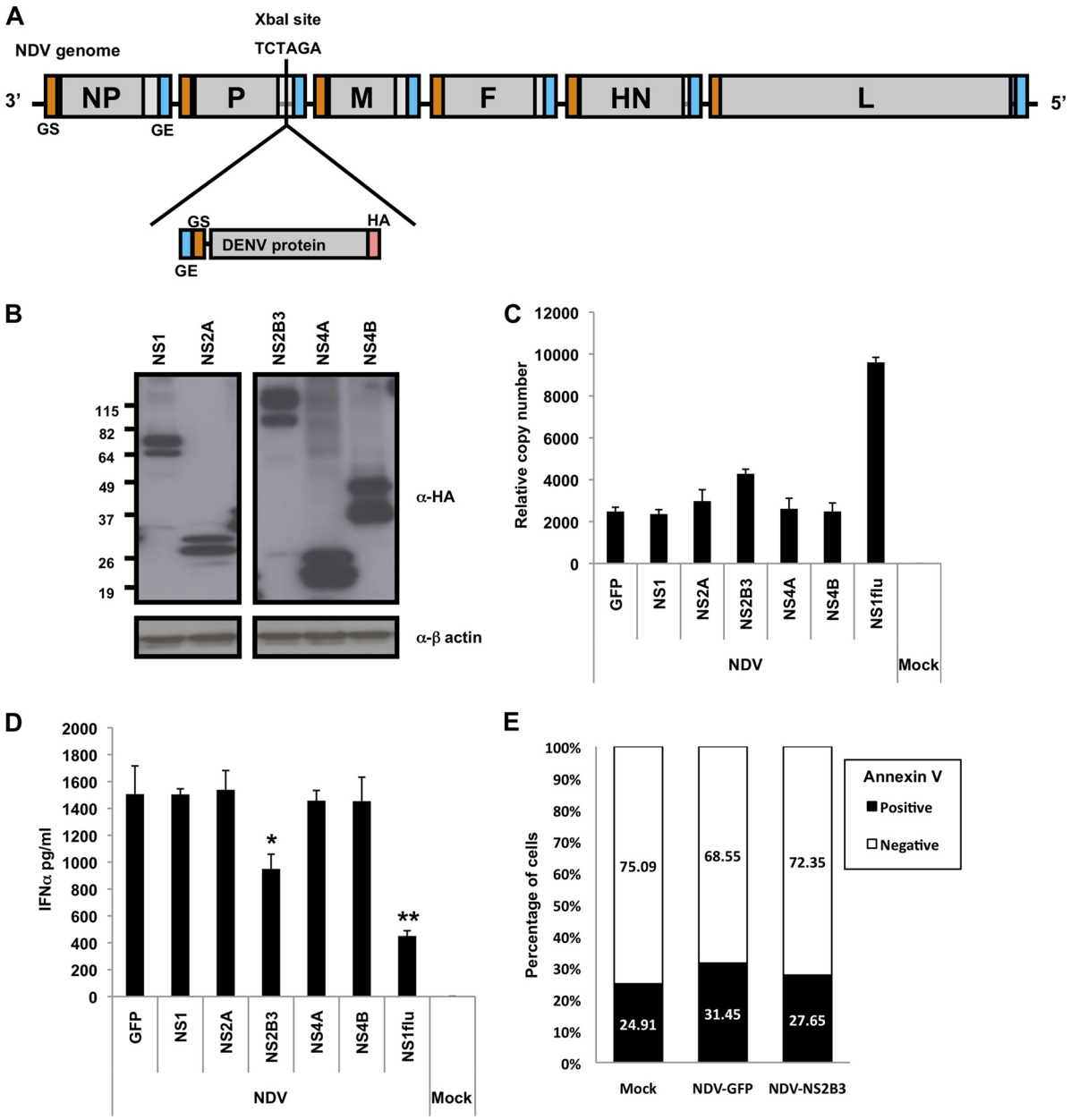

FIG. 5. DENV NS2B3 protease complex reduces type I IFN production in human DCs. (A) Schematic representation of NDV-based vector genome with the cloning site and the insert coding for the different DENV proteins. (B) Western blot analysis of protein expression 24 h after infection of DCs with NDV vector expressing DENV proteins at a MOI of 1. (C and D) Analysis of NDV RNA (C) or IFN-α protein levels (D) 24 h after infection of DCs with NDV vectors coding for DENV proteins at a MOI of 1 is also shown. (E) Analysis of The apoptotic cells by annexin V staining in mock- or NDV-infected DCs 24 h after infection. Error bars represent standard deviations for three sample replicates from a representative donor. *, $P < 0.05$; **, $P < 0.01$. GS, gene start; GE, gene end; HA, HA tag.

