Rotavirus NSP1 Protein Inhibits Interferon-Mediated STAT1 Activation

Adrish Sen,^{a,b,d} Lusijah Rott,^d Nguyen Phan,^{a,b,d} Gourab Mukherjee,^c Harry B. Greenberg^{a,b,d}

Departments of Microbiology and Immunology,^a Medicine,^b and Statistics,^c Stanford University, Stanford, California, USA; VA Palo Alto Health Care System, Palo Alto, California, USA^d

Rotavirus (RV) replicates efficiently in intestinal epithelial cells (IECs) *in vivo* despite the activation of a local host interferon (IFN) response. Previously, we demonstrated that homologous RV efficiently inhibits IFN induction in single infected and bystander villous IECs *in vivo*. Paradoxically, RV also induces significant type I IFN expression in the intestinal hematopoietic cell compartment in a relatively replication-independent manner. This suggests that RV replication and spread in IECs must occur despite exogenous stimulation of the STAT1-mediated IFN signaling pathway. Here we report that RV inhibits IFN-mediated STAT1 tyrosine 701 phosphorylation in human IECs *in vitro* and identify RV NSP1 as a direct inhibitor of the pathway. Infection of human HT29 IECs with simian (RRV) or porcine (SB1A or OSU) RV strains, which inhibit IFN induction by targeting either IFN regulatory factor 3 (IRF3) or NF- κ B, respectively, resulted in similar regulation of IFN secretion. By flow cytometric analysis at early times during infection, neither RRV nor SB1A effectively inhibited the activation of Y701-STAT1 in response to exogenously added IFN. However, at later times during infection, both RV strains efficiently inhibited IFN-mediated STAT1 activation within virus-infected cells, indicating that RV encodes inhibitors of IFN signaling targeting STAT1 phosphorylation. Expression of RV NSP1 in the absence of other viral proteins resulted in blockage of exogenous IFN-mediated STAT1 phosphorylation, and this function was conserved in NSP1 from simian, bovine, and murine RV strains. Analysis of NSP1 determinants responsible for the inhibition of IFN induction and signaling pathways revealed that these determinants are encoded on discrete domains of NSP1. Finally, we observed that at later times during infection with SB1A, there was almost complete inhibition of IFN-mediated Y701-STAT1 in bystander cells staining negative for viral antigen. This property segregated with the NSP1 gene and was observed in a simian SA11 monoreassortant that encoded porcine OSU NSP1 but not in wild-type SA11 or a reassortant encoding simian RRV NSP1.

Viral infection of host cells activates a potent innate immune response that, unless actively subverted by the invading virus, results in the establishment of an antiviral state capable of restricting viral replication and subsequent spread to other host cells (1–3). In most nonimmune cells, this antiviral state is characterized by activation of a transcriptional program that leads to the expression of hundreds of antiviral genes (4). Type I interferons (IFNs), which are secreted from infected cells and bind their cognate receptors in both an autocrine and paracrine manner, are early critical mediators of this host antiviral program in most cell types. The presence of virus in host cells is detected by a variety of distinct membrane-bound or cytoplasmic pattern recognition receptors (PRRs) that bind to pathogen-associated molecular patterns (PAMPs) including double-stranded RNA (dsRNA) and the phosphate and methyl moieties on viral RNA (2). Ligand-activated PRRs mediate the assembly of signaling complexes that ultimately result in the activation of the transcription factors IFN regulatory factor 3 (IRF3) and NF- κ B, both of which are required for the induction of type I IFNs (5). During this early phase of infection, IRF3 and NF- κ B activated by the presence of virus also mediate the transcription of several unique and overlapping sets of virus stress-induced genes (VSIGs) (6). Interferon secreted from these initially infected cells binds and activates IFN receptors, triggering a signaling cascade that results in the expression of numerous interferon-stimulated genes (ISGs) and synthesis of secondary IFN subtypes in activated cells (1, 4). The transcription of a majority of ISGs is critically dependent on IFN receptor-mediated activation of the transcription factor signal transducer and activator of transcription 1 (STAT1) as a result of its recruit-

ment to the IFN receptor and subsequent phosphorylation at a tyrosine residue (Y701) by Janus-activated kinase (JAK) (7). The phosphorylation of STAT1 at Y701 leads to the formation of a heterotrimeric complex of STAT1, STAT2, and IRF9; this complex, called interferon-stimulated gene factor 3 (ISGF3), induces the transcription of antiviral ISGs containing the STAT-responsive interferon-stimulated response element (ISRE) and/or gamma-activated sequence (GAS) promoter elements (8, 9).

Rotavirus (RV) is a nonenveloped icosahedral member of the *Reoviridae* family with a segmented dsRNA genome that encodes a total of six nonstructural and six structural proteins (10). Rotaviruses replicate predominantly in mature villous enterocytes of the small intestine and cause severe dehydrating diarrhea in infants and children below the age of 5 years, accounting for ~450,000 deaths annually (11). In addition to their importance as human pathogens, rotaviruses also infect and cause diarrheal disease in the young of many other mammalian species. One approach to rotavirus vaccine development exploits the natural attenuation of rotaviruses in a heterologous host species (i.e., a species that is not

Received 3 June 2013 Accepted 9 October 2013

Published ahead of print 16 October 2013

Address correspondence to Harry B. Greenberg, harry.greenberg@stanford.edu.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JVI.01501-13>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.01501-13

the usual host) (12). This host range restriction (HRR) is likely to be multifactorial, and several lines of evidence suggest that STAT1-dependent innate immune responses are one of the factors that restrict replication of heterologous RV at intestinal and systemic sites (13–18). In young children, a heterologous simian and two heterologous bovine RV strains are substantially attenuated for replication in the gut (19). In the suckling mouse model, the heterologous simian rotavirus RRV replicates poorly in the gut compared to the homologous murine EW strain ($\sim 10^4$ -fold less well), and this restriction of RRV is significantly alleviated in mice lacking the type I/II IFN receptors or STAT1 ($\sim 10^2$ - to 10^3 -fold increase relative to replication in wild-type mice) (14, 20). In contrast to the IFN-sensitive replication of the heterologous simian RRV, the homologous murine EW RV strain replicates to high titers in the suckling mouse intestine and is only modestly enhanced ($\sim 10^1$ -fold) in the absence of type I/II IFN receptors or STAT1 (14, 20).

As opposed to the IFN-dependent phenotypes of heterologous RV replication *in vivo*, most RV strains studied so far (e.g., simian RRV) are capable of inhibiting the induction of IFN *in vitro* (15, 21, 22). However, certain exceptions have been reported, including the bovine UK rotavirus strain in murine fibroblasts (15, 23) and several isolates of RV that encode truncated NSP1 proteins and form small plaques in interferon-competent cells in culture (24, 25). Regardless of their ability to regulate IFN secretion later during infection, rotavirus infection likely triggers a conserved signaling pathway early in infection, resulting in activation of IRF3, NF- κ B-dependent VSIGs, and possibly IFN itself (26). We and others have found that early during infection, both RRV and UK RVs activate the cytosolic PRRs RIG-I and MDA-5 in a replication-dependent manner, and subsequent signaling mediated through the mitochondrial adaptor MAVS results in induction of several IRF3- and NF- κ B-dependent VSIGs and IFNs (20, 23, 27–33). However, at later times during infection (16 to 24 h postinfection [hpi]), RRV more effectively prevents IFN secretion than UK RV. Unlike UK NSP1, RRV NSP1 degrades murine IRF3 and inhibits its transcriptional activity at a stage following the phosphorylation of IRF3 at its carboxyl terminus (23). The inability of UK NSP1 to degrade murine IRF3 in murine fibroblasts is not an inherent defect in the protein but is dependent on the IRF3 protein encoded by the host cell. UK NSP1 degrades the endogenous IRF3 expressed in simian COS7 cells, and simian IRF3 can also be efficiently degraded by UK NSP1 when transiently expressed in mouse embryonic fibroblasts (MEFs) (23). Other studies have shown that NSP1 degrades additional IRFs, including IRF5 and IRF7 (22, 25), indicating that apart from IRF3-dependent IFN induction, other IRF-specific responses may also be regulated by rotavirus during infection. Previous studies have also described NSP1-mediated inhibition of IFN induction by inhibition of RIG-I (31) and inhibition of poly(I:C)-directed IRF3-dependent transcription in the absence of any IRF3 degradation (23). Thus, the inhibition of IRF3 function by rotaviruses may involve both degradation-dependent and degradation-independent mechanisms.

Interestingly, certain rotavirus strains encode an NSP1 that fails to direct IRF3 degradation or inhibition (22, 29). The NSP1 proteins from several of these strains inhibit IFN induction by targeting the NF- κ B pathway. Graff and coworkers (29) demonstrated that porcine rotavirus OSU NSP1 is unable to degrade IRF3 but interacts with and degrades β -TrCP, an essential cofac-

tor in I κ B- α degradation and consequent NF- κ B activation. The NSP1 protein of still other RV strains may inhibit IFN induction by interacting with beta transducin repeat-containing protein (β -TrCP), but without causing its degradation, in a manner reminiscent of the A49 protein of vaccinia virus (34; M. Morelli and J. T. Patton, personal communication). In addition, certain rotaviruses sequester the NF- κ B p65 subunit in viroplasm during infection (30), potentially blocking NF- κ B signals in a β -TrCP-independent manner. Although the viral factors and mechanisms used in β -TrCP degradation-independent inhibition of NF- κ B transcriptional activity have yet to be determined, it is likely that rotavirus inhibition of NF- κ B-dependent innate signaling is critical for inhibition of the innate immune response in murine villous intestinal epithelial cells (IECs) *in vivo* (20). STAT1-deficient suckling mice can induce IFN normally but lack secondary IFN-mediated responses; in these mice, infection with murine EW results in significantly lower NF- κ B-dependent transcription and increased I κ B- α protein levels in the intestine at 16 hpi compared to infection with simian RRV (20).

Recently, using a single-cell analytical approach, we observed that EW murine RV prevents the induction of IFN transcripts in both infected and bystander villous IECs *in vivo* despite robust IFN induction in resident intestinal hematopoietic cells (20). In IECs from 5-day-old suckling mice infected with the EW strain of murine rotavirus for 16 h, NF- κ B-dependent activation of VSIG transcription was absent, but IRF3-dependent VSIG transcription was detected, indicating that EW regulates NF- κ B activity in the intestinal epithelium. Interestingly, *in vitro*, the EW NSP1 protein effectively degrades IRF3 in fibroblasts to inhibit IFN induction, likely after phosphorylation in the carboxyl-terminal region of IRF3 (23). Thus, rotavirus regulation of IRF3 and NF- κ B may be more complex than previously appreciated and is likely dependent on several factors, including timing during infection and the type of cell infected. Regardless of the manner in which IFN induction is inhibited, the finding that homologous RV replicates in the epithelium of the murine intestine to high levels despite robust IFN induction in local hematopoietic cells argues that (i) rotaviruses likely block antiviral signaling in response to exogenous IFN and (ii) such virus-mediated inhibition likely also occurs in bystander uninfected epithelial cells.

Recently, Holloway et al. (30) demonstrated that early in rotavirus infection, STAT1 function is inhibited by a conserved mechanism that allows STAT1-Y701 activation in response to exogenous IFN-mediated signaling but prevents its subsequent nuclear localization. That study did not identify a role for individually expressed NSP1, NSP3, or NSP4 in RV-mediated STAT1 functional inhibition. Since secondary responsiveness and transcription of ISGs through exogenous stimulation of IFN receptors in both infected (autocrine) and bystander (paracrine) cells are critically dependent on the phosphorylation of STAT1 at Y701, it seemed plausible that rotaviruses not only have evolved functions to inhibit IRF3 and NF- κ B activation but also might effectively prevent IFN-mediated STAT1 signaling in both infected and bystander intestinal epithelial cells. Here we report that rotavirus infection does effectively suppress exogenous IFN-directed STAT1 activation in both infected and bystander IECs late in infection and identify rotavirus NSP1 as an antagonist of IFN-mediated STAT1 Y701 phosphorylation. Our findings help explain the previously observed regulation of the host IFN response in the

intestine *in vivo* (20) and reveal a novel function of NSP1 as a STAT1 antagonist.

MATERIALS AND METHODS

Cells and viruses. Human intestinal epithelial HT29 and monkey kidney COS7 cells were purchased from the American Type Culture Collection (ATCC) and were maintained in Dulbecco's modified Eagle medium (D-MEM; Cellgro) containing 10% fetal calf serum (Invitrogen) supplemented with nonessential amino acids, glutamine, penicillin, and streptomycin (complete D-MEM). Rotavirus strains RRV, OSU, SB1A, and UK were propagated in MA104 cells, and titers were determined by a plaque assay as described previously (15). The simian SA11-4F strain and the SA11 monoreassortants SRF (encoding RRV NSP1) and SOF (encoding OSU NSP1) were generously provided by John Patton (22).

Reagents and antibodies. Poly(I-C) was purchased precomplexed with a transfection reagent [Lyovect poly(I-C); Invivogen]. The following commercial antibodies were obtained (suppliers in parentheses): IRF3 (rabbit monoclonal antibody from Cell Signaling), tubulin (mouse monoclonal from Sigma), actin (mouse monoclonal from Sigma), β -TrCP (rabbit monoclonal from Cell Signaling), phosphorylated IRF3 (pIRF3; S369) (mouse monoclonal from Cell Signaling), FLAG epitope (mouse monoclonal from Sigma), I κ B- α (rabbit monoclonal from Cell Signaling), and secondary horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit polyclonal antibodies (Amersham). Alexa 647-I κ B- α and Alexa 647-pSTAT1(Y701) antibodies used in fluorescence-activated cell sorter (FACS) analyses were purchased from BD Biosciences. The mouse monoclonal antibody to rotavirus VP6 (1E11) was conjugated to Alexa 488 by using a commercial kit (Novus Bio). Purified human IFN- β (4×10^6 U/ml; PBL Interferon Source) and purified human tumor necrosis factor alpha (TNF- α) (50 μ g/ml; Cell Signaling Technologies) were used for stimulation of cells. MG132 (Calbiochem) was used at 10 μ M for 12 h. Plasmids encoding green fluorescent protein (GFP)-NSP1 fusion proteins from RRV, UK, or EW strains were on a GFP-C1 vector backbone and were described previously (23). The RING finger (RF) mutant RRV-C59H63A was cloned by using site-directed mutagenesis (Quikchange; Stratagene), and the sequence was verified prior to use. FLAG-murine IRF3 and -human IRF3 constructs were described previously (33).

Virus infections and transfections. Cells were plated into 6-well or 24-well cluster plates and infected when confluent. Cells were washed three times with D-MEM without additives, and virus was added at the multiplicity of infection (MOI) specified and allowed to adsorb at 37°C for 1 h. Cells were then washed three times, and infection was allowed to proceed in D-MEM lacking serum for the times indicated. In experiments using poly(I-C), Lyovect poly(I-C) was added directly to culture medium at a final concentration of 1 μ g/ml. For transient transfection, HT29 or COS7 cells were seeded into 24-well plates 1 day prior to transfection and transfected with FuGENE 6 (Roche) or with Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocols. GFP-NSP1 expression was detected in cells after 48 h by fluorescence microscopy and analyzed further by FACS, immunoblotting, or luciferase assays.

Immunoblotting and microscopy. Cells were lysed in Laemmli buffer containing 2% SDS and 5% β -mercaptoethanol after two washes in phosphate-buffered saline (PBS) (pH 7.0). Cell lysis was performed at room temperature for 20 min, and lysates were passed through a 25-gauge needle to reduce viscosity. Cell lysates were boiled for 5 min, and tubes were briefly spun at $10,000 \times g$ and separated on 10% SDS-PAGE gels. Following electrophoresis, proteins were transferred onto nitrocellulose membranes (Amersham Biosciences) and detected by using the indicated antibodies. Blots were exposed to autoradiography film (Amersham) and developed by using an enhanced chemiluminescence kit (GE Healthcare). Blots were subsequently stripped and reprobed by using a ReBlot kit according to the manufacturer's instructions (Millipore). For comparison of expression levels of GFP plasmids in COS7 cells, fluorescent foci were

counted in 3 to 5 fields containing equivalent cell densities at a $\times 10$ magnification under an inverted fluorescence microscope (Nikon).

FACS analysis. Cells were plated into 24-well cluster plates and infected with RV or transfected with expression plasmids. Infected cells were harvested at 6 hpi or 16 hpi and transfected cells were harvested at 48 h after transfection for flow cytometry analysis. Cells were washed in PBS and fixed at room temperature for 10 min by using 1.6% (vol/vol) methanol-free paraformaldehyde (Electron Microscopy Services). Cells were washed in FACS staining buffer (PBS containing 0.5% [wt/vol] bovine serum albumin) and permeabilized in cold methanol at 4°C for 15 min. Cells were washed in FACS staining buffer, stained by using conjugated antibodies at 4°C for 30 min, and washed as described above, prior to analysis by flow cytometry on an LSRII or FACSCalibur instrument (BD Biosciences). The flow data were analyzed by using FlowJo software and Cytobank analysis routines. For examination of pSTAT1 changes in different populations, the measured median intensity values were transformed to an arcsinh scale with a cofactor of 150, and the arcsinh ratio of medians (stimulated/control) was calculated. An arcsinh ratio of 0.0 indicates no change, and ratios of -4.6 and $+4.6$ indicate a shift in the mean fluorescence intensity (MFI) from 100,000 to 1,000 or vice versa, as implemented in Cytobank and described previously (35). Heat maps of pSTAT1 signaling changes were derived from HT29 cells infected at an MOI of 1.0 for 6 h or 16 h and stimulated with 500 U/ml of human IFN- β for 20 min. pSTAT1-Y701 signaling changes were expressed as $\log_{10}$ (median intensity IFN stimulated/median intensity unstimulated) (36).