Active DENV NS2B3 protease is required for type I IFN inhibition. To further map this inhibition by the NS2B3 protease complex, we introduced mutations in the catalytic triad of DENV protease, S135A and H51A, that have been reported to impair protease activity (22, 55), and we observed that all the mutations introduced impaired the NS2B3 inhibitory effect (Fig. 7A), suggesting an important role for the protease activity of the NS2B3 protein as an immune antagonist. We further investigated this effect, and we observed that the expression of just the protease domain (NS2B3pro, consisting of the 40 last amino acids of NS2B and the 180 first amino acids of NS3 [55]) was enough to reduce IFN-β promoter activity, and this inhibitory effect was abolished when the S135A mutation was introduced into this protease domain construct (Fig. 7A). In all cases, the impaired protease activity of the mutated constructs was confirmed by analyzing the absence of NS2B3 self-processing by Western blots (Fig. 7B). To discard that induction of apoptosis by NS2B3, as has been shown for other flaviviruses proteases (41, 42), could play a role in the inhibitory effect

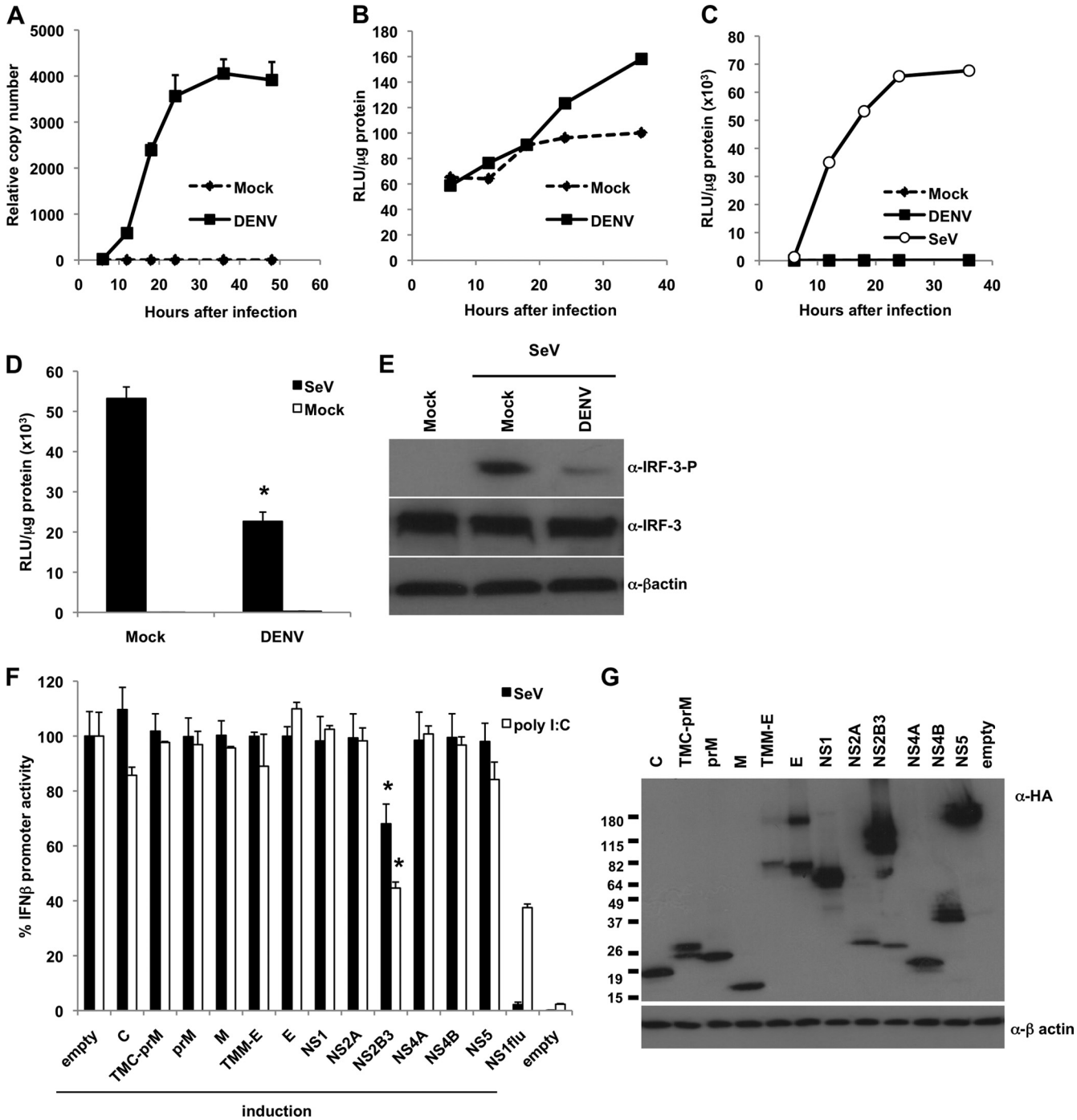

FIG. 6. DENV infection inhibits type I IFN production in 293T cells mediated by NS2B3 protein complex. The ability of DENV to inhibit IFN-α/β production was tested in a reporter assay using 293T-IFN-β-Luc cells. (A) Replication of DENV after infection of 293T-IFN-β-Luc cells with a MOI of 1. (B and C) Luciferase levels were measured different times after infection of 293T-IFN-β-Luc cells with DENV at a MOI of 1 (B) or with SeV (C). (D) 293T-IFN-β-Luc cells were infected with DENV at a MOI of 1, and 24 h later, the same cells were infected with SeV at the same MOI. Samples were collected, and IFN-β promoter activation was measured by luciferase analysis 18 h after secondary infection. (E) Analysis by Western blotting of the phosphorylated and total levels of IRF-3 in 293T-IFN-β-Luc cells infected as described above. (F) Type I IFN production antagonist assay. 293T cells were transfected with mammalian expression plasmids coding for single DENV proteins and a reporter plasmid expressing a luciferase gene under the control of the IFN-β promoter. A plasmid expressing human TLR3 was included for TLR3-mediated IFN-β induction. IFN-β production was triggered by SeV infection or poly(I:C) (25 μg/ml) treatment, and IFN-β promoter activation was measured by determining luciferase expression. (G) Western blot analysis of the expression of the different DENV proteins 24 h after transfection of 293T cells. Error bars represent standard deviations of data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

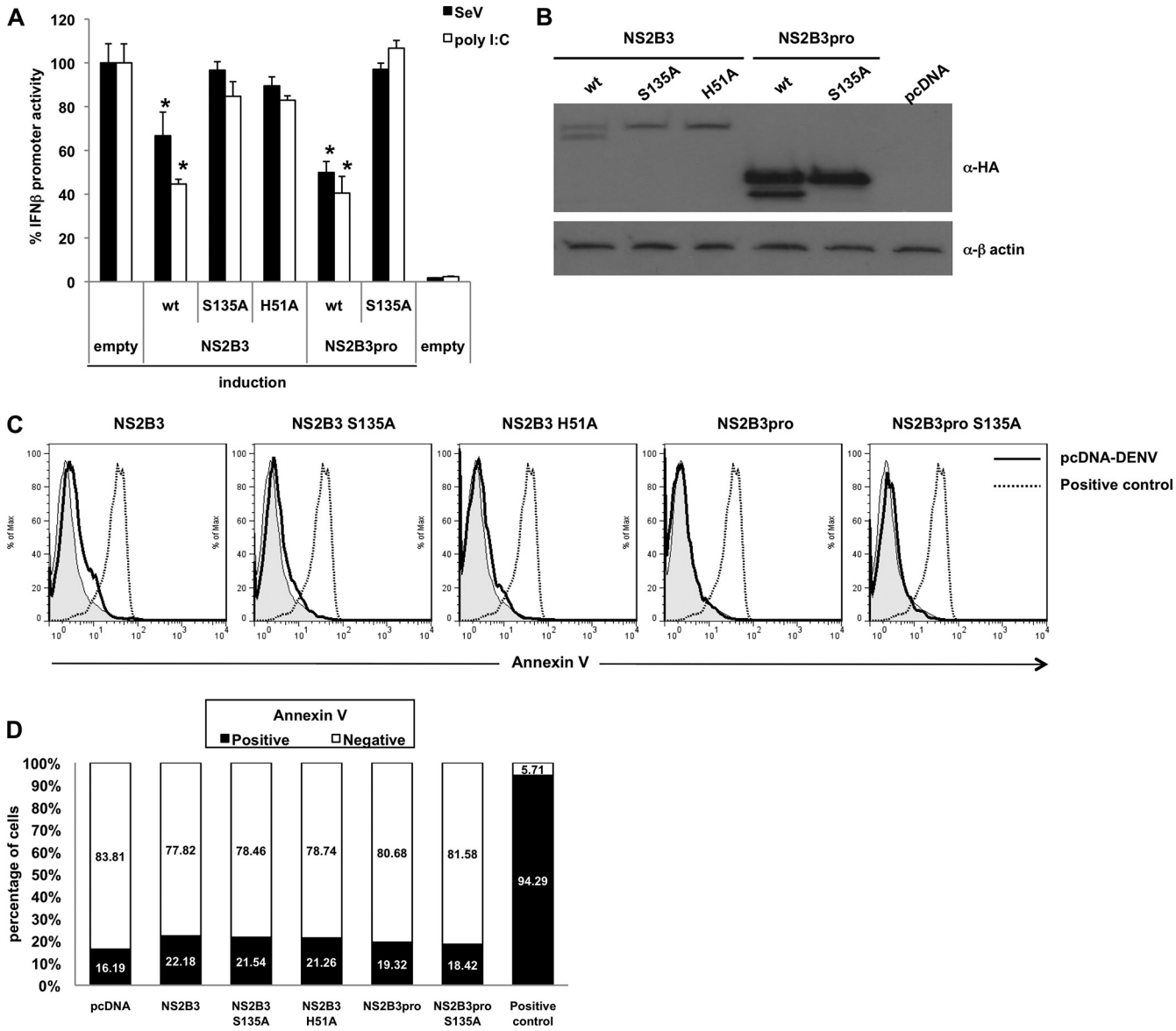

FIG. 7. Catalytically active DENV NS2B3 protease domain is required for inhibition of type I IFN production. (A) Analysis of the effect that mutations in the catalytic triad of the DENV NS2B3 protease complex and protease domain (NS2B3pro) had on IFN-β promoter activation. 293T cells were transfected as described in Methods with mutated versions of the NS2B3 protease complex (NS2B3 S135A and H51A) or the protease domain (NS2B3pro S135A), and IFN-β production was induced by SeV infection or poly(I:C) treatment; luciferase analysis was performed as already described. wt, wild type. (B) Confirmation of impaired protease activity of mutated NS2B3 protein complex and NS2B3 protease domain. (C and D) Analysis by flow cytometry (C) or quantification (D) of apoptotic cells by annexin V staining 24 h after transfection of 293T cells with the different constructs expressing the DENV NS2B3 protease complex (thick black line). A positive control (dotted line) of induction of apoptosis an anti-CD95 (FAS) antibody and a mock-transfected group (thin line and filled plot) were included.

observed at the IFN-β promoter level, we analyzed the levels of apoptotic cells (annexin V-positive cells) in 293T cells transfected with the mentioned NS2B3-expressing plasmids (Fig. 7C). The levels of transfection were similar with all plasmids coding for NS2B3 constructs, reaching 80 to 85% of transfected cells (data not shown). After quantification, no differences were observed within the groups with low levels of annexin V-positive cells, reaching about 20% of apoptotic cells (Fig. 7D). All together, these data strongly demonstrate a predominant role for the protease domain of DENV NS2B3 in the inhibition of type I IFN induction.