Luciferase assays. COS7 cells in 24-well plates were transfected in duplicate with 4 ng of *Renilla* luciferase reporter (pRL-TK; Promega), 200 ng of the firefly PRD(III) reporter, 150 ng of human IRF3 plasmid as an activator, and 200 ng of GFP or GFP-NSP1 plasmids. The total amount of DNA was normalized by using an empty vector. At 48 h posttransfection, cells were washed in PBS and lysed by using Stop and Glo Dual Luciferase Assay kit reagents as directed by the manufacturer (Promega). *Renilla* and firefly luciferase activities were measured by using a luminometer (Turner Designs), and PRD(III) activation was normalized to *Renilla* luciferase levels. Data are reported as normalized PRD(III) activity.

Interferon measurements. Cell culture supernatants were collected, and secreted human IFN- β levels were measured in duplicate by using the Verikine human IFN- β enzyme-linked immunosorbent assay (ELISA) kit (PBL Interferon Source). Amounts of secreted IFN- β were calculated from a standard curve created by using a human IFN- β standard according to the manufacturer's instructions.

RESULTS

Porcine SB1A and simian RRV strains inhibit NF- κ B and IRF3, respectively. Numerous RV strains, including simian RRV, inhibit IFN induction through degradation of IRF3 in HT29 human IECs (22). In contrast, the porcine OSU strain degrades β -TrCP and not IRF3 (22, 29). Human intestinal epithelial HT29 cells were infected with the porcine rotavirus SB1A strain or the simian rotavirus RRV strain. At 6 hpi, cells were lysed and analyzed by immunoblotting (Fig. 1A). As expected based on previous studies of the OSU strain (22, 29), infection with porcine SB1A resulted in β -TrCP depletion and no changes in levels of IRF3 compared to mock-infected cells. In contrast, RRV infection resulted in decreases in IRF3 and I κ B- α but no significant change in the levels of β -TrCP. Phosphorylation of IRF3 at S396 could be detected following infection with SB1A but not with RRV. These results indicate that SB1A and RRV employ unique NF- κ B- and IRF3-inhibitory strategies, respectively, to counter IFN induction (26).

To extend these findings, HT29 cells were infected with either RRV or SB1A for 6 h and then stimulated with 50 ng/ml TNF- α for 20 min before fixation, permeabilization, and staining for rotavirus VP6 antigen and I κ B- α and subsequent analysis by flow cy-

FIG 1 Rotavirus strains SB1A and RRV inhibit NF-κB and IRF3 pathways, respectively, early during infection of HT29 IECs. (A) HT29 cells were infected with porcine SB1A or simian RRV strains and analyzed by immunoblotting at 6 hpi. (B) HT29 cells were infected for 6 h and stimulated with TNF-α for 30 min prior to staining for levels of rotaviral protein VP6 and IκB-α and analysis by cytometry. FITC, fluorescein isothiocyanate; APC, allophycocyanin. (C) IκB-α data in panel B are plotted as mean fluorescence intensity (MFI) arcsinh ratios. For each panel, unstimulated baseline arcsinh ratios are in black, and fold changes from this value are colored according to the heat map shown at the bottom. The red dashed line indicates the baseline level of IκB-α in unstimulated mock-infected HT29 controls. Results shown are representative of at least 2 independent experiments.

tometry (Fig. 1B). In mock-infected controls, stimulation with TNF-α resulted in a significant decrease in the levels of IκB-α, indicative of NF-κB activation (Fig. 1B and C). Activation of the NF-κB pathway (and a significant decrease in the levels of IκB-α) was also observed in cultures infected with RRV or SB1A after TNF-α stimulation in those cells negative for staining of the RV VP6 antigen (VP6⁻ cells), here referred to as bystander cells (Fig. 1B and C). RRV-infected cells (VP6⁺ cells), in the absence of any stimulation, had low levels of IκB-α, similar to TNF-α-treated mock-infected cells, and treatment with TNF-α did not result in a further decrease in IκB-α levels in RRV-infected (VP6⁺) cells (Fig. 1C). In contrast, no decrease in IκB-α levels was observed in SB1A-infected cells in the absence of TNF-α stimulation (Fig. 1C), in agreement with our immunoblotting results (Fig. 1A). However, SB1A-infected cells (VP6⁺) were able to inhibit IκB-α degradation in response to the exogenous TNF-α signal compared to mock-infected controls (~14% of infected cells were negative for IκB-α, compared to ~68% of mock-infected cells) (Fig. 1B and C). Thus, SB1A differed from RRV in its abilities to prevent IκB-α degradation and inhibit TNF-α-mediated activation of the NF-κB

pathway. Together with results from previous studies (22, 29), these results demonstrate that in HT29 cells, RRV and SB1A inhibit IRF3 and NF-κB activation pathways, respectively, early during infection.

Rotavirus strains inhibit IFN-mediated pSTAT1-Y701 activation late in infection. In order to investigate whether RRV and/or SB1A infections affect the IFN signaling pathway, we first examined the early effect of infection on pSTAT1-Y701 expression in response to exogenous IFN receptor activation at 6 hpi. We used flow cytometry to distinguish regulation of STAT1 phosphorylation (pSTAT1-Y701) within RV-infected and bystander cells, as defined by staining for the VP6 RV antigen. HT29 cells were infected in duplicate at an MOI of 1.0 (low MOI) or 10.0 (high MOI). At 6 hpi, one set of cells from each MOI was stimulated with exogenous IFN-β for 20 min. Cells were then fixed, permeabilized, and stained for both rotavirus VP6 and phosphorylation at Y701-STAT1 (Fig. 2A). SB1A and RRV infection resulted in similar levels of infectivity at 6 hpi, as evidenced by comparable percentages of VP6⁺ cells (RRV, 16 to 22% VP6⁺ cells at low MOI and 96 to 97% VP6⁺ cells at high MOI; SB1A, 24 to 25%

FIG 2 At 6 hpi, rotavirus is unable to inhibit pY701-STAT1 activation in response to exogenous IFN- β stimulation. (A) HT29 cells were infected with porcine SB1A or simian RRV strains at an MOI of 1 or 10 and, at 6 hpi, were stimulated with IFN- β for 20 min prior to staining for levels of RV VP6 and pY701-STAT1 and analysis by cytometry. (B) The pY701-STAT1 data in panel A are plotted as MFI arcsinh ratios of stimulated to unstimulated cells. In each panel, the unstimulated baseline level of pY701-STAT1 in mock-infected HT29 cells is indicated by a dashed red line. Results shown are representative of 3 independent experiments.

VP6⁺ cells at low MOI and 96 to 97% VP6⁺ cells at high MOI (Fig. 2A). In the absence of any exogenous IFN- β stimulation (Fig. 2A, column 1 in each MOI panel), a subpopulation of SB1A-infected cells at 6 hpi contained higher levels of STAT1 phosphorylated on Y701 than did mock-infected controls or bystander cells (~31% of SB1A-VP6⁺ cells at low MOI and ~63% at high MOI were pY701-STAT1⁺) (Fig. 2A). A similar effect on pSTAT1-Y701 levels was observed for unstimulated RRV VP6⁺ cells at the higher MOI (~7% of cells were pY701-STAT1⁺), but STAT1-Y701 activation was considerably weaker with RRV infection than in the case of SB1A. Activation was not observed for SB1A and RRV bystander cells, with the exception of a small number of SB1A bystander cells at the low MOI, ~2.5% of which contained pY701-STAT1 levels above baseline unstimulated levels (Fig. 2A).

In order to examine whether SB1A and RRV could prevent STAT1 activation in response to exogenous IFN-mediated receptor stimulation at 6 hpi, cells were stimulated with purified IFN- β (500 U/ml) for 20 min prior to fixation and analyzed by cytometry (Fig. 2A and B). Mock-infected control cells responded to IFN- β stimulation with phosphorylation of STAT1-Y701, and similar activation was also observed in RRV-infected and RRV bystander cell populations at both MOIs. In SB1A-infected cells, ~31 to 63% of which had elevated levels of pY701-STAT1 in the absence of IFN- β stimulation, treatment with exogenous IFN resulted in an incremental increase in pY701-STAT1 expression at both MOIs, accompanied by a decrease in the percentage of cells belonging to the SB1A-VP6⁺ pY701-STAT1⁻ population. Bystander cells in the SB1A culture also responded to IFN- β at 6 hpi, similar to bystander RRV- or mock-infected control populations. Thus, at 6 hpi, neither SB1A nor RRV induced significant phosphorylation of STAT1 on Y701 in bystander cells, and neither strain effectively blocked pY701-STAT1 activation following exogenous IFN recep-

tor stimulation (see Statistical Analysis 1 in the supplemental material).

Previous studies (23, 27, 33) reported that RV induces an early antiviral response but that expression of viral nonstructural proteins leads to an inhibition of innate responses later during infection. Therefore, we next examined the ability of SB1A and RRV to inhibit IFN signaling later in infection. HT29 cells were infected with SB1A or RRV (MOI of 1) and analyzed by flow cytometry at 16 hpi for levels of rotavirus VP6 antigen and pY701-STAT1. As shown in Fig. 3A, in the absence of exogenous IFN stimulation, neither RV strain induced detectable STAT1 phosphorylation at 16 hpi in bystander VP6⁻ cell populations (Fig. 3A, row 1); levels were similar to those in mock-infected controls. However, at 16 hpi, 2 discrete populations of SB1A-VP6-positive cells could be identified, and approximately one-half (~47%) of all SB1A-positive cells contained significantly higher levels of pY701-STAT1 than either RRV-positive cells or mock-infected controls (90% level of significance for the null hypothesis based on a log-linear model [see Statistical Analysis 1 in the supplemental material]). The presence of 2 distinct populations of SB1A-infected cells differing in their pY701-STAT1 levels was thus observed at both early and later times in infection (Fig. 2A and 3A), indicating that SB1A leads to direct and prolonged STAT1 phosphorylation in the absence of exogenous IFN stimulation, but this occurs only in a subset of infected intestinal cells.

In order to examine whether RV infection can block exogenous IFN-stimulated pY701-STAT1 activation late in infection, SB1A-infected cells, RRV-infected cells, or mock-infected controls were stimulated with increasing doses of purified IFN- β (500 U/ml, 1,000 U/ml, and 2,000 U/ml) for 20 min prior to analysis by cytometry. As shown in Fig. 3A and B, the level of pSTAT1-Y701 increased in uninfected controls in a dose-dependent manner at

FIG 3 At 16 hpi, the indicated rotavirus strains effectively inhibit pY701-STAT1 activation in response to exogenous IFN- β stimulation. (A) HT29 cells were infected with porcine SB1A or simian RRV strains at an MOI of 1.0 and, at 16 hpi, were stimulated with IFN- β at the indicated concentrations for 20 min prior to staining for levels of RV VP6 and pY701-STAT1 and analysis by cytometry. (B) The pY701-STAT1 data in panel A are plotted as MFI arcsinh ratios. In each panel, the unstimulated baseline level of pY701-STAT1 in mock-infected HT29 cells is indicated by a dashed red line. (C) Heat map summarizing the pSTAT1-Y701 signaling changes observed in mock-infected, infected (Inf), and bystander (Bys) cell populations of HT29 cells infected at an MOI of 1.0 and stimulated with 500 U/ml of IFN at either 6 hpi or 16 hpi. Median intensities of pY701 were transformed as described in Materials and Methods to derive fold changes in STAT1-Y701 levels. Results shown are representative of 3 independent experiments. (D) HT29 cells were infected with the indicated RV strains or transfected with poly(I:C), and secreted IFN- β was measured in supernatants at 16 hpi by ELISA (**, $P < 0.005$; ns, nonsignificant [$P > 0.5$]). Results are representative of 2 independent experiments.

500 U/ml and 1,000 U/ml of IFN and did not increase further with 2,000 U/ml of stimulant, likely reflecting that receptor saturation was reached at 1,000 U/ml. In contrast, RRV-infected (VP6⁺) cells efficiently inhibited the activation of STAT1 at all doses of IFN tested. In SB1A-infected (VP6⁺) cells (that were either pY701-STAT1⁺ or pY701-STAT1⁻ in roughly equal ratios in the absence of exogenous IFN), the addition of IFN did not result in an appreciable dose-dependent increase in pY701-STAT1 expression, and the proportion of pSTAT1⁺ to pSTAT1⁻ cells remained fairly constant (~53%, ~48%, and ~47% of SB1A-VP6⁺ cells were pY701-STAT1 negative following IFN stimulation at the 3 doses) (Fig. 3B). Thus, cells infected with SB1A were highly resistant to any further IFN-mediated pY701-STAT1 activation regardless of their basal phosphorylation status of Y701-STAT1. Of note, SB1A cells that were VP6 negative (bystanders) were also almost completely refractory to IFN-mediated STAT1 activation even at the highest dose of IFN used (at 2,000 U/ml of stimulant, only ~4% of all SB1A-VP6⁺ cells were pY701-STAT1⁺, compared to ~62% of all mock-infected control cells). A weaker inhibitory effect on bystanders was also observed for the RRV-infected cultures (~35% of all RRV-VP6⁺ cells were pY701-STAT1 positive). Taken together, these findings demonstrate that both SB1A and RRV in-

hibit IFN receptor-mediated signaling in infected and bystander cell populations late in infection despite their different effects on STAT1 phosphorylation early in infection (Fig. 3C). Of note, despite differences in pY701-STAT1 in unstimulated RRV-VP6⁺ and SB1A-VP6⁺ cells, IFN- β secretion by HT29 cells at 16 h following infection with RRV, SB1A, or another β -TrCP-degrading porcine RV strain, OSU, was not significantly different (Fig. 3D), indicating that both RRV and SB1A regulate IFN- β secretion similarly late in infection.

Comparison of the results from SB1A and RRV infection at 6 hpi and 16 hpi (Fig. 2 and 3C) revealed at least three distinct effects on STAT1: direct inhibition of pY701-STAT1 within infected (VP6⁺) cells (in all infected cells by RRV and in ~50% of infected cells by SB1A late in infection), direct and prolonged activation of pY701-STAT1 in the absence of exogenous IFN stimulation (in a subset of SB1A-infected cells early and late in infection), and remote inhibitory effects on pY701-STAT1 in bystander (VP6⁻) cells late in infection (by SB1A and, to a lesser extent, RRV) (90% level of significance for the null hypotheses based on a log-linear model [see Statistical Analysis 1 in the supplemental material]).

The rotavirus NSP1 protein is an inhibitor of pY701-STAT1 activation in response to IFN receptor stimulation. We next fo-

FIG 4 The rotavirus NSP1 protein is an inhibitor of pY701-STAT1 activation in response to exogenous IFN-β stimulation. (A) COS7 cells were transfected with GFP-NSP1- or GFP-expressing plasmids and with a plasmid expressing FLAG-tagged murine IRF3, and at 48 h posttransfection, cells were analyzed by immunoblotting. (B) HT29 cells were transfected with plasmids expressing GFP-NSP1 from the RRV, UK, or EW RV strain and either treated with MG132 for 12 h at 36 h posttransfection or stimulated with 500 U/ml of IFN-β for 20 min at 48 h posttransfection prior to analysis by cytometry. (C) The pY701-STAT1 data in panel B are plotted as MFI arcsinh ratios. In each panel, the unstimulated baseline level of pY701-STAT1 in GFP-RRV NSP1[−] cells is indicated by a dashed red line. Results are representative of 2 independent experiments.

cused on identifying the RV factor(s) involved in the direct inhibition of interferon-mediated STAT1 phosphorylation that occurs within infected cells late in infection. One potential candidate for the inhibitory effect on STAT1 activation in infected cells is the NSP1 protein, which has been found to cosegregate with the STAT1-dependent replication and virulence phenotypes both *in vitro* and *in vivo* (13, 15, 16). Therefore, we examined whether recombinant NSP1 expressed in the absence of other RV proteins could directly inhibit pY701-STAT1 activation in response to exogenous IFN receptor stimulation. The region encoding the RRV NSP1 protein was cloned into a mammalian expression vector as a C-terminal fusion to GFP and overexpressed by transfection. The GFP-RRV NSP1 fusion protein was functional, as it effectively degraded simian/human and murine IRF3 proteins in COS7 cells (Fig. 4A) in a previously described assay (23). We next used flow cytometry to examine pY701-STAT1 activation in HT29 cells transfected with the GFP-NSP1 construct following IFN-β stimulation. Since HT29 cells are poorly transfectable, flow cytometry allowed us to specifically resolve signaling events within the minority of cells that expressed GFP-NSP1, as indicated by analysis of GFP-positive cells.

As shown in Fig. 4B, expression of GFP-RRV NSP1 in HT29 cells was detected by flow cytometry in ~6 to 7% of cells at 48 h after transfection. Inhibition of the proteasome with MG132 for 8 h prior to analysis resulted in an approximately 3-fold increase in NSP1 levels (Fig. 4B), indicating that the low-level GFP-NSP1 expression was due in part to proteasomal degradation of NSP1. In the absence of exogenous IFN-β stimulation, there was no detectable pY701-STAT1 activation in either GFP-NSP1⁺ or GFP-

NSP1[−] cells. Following MG132 treatment, there was no detectable increase in pY701-STAT1 levels, demonstrating that in resting cells, RRV NSP1 neither activates nor proteasomally degrades pY701-STAT1. When cells were stimulated with IFN-β for 20 min at 48 h after GFP-RRV NSP1 transfection, GFP-NSP1[−] cells (bystander cells) responded to IFN stimulation with a marked increase in pSTAT1-Y701 levels (Fig. 4B and C). In contrast, in cells expressing the GFP-NSP1 protein, IFN-mediated activation of pY701-STAT1 was effectively inhibited.