DISCUSSION

Since DCs are an important link between the innate and adaptive immune responses in humans (3, 13, 23) and they have been described as target cells for DENV infection *in vivo*, we studied the effect that DENV infection had on type I IFN

production in human monocyte-derived DCs. There is some evidence about how DENV evades immune responses, in particular, type I IFN signaling (2, 31, 32). However, little is known about how DENV evades innate immune responses in primary human immune cells in order to produce disease in humans. We recently reported that DENV-infected human DCs did not produce type I IFN, resulting in an impaired ability of DCs to prime T cells (44). Since most of the studies looking at type I IFN production with DENV have been done using low MOIs (20, 49), here we further investigated the effect that the dose of DENV could have on IFN-α/β production. We observed a complete inhibition of IFN-α/β production in DENV-infected DCs at both the RNA and protein levels (Fig. 1C, D, and E) even when large amounts of DENV were used and almost 80% of the cells were infected (Fig. 1A). We observed a similar result after infection of 293T-IFN-β-Luc cells with DENV, where DENV was able to replicate efficiently without the activation of the IFN-β promoter (Fig. 6A to C). These results clearly indicate that DENV blocks or does not induce the IFN pathway in infected cells. Several viruses, including some flaviviruses, have been reported to block IFN production in infected cells as an efficient immune evasion mechanism by interfering with the pathway at different levels (4, 9, 51). However, there is still little evidence about how DENV could be interrupting this pathway. Our recent report showed a lack of IRF-3 phosphorylation after DENV infection of human DCs (44), providing an explanation for the absence of IFN-α/β production seen in those cells.

To further investigate if DENV infection induced a general inhibition of the type I IFN pathway in DCs, we here used NDV and other well-characterized strong stimuli as a trigger of IFN-α/β induction. The fact that NDV was able to infect both DENV-infected and neighboring noninfected DCs (Fig. 3) provides further evidence about the absence of IFN-α/β production after DENV infection of those DCs, since IFN-α/β production would generate an antiviral state, especially in neighboring noninfected cells, where DENV is not present to antagonize IFN signaling, that would impair subsequent infections by other viruses, such as NDV (4, 51). Interestingly, DENV-preinfected DCs supported NDV replication better than mock-preinfected DCs, although the number of NDV-infected cells did not change (Fig. 3). This replication enhancement might be due to an IFN antagonist effect of DENV in those cells, including the absence of type I IFN production and the effects of the DENV NS4B and NS5 proteins in the prevention of the antiviral state by antagonism of IFN-dependent JAK-STAT signaling (2, 31, 33, 34). This finding could have implications for the susceptibility of DENV-infected patients to other blood pathogens, such as HIV or hepatitis C virus (HCV), since a lack of IFN production in those cells may allow a secondary infection to progress more efficiently. The observation that type I IFN gene expression is reduced in DENV-infected DCs and also in 293T cells (Fig. 2A and B and 6D) after triggering of IFN-α/β production clearly indicates that DENV infection interferes with this pathway. Also supporting this observation is the requirement of DENV replication for this inhibition, since UV inactivation of DENV totally abolishes this inhibition (Fig. 2B). Since this inhibition was observed after IFN-α/β induction by different pathways (Fig. 2F), including RIG-I, MDA5, and TLR3, it could indicate that DENV interferes with these pathways by targeting a common component or that DENV may encode additional IFN antagonists targeting each one of those pathways at different levels, as has been described for the WNV E, NS1, and NS2A proteins (9). In general, virally encoded proteins may have several functions, and the same viral immune antagonist can interfere with several pathways (4, 51). As an example, the influenza A NS1 protein has multiple functions, such as the inhibition of the type I IFN system in infected cells, binding and sequestration of dsRNA, interference with host mRNA processing, facilitation of preferential viral mRNA translation, and inhibition of DC activation (12).

Our data showing DENV interference with IFN-α/β production in infected DCs even at the IFN-α/β RNA level (Fig. 2A) suggest that DENV infection interferes with the type I IFN production pathway at an upstream step before the induction of gene expression and not at the protein level. The reduction of IRF-3 phosphorylation observed after NDV infection in previously DENV-infected DCs compared to results for only NDV-infected ones (Fig. 2C) or in DENV-infected 293T cells after SeV infection (Fig. 6E) supports this hypothesis. Since some DCs exposed to DENV were not infected with DENV but were subsequently infected with NDV (Fig. 3D), which is able to induce a strong IRF-3 phosphorylation (44), it may be difficult to show a strong reduction of phosphorylated IRF-3 after NDV infection of previously DENV-infected DCs by Western blotting. Also, it might be difficult to distinguish the contribution to the IRF-3 phosphorylation of each population present in the group of doubly infected DCs. Actually, the 37% reduction observed after quantification of the Western blot densitometry should correspond to the number of DCs that are coinfected with the two viruses (Fig. 3D). If we assume that equal levels of NDV infection induce similar levels of IRF-3 phosphorylation, the difference observed in IRF-3 phosphorylation between NDV-infected DCs and DENV-NDV-infected DCs cannot be due to a difference in NDV infection levels, since the percentages of NDV-infected cells are similar (Fig. 3D). Thus, the reduction must be taking place in doubly DENV- and NDV-infected DCs, which are around one-third of the total NDV-infected DCs. Several attempts to address this issue by isolating RNA from sorted cells proved to be unsuccessful due to the poor quality of the RNA after such an aggressive procedure, in which cells needed to also be permeabilized for DENV-specific staining. Nevertheless, we have shown a dramatic reduction of IRF-3 phosphorylation in DENV-infected 293T cells subsequently infected with SeV compared to results for mock-infected 293T cells after SeV secondary infection (Fig. 6E). This strong reduction can probably be related to the fact that DENV is able to infect around 80% of the 293T cells, increasing the amount of cells that would be able to interfere with the induction of type I IFN elicited by SeV infection.

Furthermore, we provide evidence that DENV-infected DCs are the cells directly involved in this inhibition of type I IFN production. First, increasing the MOI of DENV to infect DCs correlated with a higher reduction of IFN-α/β levels after NDV infection. Although the inhibition seen after infection with a MOI of 25 did not differ from that seen with five times less DENV (Fig. 2D), these results were in accordance with the levels of infectivity, where no difference in the number of infected cells was observed with those two MOIs (Fig. 1A). Second, the reduction of type I IFN gene expression was not

observed in bystander cells, using a transwell system of paracrine activation of DCs (Fig. 4) (7). We did not observe differences in IFN-α/β gene expression or protein production after triggering of the IFN pathway with NDV in noninfected DCs cultured in transwell plates sharing the medium with DENV-infected DCs (Fig. 4D and E). Thus, we have demonstrated that the inhibition of type I IFN production by DENV occurs only in DCs directly infected with DENV. These results, together with the observation that DENV replication was required for this inhibitory effect, provide some evidence indicating that the reduction in IFN-α/β gene expression after NDV infection in human DCs is mediated by DENV and/or a replication-dependent DENV-induced cellular factor. The inhibition of type I IFN observed when NDV infection was performed 2 h after DENV infection (Fig. 2E) could be mediated by DENV proteins, since DENV replication has been reported to take place as early as 3 h after infection (27). Thus, because protein translation is required for RNA replication, DENV viral proteins have to be expressed earlier, and thus, a minimum 2-h lapse between infections is needed.

In order to investigate the possible mechanism developed by DENV to interfere with type I IFN production, we tested the potential IFN-α/β antagonist activity of several DENV proteins expressed individually using two different systems. First, DENV proteins were expressed from an NDV vector in human DCs (Fig. 5), and second, they were expressed from a mammalian expression vector in 293T cells (Fig. 6F). The 35% inhibition observed in IFN-β promoter activity in transfected 293T cells achieved by the NS2B3 protein complex (Fig. 6F) after induction of IFN-β production through cytosolic receptors (SeV induction) or TLR3 [poly(I:C) induction] correlates with the inhibition observed after infection with an NDV vector expressing the same construct in DCs (Fig. 5D) and with the inhibition observed after SeV infection of DENV-infected DCs and 293T cells (Fig. 2F and 6D). Both NDV and SeV are very strong IFN inducers in DCs, and thus, the potential IFN antagonist of DENV may not be able to completely block such a robust induction. Recently it has been found that DENV replication and assembly are likely taking place in DENV-induced vesicles derived from the ER (52), which could disguise the RNA from detection, leading to poor IFN induction that could be antagonized by a weak antagonist. The lower inhibition observed when the protease complex was expressed from NDV in comparison with the inhibition observed when the cells were previously infected with DENV and then challenged with NDV (compare Fig. 5D with Fig. 2A) could be explained by the earlier expression of DENV proteins in the latter system, probably before IFN-α/β production triggered by NDV infection. However, during NDV-NS2B3 infection, IFN-α/β production is induced at the same time as or earlier than protein expression, since NDV is a negative-strand virus. Additionally, the requirement of other proteins, specific protein processing, and/or DENV factors in order to achieve an inhibition such as the one observed with DENV in both DCs and 293T cells cannot be discarded (Fig. 2A and 6D). As an example, a specific protein processing and maturation requirement has been described to be critical for NS5 IFN antagonist function (2). Nevertheless, the fact that mutations in the catalytic site of the DENV NS2B3 protease complex impaired the inhibitory effect on type I IFN production and that the expression of the protease domain alone maintained that inhibition strongly demonstrates a predominant role for this protease domain. It is possible that cleavage of some factor related to the IFN production pathway by the NS2B3 protease complex is required for the inhibition of type I IFN production. This type of viral evasion strategy has been described for other viruses of the flaviviridae family, like HCV, whose protease is able to cleave the IPS-1 adaptor protein (25, 29).