In order to confirm and extend these observations, we cloned and expressed GFP-NSP1 proteins from the bovine UK and murine EW strains that, like RRV, inhibit IRF3 in HT29 cells (22). Both UK and EW GFP-NSP1 protein levels were increased in the presence of the proteasomal inhibitor MG132, and their expression in HT29 cells effectively inhibited activation of pY701-STAT1 in response to IFN receptor stimulation (Fig. 4B and C). These results demonstrate that NSP1 from certain RV strains, expressed by itself, is an efficient inhibitor of STAT1 Y701 phosphorylation in response to exogenous IFN-β stimulation. This property of NSP1 is conserved among at least three RV strains with the ability to degrade IRF3 and that encode NSP1s that are themselves subject to proteasomal degradation.

STAT1 inhibition by NSP1 does not require the RING finger motif. Although the RRV, UK, and EW RV NSP1 proteins are highly divergent at the level of amino acid sequence, they all contain a conserved RING finger (RF)-like motif that forms two predicted zinc-coordinating loops (Fig. 5A). In order to examine whether this conserved motif has a role in STAT1 inhibition, we mutated amino acids C59 and H63 within each of the two pre-

FIG 5 NSP1-mediated IRF3 and pY701-STAT1 inhibition are encoded on discrete domains. (A) Predicted RING finger motif in the RRV NSP1 protein with mutations in the two Zn-coordinating centers, indicated by black arrows. (B) Plasmids encoding GFP or the GFP-NSP1 construct were transfected into COS7 cells, and protein stability was examined by microscopy at 48 h posttransfection. Cells were treated with MG132 for 12 h. (C) The effect of transient expression of GFP- or GFP-NSP1-encoding plasmids on IRF3-dependent luciferase reporter activity was evaluated in COS7 cells. (D) COS7 cells were transfected with GFP- or GFP-NSP1-expressing plasmids. At 48 h posttransfection, cells were analyzed by immunoblotting. (E) HT29 cells were transfected with plasmids expressing GFP, GFP-RRV NSP1, GFP-C59H63A-RRV NSP1, GFP-EW NSP1, or GFP-UK NSP1 and stimulated with IFN-β or IL-6 for 20 min at 48 h posttransfection. Cells were analyzed by flow cytometry. (F) The data in panel E are plotted as MFI arcsinh ratios. Results are representative of at least 2 independent experiments. eGFP, enhanced GFP.

dicted loops of the RRV protein to alanine (Fig. 5A). Plasmids encoding GFP, wild-type GFP-RRV NSP1, mutant C59H63A, and GFP-UK NSP1 were transiently expressed in COS7 cells, and their expression levels were compared in the presence or absence of MG132. As shown in Fig. 5B, neither GFP nor GFP-UK NSP1 expression was substantially altered in COS7 cells following MG132 treatment. In contrast, the expression patterns of both the wild-type and mutant proteins were MG132 sensitive (i.e., NSP1 expression increased substantially after MG132 treatment), indicating that the NSP1 RF mutations did not affect proteasomal degradation of the protein (Fig. 5B).

In order to examine the effect of these mutations on the IRF3-inhibitory function of NSP1, we cotransfected COS7 cells with an IRF3-dependent pRD(III)-Luc reporter and plasmids encoding GFP, RRV NSP1, UK NSP1, or the C59H63A mutant. At 36 h posttransfection, cells were transfected with poly(I-C), and lucif-

erase activity was measured 12 h later. Unlike GFP-RRV NSP1 or UK NSP1, the C59H63A mutant was unable to inhibit IRF3 activity in response to poly(I-C) stimulation (Fig. 5C). Similarly, expression of GFP-RRV NSP1, but not of the C59H63A mutant, led to efficient degradation of IRF3 in COS7 cells (Fig. 5D). These results confirm and extend prior findings (37) and demonstrate that the RING finger plays an important role in inhibiting IRF3 function.

Next, we examined the role of the RING finger motif in STAT1 inhibition. HT29 cells were transfected with plasmids encoding GFP, GFP-RRV NSP1, the C59H63A mutant, GFP-EW NSP1, and GFP-UK NSP1. At 48 h posttransfection, cells were stimulated with IFN-β (500 U/ml) or an unrelated cytokine, interleukin-6 (IL-6) (25 ng/ml), for 20 min and then analyzed by flow cytometry for the levels of NSP1 and pY701-STAT1. As shown in Fig. 5E and F, in cells transfected with the empty vector, both GFP⁺ and GFP⁻

FIG 6 Effect of SA11 mono reassortants encoding simian and porcine NSP1 proteins on the activation of STAT1. (A) HT29 cells were infected with SA11 (MOI of 0.8), or the reassortants SRF and SOF (MOI of 1.2) and, at 16 hpi, were stimulated with 500 U/ml IFN- β for 20 min prior to staining for levels of RV VP6 and pY701-STAT1 and analysis by cytometry. (B) The pY701-STAT1 data in panel A are plotted as MFI arcsinh ratios. In each panel, the unstimulated baseline level of pY701-STAT1 in mock-infected HT29 cells is indicated by a dashed red line. Results are representative of 2 independent experiments.

populations responded to IFN- β , but not to IL-6, stimulation with similar increases in pY701-STAT1 expression levels (Fig. 5F, row 1). In contrast, in cells transfected with GFP-RRV NSP1, STAT1 activation was completely inhibited in response to IFN- β in cells expressing NSP1 (Fig. 5F, row 2). The cells without NSP1 expression showed a robust response similar in magnitude to that of controls, indicating that the inhibition of pY701-STAT1 was a direct consequence of NSP1 expression. A generally similar pattern of NSP1-mediated inhibition of IFN-mediated STAT1 phosphorylation was also observed upon expression of the NSP1 RF mutant, although a small population of cells expressing mutant NSP1 contained STAT1 phosphorylated at Y701 (Fig. 5F, row 3). As described above (Fig. 4B) and similar to RRV NSP1, cells expressing the EW NSP1 and UK NSP1 proteins were also able to inhibit IFN-mediated STAT1 phosphorylation (Fig. 5F, rows 4 and 5). These effects of NSP1 were specific to IFN- β -mediated STAT1 phosphorylation and were not observed when cells were stimulated with IL-6. Collectively, these results indicate that the RING finger motif is critical for initial inhibition of IFN induction at the level of IRF3 degradation, but it is not essential for NSP1-mediated inhibition of STAT1 activation. Further studies will be required to identify homologous residues and domains in NSP1 that mediate this novel STAT1-inhibitory function.

Although our results clearly identify RV inhibition of STAT1-Y701 phosphorylation during infection and the ability of NSP1 to block this critical step in the IFN response, several questions remain to be explored, including whether NSP1 encoded by porcine

RV strains is also able to inhibit pY701-STAT1 in the absence of other RV proteins and the manner in which RV influences the IFN responsiveness of VP6⁺ bystander cells later in infection. In preliminary experiments to address these questions, we compared the effects of SA11 and the monogenic reassortant strains SRF (SA11 encoding RRV NSP1) and SOF (SA11 encoding OSU NSP1) on IFN-mediated Y701-STAT1 activation (22). HT29 cells were infected with SA11 (MOI of ~ 0.8) or the reassortants SRF and SOF (MOI of ~ 1.2) and, at 16 hpi, stimulated with exogenous IFN for 20 min and analyzed by flow cytometry (Fig. 6). Infection with SOF (but not SA11 or SRF) resulted in a subpopulation of cells that were positive for pY701-STAT1 in the absence of any stimulation ($\sim 6\%$ of SOF-infected cells and $\sim 2.5\%$ of SOF bystander cells were pSTAT1⁺). Following exogenous stimulation with IFN, VP6⁺ cells from SA11, SRF, or SOF infections similarly inhibited Y701-STAT1 activation (Fig. 6A and B). Interestingly, infection with SOF also resulted in an inhibition of Y701-STAT1 phosphorylation in SOF bystander cells (Fig. 6B, row 4), similar to our findings during SB1A infections (Fig. 3). These results demonstrate that in the context of viral infection, porcine OSU NSP1 can functionally replace simian NSP1 to inhibit exogenous IFN-mediated Y701-STAT1 activation in infected IECs. Although further studies are required to directly examine the effects of SB1A NSP1 expressed on its own, one intriguing possibility based on these preliminary findings is that SB1A NSP1 orchestrates an indirect inhibitory effect on STAT1 signaling in bystander cells, as observed during infection with porcine RV.

DISCUSSION

During infection, rotavirus inhibits the induction of IFN by blocking the function of either IRF3 or NF- κ B; in the majority of strains studied to date, NSP1 proteins mediate IRF3 degradation (22, 26). Our present data (Fig. 1A) indicate that in HT29 human intestinal epithelial cells, porcine RV SB1A mediated β -TrCP degradation as early as 6 hpi. In contrast, infection with simian RRV did not result in altered β -TrCP levels but instead led to a reduction of IRF3 levels. The difference in β -TrCP levels at 6 hpi correlated well with the ability of SB1A, but not RRV, to inhibit I κ B- α degradation in response to exogenous TNF- α stimulation (Fig. 1B and C). Despite these differences, we did not find a significant difference between RRV and SB1A (or the closely related porcine OSU strain) in their abilities to regulate IFN secretion at 16 hpi (Fig. 3D). Therefore, in HT29 cells, RRV and SB1A appear to employ mutually exclusive, but equally effective, strategies for targeting IRF3 and NF- κ B function at 6 hpi to regulate the induction of IFN.

Although rotaviral inhibition of the IFN induction pathways has been examined in several studies, comparatively little is known about the manner in which different strains might regulate innate responses to exogenous IFN. It is estimated that one infectious particle of homologous RV can lead to full-blown diarrheal disease and extensive viral replication in wild-type suckling mice with intact type I, II, and III IFN responses (13). This remarkable lack of sensitivity of homologous RV to the host IFN responses, along with substantial induction of IFN in local hematopoietic cells during viral replication in the intestinal villous epithelium (20), strongly supports the hypothesis that RV might also possess strategies to subvert innate responses in infected and bystander epithelial populations that are exposed to IFN.

Following binding to their cognate receptor, type I IFNs induce tyrosine phosphorylation of STAT1 and STAT2, which form a trimolecular complex together with IRF9, called ISGF3. This complex binds to the ISREs in several ISGs (9). The phosphorylation of STAT1 on Y701 is essential for formation of the ISGF3 complex and subsequent antiviral gene transcription (8). Like type I IFN, type III IFNs exert their primary antiviral activity through the pY701-STAT1/ISGF3 axis (5). The formation of STAT1 homodimers following tyrosine phosphorylation is also pivotal in the transcriptional response of GAS promoter-containing genes following stimulation of the type II IFN receptor (38). Thus, the antiviral response downstream of several IFN subtypes is dependent on phosphorylation of STAT1 at Y701 (39). Therefore, we first examined the ability of the porcine SB1A and simian RRV RV strains to inhibit pY701-STAT1 activation in response to exogenous IFN stimulation early in infection (6 hpi) (Fig. 2). Although by 6 hpi, both SB1A and RRV had depleted their respective IFN induction mediators, β -TrCP and IRF3 (Fig. 1A), neither virus significantly inhibited exogenous IFN-mediated STAT1 phosphorylation in infected or uninfected bystander cells at this stage of infection (Fig. 2). In the absence of exogenous pathway stimulation, tyrosine phosphorylation was not detected in bystander cells from SB1A- or RRV-infected cultures (as might be expected if substantial and sustained IFN secretion was present in the cultures at 6 hpi) (Fig. 2), suggesting that at 6 hpi, RV encodes effective strategies to avoid the activation of STAT1 in bystander cells.

In our experiments, we observed that both RRV and SB1A weakly inhibited phosphorylation of STAT1 at Y701 at 6 hpi in infected and bystander populations, but these effects were consid-

erably amplified at 16 hpi (Fig. 3C), suggesting that inhibition of IFN-mediated STAT1 phosphorylation is most effective at later times during infection. It was previously reported that at 6 hpi, RV-infected cells prevent STAT1 and STAT2 nuclear localization following exogenous IFN stimulation (30). Thus, in addition to the induction and release of exogenous IFN, at 6 hpi, RV may interfere with STAT1 function by blocking its nuclear localization, a step that occurs subsequent to Y701 activation. Although the viral and host factors involved in this early regulation of STAT by RV are currently unknown, our results offer some insight into this process. A subpopulation of SB1A-infected cells displayed abnormally elevated levels of pY701-STAT1 at 6 hpi (Fig. 2 and 6), and this activation was similarly sustained through 16 hpi (Fig. 3). Interestingly, the level of pY701-STAT1 activation was highest in those cells that stained strongly for VP6, supporting the conclusion that STAT1 activation is an effect of SB1A replication rather than IFN receptor activation. In contrast, ~50% of SB1A-infected cells did not contain detectable STAT1 activation and exhibited a continuum in their expression levels of VP6. How a significant proportion of SB1A-infected cells at 16 h avoid STAT1 activation despite exogenous IFN stimulation is an interesting question raised by our results. The absence of further activation of pY701 in the two SB1A-infected cell subpopulations as well as in bystander cells at later times following stimulation with increasing doses of IFN, as well as comparable levels of secreted IFN in unstimulated infected cells at 16 h (Fig. 3), demonstrates the effectiveness of this viral strategy in preventing IFN-mediated STAT1 signaling and subsequent STAT1-dependent IFN amplification. Identification of the mechanisms underlying SB1A regulation of STAT1 activation, including the effect of β -TrCP-degrading NSP1 proteins on STAT1 signaling, will require further experiments.

In clear contrast to findings early during rotavirus infection, at 16 hpi, we found that RV-infected cells effectively resist exogenous IFN-mediated pY701-STAT1 activation (Fig. 3). In the absence of any exogenous stimulation, pY701-STAT1 expression in bystander cells of RRV- or SB1A-infected monolayers was comparable to levels in mock-infected cells (Fig. 3A) and similar to findings at 6 hpi (Fig. 2A). However, in approximately 50% of infected cells, SB1A induced STAT1 tyrosine phosphorylation to levels approaching those observed following stimulation of uninfected HT29 cells with 2,000 U/ml of exogenous IFN- β (Fig. 3A); levels in the remainder of SB1A-infected cells, as well as in all RRV-infected cells, were significantly lower and were similar to pY701-STAT1 staining in resting mock-infected HT29 cells. These differences between SB1A- and RRV-induced pY701-STAT1 were observed at both 6 hpi and 16 hpi, suggesting that SB1A infection leads to early activation of STAT1 exclusively within a subset of infected cells, and this activation is sustained later during infection. At 16 hpi, when cells were exogenously stimulated with increasing doses of IFN- β , neither SB1A- nor RRV-infected cells displayed an appreciable increase in pY701-STAT1 activation over baseline levels in their respective unstimulated populations (Fig. 3C). Thus, in contrast to the inability of RRV to inhibit IFN-mediated STAT1 activation at 6 hpi, at a later time postinfection, RRV infection efficiently suppressed the exogenous IFN signaling pathway. Sustained activation of pY701-STAT1, which was observed among SB1A-infected HT29 cells, has been associated with antiapoptotic and proliferative effects in the context of oncogenic viruses, such as acute myeloid lymphoma and Epstein-Barr virus-associated malignancies (39). It will be of interest to determine

whether SB1A-induced STAT1 activation results in similar anti-apoptotic functional consequences during infection.

An intriguing finding from our study is that although SB1A VP6⁻ cells (bystanders) responded to exogenous IFN stimulation at 6 hpi (Fig. 2), by 16 hpi, they were completely refractory to pathway stimulation (Fig. 3C), irrespective of the IFN dose. This phenomenon was also observed following infection with SOF, an SA11-derived monoreassortant strain encoding porcine OSU NSP1. A similarly significant, but weaker, effect was observed in RRV bystander cells at 16 hpi as well (Fig. 3B). These results suggest that rotavirus infection not only is able to directly inhibit the phosphorylation of pY701-STAT1 in infected cells but also can effectively mediate such inhibition in uninfected (i.e., VP6-negative) cells late in infection. The fact that pY701-STAT1 activation is not inhibited at early times postinfection (6 hpi) in either VP6-positive or -negative cells, and the potent repression of Y701 in SB1A and SOF VP6-negative cells, even at high doses of IFN- β , suggests that the factor(s) that mediates this effect originates from infected cells between 6 and 16 hpi. Similar remote effects of RV infection on bystander interferon responses were recently observed in suckling mice infected with the EW strain of murine RV (20). No amplification of IFN transcripts was observed in bystander villous IECs in these mice, despite exogenous IFN induction in the intestinal hematopoietic cell compartment. The finding that SOF, but not the closely related strains SRF and SA11, is able to mediate such inhibition in VP6-negative HT29 cells (Fig. 6) strongly supports the conclusion that NSP1 plays a role in influencing the ability of bystander IECs to respond to IFN. Alternatively, several cytokines and at least one RV nonstructural protein, NSP4, are released from RV-infected IECs and could also potentially mediate remote inhibition of STAT1 signaling (40, 41). We are currently investigating the nature of the RV-mediated signal that regulates IFN responsiveness in bystander (uninfected) cells.