Since it has been reported that expression of NS2B3 from other flaviviruses can induce apoptosis (41, 42), we performed some experiments to investigate if DENV NS2B3 induced apoptosis and if that may have some effect on the inhibition of type I IFN by NS2B3. None of the cells infected or transfected with vectors coding the NS2B3 protein complex or its mutants showed higher levels of apoptosis than the ones transfected with the controls (Fig. 5E and 7D).

In summary, this work provides new evidences regarding the mechanisms that DENV has developed to evade the immune response in humans. We have clearly demonstrated that DENV infection interferes with the production of type I IFN in human DCs, resulting in a reduction in the IFN-α/β gene expression mediated by a catalytically active NS2B3 protease complex. Also, this inhibition of type I IFN production is DENV replication dependent and without a bystander effect. These data strongly support the hypothesis that DENV is able to manipulate DCs in order to establish infection in humans. The inhibition of type I IFN production by infected DCs is likely to result in the generation of inefficient adaptive immune responses to this virus in humans and to facilitate subsequent infections by other viruses that may be sensitive to the antiviral effects of type I IFN.

ACKNOWLEDGMENTS

We thank Adolfo García-Sastre for invaluable suggestions and comments. We also thank Christopher F. Basler, Maudry Laurent-Rolle, and Cristian Smerdou for protocols and reagents and all the members of the Fernandez-Sesma laboratory for suggestions and comments.

This work was funded by 1R01AI073450 (to A.F.-S.) and a Ramon Areces Foundation Fellowship (to J.R.R.-M.).

J.R.R.-M. designed and performed experiments, analyzed data, and wrote the manuscript. A.B.-V. and D.B.-R. performed experiments. J. Ashour and J. Ayllon provided reagents. A.F.-S. designed experiments, analyzed data, wrote the manuscript, and supervised the project.

We have no competing financial interests to declare.

REFERENCES

1. **Akira, S., S. Uematsu, and O. Takeuchi.** 2006. Pathogen recognition and innate immunity. Cell **124:**783–801.
2. **Ashour, J., M. Laurent-Rolle, P. Y. Shi, and A. Garcia-Sastre.** 2009. NS5 of dengue virus mediates STAT2 binding and degradation. J. Virol. **83:**5408–5418.
3. **Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka.** 2000. Immunobiology of dendritic cells. Annu. Rev. Immunol. **18:**767–811.
4. **Basler, C. F., and A. Garcia-Sastre.** 2002. Viruses and the type I interferon antiviral system: induction and evasion. Int. Rev. Immunol. **21:**305–337.
5. **Basler, C. F., X. Wang, E. Muhlberger, V. Volchkov, J. Paragas, H. D. Klenk, A. Garcia-Sastre, and P. Palese.** 2000. The Ebola virus VP35 protein functions as a type I IFN antagonist. Proc. Natl. Acad. Sci. U. S. A. **97:**12289–12294.
6. **Baum, A., and A. Garcia-Sastre.** 2010. Induction of type I interferon by RNA viruses: cellular receptors and their substrates. Amino Acids **38:**1283–1299.
7. **Borderia, A. V., B. M. Hartmann, A. Fernandez-Sesma, T. M. Moran, and S. C. Sealfon.** 2008. Antiviral-activated dendritic cells: a paracrine-induced response state. J. Immunol. **181:**6872–6881.
8. **Clyde, K., J. L. Kyle, and E. Harris.** 2006. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J. Virol. **80:**11418–11431.