Most RV strains studied to date degrade IRF3 rather than β -TrCP during infection to subvert IFN induction (22). Our results demonstrate that cells infected with the IRF3-degradative strain RRV effectively inhibit activation of Y701 on STAT1 in response to the presence of exogenous IFN. Since innate immune responses mediated by several different IFN subtypes (types I, II, and III) converge at this critical signaling step, identification of the viral factor(s) that mediates the inhibition of Y701 phosphorylation is crucial to an understanding of the basis for rotavirus resistance/sensitivity to the host interferon response. A likely candidate for inhibition of STAT1 phosphorylation on Y701 was NSP1, whose cognate gene (gene 5) cosegregates with STAT1- and IFN-receptor-dependent replication and virulence phenotypes *in vivo* and *in vitro* (13–16, 23). In order to examine a possible role for NSP1 in STAT1 inhibition, we expressed the NSP1 proteins from three rotavirus strains (simian RRV, bovine UK, and murine EW) that share the common property of IRF3 degradation in HT29 cells (22) as GFP-tagged fusion proteins (23). Previous studies reported that NSP1 is a poorly expressed protein due to inefficient recruitment of ribosomes on NSP1 transcripts (42) and due to posttranslational NSP1 proteasomal degradation (23, 37, 43). Therefore, we chose a single-cell analytical approach to directly compare the pY701-STAT1 status within NSP1-expressing cells in the presence or absence of exogenous stimulation (Fig. 4). When pY701-STAT1 activation in response to IFN stimulation was measured in cells expressing NSP1 by flow cytometry, we found that

the NSP1 proteins encoded by RRV, UK, and EW effectively inhibited the STAT1 pathway (Fig. 4B and C). Our data also demonstrate that porcine OSU NSP1 is capable of inhibiting IFN-mediated STAT1 activation within infected cells in the context of other viral proteins (Fig. 6). Whether porcine NSP1 proteins expressed by themselves are similarly functional will require further studies.

Although the 55-kDa NSP1 protein sequences from different rotavirus strains are highly divergent, all RV NSP1 proteins examined to date contain an N-terminal RING finger motif with conserved spacing of the predicted divalent cation coordinating cysteine and histidine sites (26). Mutagenesis of C59 and H63 of RRV NSP1, in the second and first predicted Zn-binding sites of this domain, respectively, resulted in an NSP1 mutant whose expression was MG132 sensitive and hence not significantly less unstable than the wild-type protein in COS7 cells (Fig. 5B). However, proteasomal degradation of IRF3 was completely abolished in COS7 cells that expressed mutant, but not wild-type, NSP1 (Fig. 5C and D). These results are in agreement with previous observations that disruption of residues within the RING motif led to diminished IRF3 interaction and degradation (37). On the other hand, the RRV RING finger mutant retained the ability to inhibit IFN-mediated pY701-STAT1 activation in HT29 cells (Fig. 5E and F). Therefore, the ability of NSP1 to inhibit IFN induction versus IFN signaling pathways appears to be encoded by discrete domains of the protein. In our experiments using the SA11 reassortants SRF and SOF, expression of OSU (but not SA11 or RRV) NSP1 in the SA11 genetic background significantly altered the ability of “bystander” (i.e., non-VP6-expressing) cells to respond to exogenous IFN stimulation (Fig. 6), indicating that certain NSP1 proteins and/or host factors regulated by their expression during virus infection may mediate the remote effect on STAT1-Y701 signaling observed at 16 hpi (Fig. 3). However, transient expression of NSP1 encoded by the RRV, EW, or UK strain did not significantly alter STAT1 activation in NSP1⁻ cells (Fig. 4 and 5), indicating that these NSP1 proteins are unable to direct STAT1 inhibition in bystander cells. In contrast, both SOF and transiently expressed RRV/EW/UK NSP1 proteins similarly inhibited Y701-STAT1 phosphorylation directly within cells that were either VP6⁺ or NSP1⁺, respectively. We plan to examine whether NSP1 encoded by the SB1A and/or OSU strain is sufficient to inhibit IFN-mediated STAT1 signaling in NSP1⁻ populations in future studies. Since NSP1 mediates both the depletion of β -TrCP/IRF3 (25, 29) and the inhibition of IFN-directed STAT1 activation (Fig. 4 and 5), which occur at distinct times during infection, we hypothesize that the various NSP1 functions taking place during the course of infection may be subject to currently unknown temporal regulatory mechanisms that determine pathway-specific inhibition.

The ability of RV to regulate STATs was first observed by Holloway and coworkers, who found that RV blocks type I and II IFN-induced gene expression by preventing nuclear accumulation of STAT1 and STAT2 (30). In that study, at 2 to 12 hpi, simian RRV and human Wa RVs failed to inhibit Y701-STAT1 phosphorylation mediated by exogenous IFN- α . In contrast, human Wa RV was shown to activate pY701-STAT1 in the absence of exogenous stimulation, and this effect was ascribed to IFN induced during Wa infection (30). Both RRV and Wa effectively prevented nuclear accumulation of STAT1 and STAT2 in response to exogenous IFN stimulation at 6 hpi based on immunomicroscopy of total STAT proteins. Finally, RRV nonstructural proteins NSP1,

NSP3, and NSP4 did not inhibit expression from an ISRE-luciferase reporter following the addition of exogenous IFN- α .

Our experimental approach differs from the previous study (30) in several important ways that could account for the apparently contradictory results. Holloway et al. examined averaged effects in monolayers of cells infected with rotavirus to determine whether an inhibition of exogenous IFN-mediated STAT1-Y701 phosphorylation occurs during the 2- to 12-hpi time frame. We found that following infection with either RRV or SB1A, inhibition of pY701-STAT1 in response to stimulation did not occur until 16 hpi and then only within specific subpopulations of HT29 cells (Fig. 3). Thus, RV effects on STAT1 activation are most readily apparent when STAT status is assessed at the single-cell level. In the study by Holloway et al. (30), the ability of nonstructural proteins to inhibit STAT1-mediated transcription was assessed by using an “averaged” rather than a single-cell signal approach. Despite these differences, our results are in general agreement with the observations by Holloway et al. (30), including the inability of RV at 6 hpi to inhibit the activation of pY701-STAT1 in response to IFN and the activation of pY701-STAT1 by some RV strains in the absence of any exogenous IFN stimulation.

The two discrete innate immunity-inhibitory functions of rotavirus NSP1 allow it to effectively inhibit both host IFN induction and amplification signaling pathways. Previous studies uncovered an NSP1-dependent phenotype for rotavirus replication, as assessed by both viral titer and plaque size, reflecting the ability of NSP1 to regulate virus growth and subsequent spread to adjacent bystander cells (26). Initial infection of epithelial cells at a low MOI occurs only in a subset of cells, resulting in the activation of early IFN induction signaling pathways, the expression of subsets of VSIGs (20), and some level of IFN secretion (27, 33). *In vivo*, RV infection also resulted in a robust IFN response in the nonepithelial hematopoietic cell compartment (20). The finding that inhibition of both IFN secretion and IFN-mediated signaling converge on the NSP1 protein indicates that it is a multipurpose viral antagonist of the host innate immune response. Thus, NSP1 is a viral factor that is capable of inhibiting the IFN induction pathway in different cell types by targeting cell-type-specific IRFs and of negating the antiviral effects of the multiple downstream IFNs (types I, II, and III) that rely on successful tyrosine activation of STAT1.

Several interesting questions are raised by our findings. The mechanism by which SB1A regulates STAT1 signaling and the identities of remote inhibitory factors that influence STAT1 signaling in uninfected bystander cells remain to be determined. Our data, when combined with recent observations that a robust early innate response correlates with live attenuated vaccine immunogenicity (44, 45), suggest that it might be possible to further enhance reassortant rotavirus vaccine strain immunogenicity by diminishing the ability of the strains to inhibit innate responses by incorporating a relevant NSP1 gene.

ACKNOWLEDGMENTS

We thank Ningguo Feng and Nandini Sen for helpful suggestions, Grace Laidlaw for technical assistance and comments, and Joyce Troiano for administrative assistance.

This study was supported in part by a VA merit award and NIH grants R01 AI021362-26, P30DK56339, and AI057229.