9. **Diamond, M. S.** 2009. Mechanisms of evasion of the type I interferon antiviral response by flaviviruses. J. Interferon Cytokine Res. **29:**521–530.
10. **Diamond, M. S., D. Edgil, T. G. Roberts, B. Lu, and E. Harris.** 2000. Infection of human cells by dengue virus is modulated by different cell types and viral strains. J. Virol. **74:**7814–7823.
11. **Diamond, M. S., T. G. Roberts, D. Edgil, B. Lu, J. Ernst, and E. Harris.** 2000. Modulation of Dengue virus infection in human cells by alpha, beta, and gamma interferons. J. Virol. **74:**4957–4966.
12. **Fernandez-Sesma, A.** 2007. The influenza virus NS1 protein: inhibitor of innate and adaptive immunity. Infect. Disord. Drug Targets **7:**336–343.
13. **Fernandez-Sesma, A., S. Marukian, B. J. Ebersole, D. Kaminski, M. S. Park, T. Yuen, S. C. Sealfon, A. Garcia-Sastre, and T. M. Moran.** 2006. Influenza virus evades innate and adaptive immunity via the NS1 protein. J. Virol. **80:**6295–6304.
14. **Gale, M., Jr., and G. C. Sen.** 2009. Viral evasion of the interferon system. J. Interferon Cytokine Res. **29:**475–476.
15. **Garcia-Sastre, A., and C. A. Biron.** 2006. Type 1 interferons and the virus-host relationship: a lesson in detente. Science **312:**879–882.
16. **Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster.** 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology **252:**324–330.
17. **Gubler, D. J.** 1998. Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev. **11:**480–496.
18. **Hidmark, A. S., G. M. McInerney, E. K. Nordstrom, I. Douagi, K. M. Werner, P. Liljestrom, and G. B. Karlsson Hedestam.** 2005. Early alpha/beta interferon production by myeloid dendritic cells in response to UV-inactivated virus requires viral entry and interferon regulatory factor 3 but not MyD88. J. Virol. **79:**10376–10385.
19. **Ho, L. J., L. F. Hung, C. Y. Weng, W. L. Wu, P. Chou, Y. L. Lin, D. M. Chang, T. Y. Tai, and J. H. Lai.** 2005. Dengue virus type 2 antagonizes IFN-alpha but not IFN-gamma antiviral effect via down-regulating Tyk2-STAT signaling in the human dendritic cell. J. Immunol. **174:**8163–8172.
20. **Ho, L. J., J. J. Wang, M. F. Shaio, C. L. Kao, D. M. Chang, S. W. Han, and J. H. Lai.** 2001. Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. J. Immunol. **166:**1499–1506.
21. **Jones, M., A. Davidson, L. Hibbert, P. Gruenwald, J. Schlaak, S. Ball, G. R. Foster, and M. Jacobs.** 2005. Dengue virus inhibits alpha interferon signaling by reducing STAT2 expression. J. Virol. **79:**5414–5420.
22. **Khumthong, R., C. Angsuthanasombat, S. Panyim, and G. Katzenmeier.** 2002. In vitro determination of dengue virus type 2 NS2B-NS3 protease activity with fluorescent peptide substrates. J. Biochem. Mol. Biol. **35:**206–212.
23. **Le Bon, A., and D. F. Tough.** 2002. Links between innate and adaptive immunity via type I interferon. Curr. Opin. Immunol. **14:**432–436.
24. **Li, W. X., H. Li, R. Lu, F. Li, M. Dus, P. Atkinson, E. W. Brydon, K. L. Johnson, A. Garcia-Sastre, L. A. Ball, P. Palese, and S. W. Ding.** 2004. Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. Proc. Natl. Acad. Sci. U. S. A. **101:**1350–1355.
25. **Li, X. D., L. Sun, R. B. Seth, G. Pineda, and Z. J. Chen.** 2005. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc. Natl. Acad. Sci. U. S. A. **102:**17717–17722.
26. **Lin, R. J., C. L. Liao, E. Lin, and Y. L. Lin.** 2004. Blocking of the alpha interferon-induced Jak-Stat signaling pathway by Japanese encephalitis virus infection. J. Virol. **78:**9285–9294.
27. **Lindenbach, B. D., and C. M. Rice.** 2003. Molecular biology of flaviviruses. Adv. Virus Res. **59:**23–61.
28. **Loo, Y. M., J. Fornek, N. Crochet, G. Bajwa, O. Perwitasari, L. Martinez-Sobrido, S. Akira, M. A. Gill, A. Garcia-Sastre, M. G. Katze, and M. Gale, Jr.** 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J. Virol. **82:**335–345.
29. **Loo, Y. M., D. M. Owen, K. Li, A. K. Erickson, C. L. Johnson, P. M. Fish, D. S. Carney, T. Wang, H. Ishida, M. Yoneyama, T. Fujita, T. Saito, W. M. Lee, C. H. Hagedorn, D. T. Lau, S. A. Weinman, S. M. Lemon, and M. Gale, Jr.** 2006. Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. Proc. Natl. Acad. Sci. U. S. A. **103:**6001–6006.
30. **Lopez, C. B., B. Moltedo, L. Alexopoulou, L. Bonifaz, R. Flavell, and T. M. Moran.** 2004. Toll like receptor-Independent induction of dendritic cell maturation and adaptive immunity by negative-strand RNA viruses. J. Immunol. **173:**6882–6889.
31. **Mazzon, M., M. Jones, A. Davidson, B. Chain, and M. Jacobs.** 2009. Dengue virus NS5 inhibits interferon-alpha signaling by blocking signal transducer and activator of transcription 2 phosphorylation. J. Infect. Dis. **200:**1261–1270.
32. **Munoz-Jordan, J. L.** 2010. Subversion of interferon by dengue virus. Curr. Top. Microbiol. Immunol. **338:**35–44.
33. **Munoz-Jordan, J. L., M. Laurent-Rolle, J. Ashour, L. Martinez-Sobrido, M. Ashok, W. I. Lipkin, and A. Garcia-Sastre.** 2005. Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J. Virol. **79:**8004–8013.
34. **Munoz-Jordan, J. L., G. G. Sanchez-Burgos, M. Laurent-Rolle, and A. Garcia-Sastre.** 2003. Inhibition of interferon signaling by dengue virus. Proc. Natl. Acad. Sci. U. S. A. **100:**14333–14338.
35. **Nakaya, T., J. Cros, M. S. Park, Y. Nakaya, H. Zheng, A. Sagrera, E. Villar, A. Garcia-Sastre, and P. Palese.** 2001. Recombinant Newcastle disease virus as a vaccine vector. J. Virol. **75:**11868–11873.
36. **Navarro-Sanchez, E., R. Altmeyer, A. Amara, O. Schwartz, F. Fieschi, J. L. Virelizier, F. Arenzana-Seisdedos, and P. Despres.** 2003. Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. EMBO Rep. **4:**723–728.
37. **Nightingale, Z. D., C. Patkar, and A. L. Rothman.** 2008. Viral replication and paracrine effects result in distinct, functional responses of dendritic cells following infection with dengue 2 virus. J. Leukoc. Biol. **84:**1028–1038.
38. **Palmer, D. R., P. Sun, C. Celluzzi, J. Bisbing, S. Pang, W. Sun, M. A. Marovich, and T. Burgess.** 2005. Differential effects of dengue virus on infected and bystander dendritic cells. J. Virol. **79:**2432–2439.
39. **Park, M. S., M. L. Shaw, J. Munoz-Jordan, J. F. Cros, T. Nakaya, N. Bouvier, P. Palese, A. Garcia-Sastre, and C. F. Basler.** 2003. Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. J. Virol. **77:**1501–1511.
40. **Park, M. S., J. Steel, A. Garcia-Sastre, D. Swayne, and P. Palese.** 2006. Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease. Proc. Natl. Acad. Sci. U. S. A. **103:**8203–8208.
41. **Prikhod'ko, G. G., E. A. Prikhod'ko, A. G. Pletnev, and J. I. Cohen.** 2002. Langat flavivirus protease NS3 binds caspase-8 and induces apoptosis. J. Virol. **76:**5701–5710.
42. **Ramanathan, M. P., J. A. Chambers, P. Pankhong, M. Chattergoon, W. Attatippaholkun, K. Dang, N. Shah, and D. B. Weiner.** 2006. Host cell killing by the West Nile virus NS2B-NS3 proteolytic complex: NS3 alone is sufficient to recruit caspase-8-based apoptotic pathway. Virology **345:**56–72.
43. **Randall, R. E., and S. Goodbourn.** 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. **89:**1–47.
44. **Rodriguez-Madoz, J. R., D. Bernal-Rubio, D. Kaminski, K. Boyd, and A. Fernandez-Sesma.** 17 February 2010. Dengue virus inhibits the production of type I interferon in primary human dendritic cells. J. Virol. doi:10.1128/JVI.02514-09.
45. **Rodriguez-Madoz, J. R., J. Prieto, and C. Smerdou.** 2005. Semliki forest virus vectors engineered to express higher IL-12 levels induce efficient elimination of murine colon adenocarcinomas. Mol. Ther. **12:**153–163.
46. **Schulz, O., A. Pichlmair, J. Rehwinkel, N. C. Rogers, D. Scheuner, H. Kato, O. Takeuchi, S. Akira, R. J. Kaufman, and C. Reis e Sousa.** 2010. Protein kinase R contributes to immunity against specific viruses by regulating interferon mRNA integrity. Cell Host Microbe **7:**354–361.
47. **Shaw, M. L., W. B. Cardenas, D. Zamarin, P. Palese, and C. F. Basler.** 2005. Nuclear localization of the Nipah virus W protein allows for inhibition of both virus- and toll-like receptor 3-triggered signaling pathways. J. Virol. **79:**6078–6088.
48. **Smerdou, C., and P. Liljestrom.** 1999. Two-helper RNA system for production of recombinant Semliki forest virus particles. J. Virol. **73:**1092–1098.
49. **Sun, P., S. Fernandez, M. A. Marovich, D. R. Palmer, C. M. Celluzzi, K. Boonnak, Z. Liang, H. Subramanian, K. R. Porter, W. Sun, and T. H. Burgess.** 2009. Functional characterization of ex vivo blood myeloid and plasmacytoid dendritic cells after infection with dengue virus. Virology **383:**207–215.
50. **Tassaneetrithep, B., T. H. Burgess, A. Granelli-Piperno, C. Trumpfheller, J. Finke, W. Sun, M. A. Eller, K. Pattanapanyasat, S. Sarasombath, D. L. Birx, R. M. Steinman, S. Schlesinger, and M. A. Marovich.** 2003. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J. Exp. Med. **197:**823–829.
51. **Weber, F., and O. Haller.** 2007. Viral suppression of the interferon system. Biochimie **89:**836–842.
52. **Welsch, S., S. Miller, I. Romero-Brey, A. Merz, C. K. Bleck, P. Walther, S. D. Fuller, C. Antony, J. Krijnse-Locker, and R. Bartenschlager.** 2009. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell Host Microbe **5:**365–375.
53. **WHO.** 1997. Dengue hemorragic fever: diagnosis, treatment and control, 2nd ed. WHO, Geneva, Switzerland.
54. **Wu, S. J., G. Grouard-Vogel, W. Sun, J. R. Mascola, E. Brachtel, R. Putvatana, M. K. Louder, L. Filgueira, M. A. Marovich, H. K. Wong, A. Blauvelt, G. S. Murphy, M. L. Robb, B. L. Innes, D. L. Birx, C. G. Hayes, and S. S. Frankel.** 2000. Human skin Langerhans cells are targets of dengue virus infection. Nat. Med. **6:**816–820.
55. **Yusof, R., S. Clum, M. Wetzel, H. M. Murthy, and R. Padmanabhan.** 2000. Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids in vitro. J. Biol. Chem. **275:**9963–9969.
56. **Zhou, H., N. J. Singh, and K. S. Kim.** 2006. Homology modeling and molecular dynamics study of West Nile virus NS3 protease: a molecular basis for the catalytic activity increased by the NS2B cofactor. Proteins **65:**692–701.