REFERENCES

- Randall RE, Goodbourn S. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J. Gen. Virol.* 89:1–47. <http://dx.doi.org/10.1099/vir.0.83391-0>.
- Thompson MR, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA. 2011. Pattern recognition receptors and the innate immune response to viral infection. *Viruses* 3:920–940. <http://dx.doi.org/10.3390/v3060920>.
- Versteeg GA, Garcia-Sastre A. 2010. Viral tricks to grid-lock the type I interferon system. *Curr. Opin. Microbiol.* 13:508–516. <http://dx.doi.org/10.1016/j.mib.2010.05.009>.
- Borden EC, Williams BR. 2011. Interferon-stimulated genes and their protein products: what and how? *J. Interferon Cytokine Res.* 31:1–4. <http://dx.doi.org/10.1089/jir.2010.0129>.
- Iversen MB, Paludan SR. 2010. Mechanisms of type III interferon expression. *J. Interferon Cytokine Res.* 30:573–578. <http://dx.doi.org/10.1089/jir.2010.0063>.
- Andersen J, VanScoy S, Cheng TF, Gomez D, Reich NC. 2008. IRF-3-dependent and augmented target genes during viral infection. *Genes Immun.* 9:168–175. <http://dx.doi.org/10.1038/sj.gene.6364449>.
- Ramana CV, Chatterjee-Kishore M, Nguyen H, Stark GR. 2000. Complex roles of Stat1 in regulating gene expression. *Oncogene* 19:2619–2627. <http://dx.doi.org/10.1038/sj.onc.1203525>.
- Harrison DA. 2012. The Jak/STAT pathway. *Cold Spring Harb. Perspect. Biol.* 4:a011205. <http://dx.doi.org/10.1101/cshperspect.a011205>.
- Stark GR, Darnell JE, Jr. 2012. The JAK-STAT pathway at twenty. *Immunity* 36:503–514. <http://dx.doi.org/10.1016/j.immuni.2012.03.013>.
- Trask SD, Ogden KM, Patton JT. 2012. Interactions among capsid proteins orchestrate rotavirus particle functions. *Curr. Opin. Virol.* 2:373–379. <http://dx.doi.org/10.1016/j.coviro.2012.04.005>.
- Babji S, Kang G. 2012. Rotavirus vaccination in developing countries. *Curr. Opin. Virol.* 2:443–448. <http://dx.doi.org/10.1016/j.coviro.2012.05.005>.
- Angel J, Franco MA, Greenberg HB. 2012. Rotavirus immune responses and correlates of protection. *Curr. Opin. Virol.* 2:419–425. <http://dx.doi.org/10.1016/j.coviro.2012.05.003>.
- Broome RL, Vo PT, Ward RL, Clark HF, Greenberg HB. 1993. Murine rotavirus genes encoding outer capsid proteins VP4 and VP7 are not major determinants of host range restriction and virulence. *J. Virol.* 67:2448–2455.
- Feng N, Kim B, Fenaux M, Nguyen H, Vo P, Omary MB, Greenberg HB. 2008. Role of interferon in homologous and heterologous rotavirus infection in the intestines and extraintestinal organs of suckling mice. *J. Virol.* 82:7578–7590. <http://dx.doi.org/10.1128/JVI.00391-08>.
- Feng N, Sen A, Nguyen H, Vo P, Hoshino Y, Deal EM, Greenberg HB. 2009. Variation in antagonism of the interferon response to rotavirus NSP1 results in differential infectivity in mouse embryonic fibroblasts. *J. Virol.* 83:6987–6994. <http://dx.doi.org/10.1128/JVI.00585-09>.
- Feng N, Sen A, Wolf M, Vo P, Hoshino Y, Greenberg HB. 2011. Roles of VP4 and NSP1 in determining the distinctive replication capacities of simian rotavirus RRV and bovine rotavirus UK in the mouse biliary tract. *J. Virol.* 85:2686–2694. <http://dx.doi.org/10.1128/JVI.02408-10>.
- Franco MA, Greenberg HB. 1999. Immunity to rotavirus infection in mice. *J. Infect. Dis.* 179(Suppl 3):S466–S469.
- Greenberg HB, Estes MK. 2009. Rotaviruses: from pathogenesis to vaccination. *Gastroenterology* 136:1939–1951. <http://dx.doi.org/10.1053/j.gastro.2009.02.076>.
- Anderson EJ. 2008. Rotavirus vaccines: viral shedding and risk of transmission. *Lancet Infect. Dis.* 8:642–649. [http://dx.doi.org/10.1016/S1473-3099\(08\)70231-7](http://dx.doi.org/10.1016/S1473-3099(08)70231-7).
- Sen A, Rothenberg ME, Mukherjee G, Feng N, Kalisky T, Nair N, Johnstone IM, Clarke MF, Greenberg HB. 2012. Innate immune response to homologous rotavirus infection in the small intestinal villous epithelium at single-cell resolution. *Proc. Natl. Acad. Sci. U. S. A.* 109:20667–20672. <http://dx.doi.org/10.1073/pnas.1212188109>.
- Arnold MM, Patton JT. 2009. Rotavirus antagonism of the innate immune response. *Viruses* 1:1035–1056. <http://dx.doi.org/10.3390/v1031035>.
- Arnold MM, Patton JT. 2011. Diversity of interferon antagonist activities mediated by NSP1 proteins of different rotavirus strains. *J. Virol.* 85:1970–1979. <http://dx.doi.org/10.1128/JVI.01801-10>.
- Sen A, Feng N, Ettayebi K, Hardy ME, Greenberg HB. 2009. IRF3 inhibition by rotavirus NSP1 is host cell and virus strain dependent but

- independent of NSP1 proteasomal degradation. *J. Virol.* 83:10322–10335. <http://dx.doi.org/10.1128/JVI.01186-09>.
24. Barro M, Patton JT. 2005. Rotavirus nonstructural protein 1 subverts innate immune response by inducing degradation of IFN regulatory factor 3. *Proc. Natl. Acad. Sci. U. S. A.* 102:4114–4119. <http://dx.doi.org/10.1073/pnas.0408376102>.
 25. Barro M, Patton JT. 2007. Rotavirus NSP1 inhibits expression of type I interferon by antagonizing the function of interferon regulatory factors IRF3, IRF5, and IRF7. *J. Virol.* 81:4473–4481. <http://dx.doi.org/10.1128/JVI.02498-06>.
 26. Arnold MM, Sen A, Greenberg HB, Patton JT. 2013. The battle between rotavirus and its host for control of the interferon signaling pathway. *PLoS Pathog.* 9:e1003064. <http://dx.doi.org/10.1371/journal.ppat.1003064>.
 27. Broquet AH, Hirata Y, McAllister CS, Kagnoff MF. 2011. RIG-I/MDA5/MAVS are required to signal a protective IFN response in rotavirus-infected intestinal epithelium. *J. Immunol.* 186:1618–1626. <http://dx.doi.org/10.4049/jimmunol.1002862>.
 28. Cuadras MA, Feigelsstock DA, An S, Greenberg HB. 2002. Gene expression pattern in Caco-2 cells following rotavirus infection. *J. Virol.* 76:4467–4482. <http://dx.doi.org/10.1128/JVI.76.9.4467-4482.2002>.
 29. Graff JW, Ettayebi K, Hardy ME. 2009. Rotavirus NSP1 inhibits NF- κ B activation by inducing proteasome-dependent degradation of beta-TrCP: a novel mechanism of IFN antagonism. *PLoS Pathog.* 5:e1000280. <http://dx.doi.org/10.1371/journal.ppat.1000280>.
 30. Holloway G, Truong TT, Coulson BS. 2009. Rotavirus antagonizes cellular antiviral responses by inhibiting the nuclear accumulation of STAT1, STAT2, and NF- κ B. *J. Virol.* 83:4942–4951. <http://dx.doi.org/10.1128/JVI.01450-08>.
 31. Qin L, Ren L, Zhou Z, Lei X, Chen L, Xue Q, Liu X, Wang J, Hung T. 2011. Rotavirus nonstructural protein 1 antagonizes innate immune response by interacting with retinoic acid inducible gene I. *J. Virol.* 85:526. <http://dx.doi.org/10.1186/1743-422X-8-526>.
 32. Rollo EE, Kumar KP, Reich NC, Cohen J, Angel J, Greenberg HB, Sheth R, Anderson J, Oh B, Hempson SJ, Mackow ER, Shaw RD. 1999. The epithelial cell response to rotavirus infection. *J. Immunol.* 163:4442–4452.
 33. Sen A, Pruijssers AJ, Dermody TS, Garcia-Sastre A, Greenberg HB. 2011. The early interferon response to rotavirus is regulated by PKR and depends on MAVS/IPS-1, RIG-I, MDA-5, and IRF3. *J. Virol.* 85:3717–3732. <http://dx.doi.org/10.1128/JVI.02634-10>.
 34. Mansur DS, Maluquer de Motes C, Unterholzner L, Sumner RP, Ferguson BJ, Ren H, Strnadova P, Bowie AG, Smith GL. 2013. Poxvirus targeting of E3 ligase beta-TrCP by molecular mimicry: a mechanism to inhibit NF- κ B activation and promote immune evasion and virulence. *PLoS Pathog.* 9:e1003183. <http://dx.doi.org/10.1371/journal.ppat.1003183>.
 35. Kotecha N, Krutzyk PO, Irish JM. 2010. Web-based analysis and publication of flow cytometry experiments. *Curr. Protoc. Cytom.* Chapter 10: Unit 10.17. <http://dx.doi.org/10.1002/0471142956.cy1017s53>.
 36. Irish JM, Myklebust JH, Alizadeh AA, Houot R, Sharman JP, Czerwinski DK, Nolan GP, Levy R. 2010. B-cell signaling networks reveal a negative prognostic human lymphoma cell subset that emerges during tumor progression. *Proc. Natl. Acad. Sci. U. S. A.* 107:12747–12754. <http://dx.doi.org/10.1073/pnas.1002057107>.
 37. Graff JW, Ewen J, Ettayebi K, Hardy ME. 2007. Zinc-binding domain of rotavirus NSP1 is required for proteasome-dependent degradation of IRF3 and autoregulatory NSP1 stability. *J. Gen. Virol.* 88:613–620. <http://dx.doi.org/10.1099/vir.0.82255-0>.
 38. Ramsauer K, Farlik M, Zupkovitz G, Seiser C, Kroger A, Hauser H, Decker T. 2007. Distinct modes of action applied by transcription factors STAT1 and IRF1 to initiate transcription of the IFN- γ -inducible gbp2 gene. *Proc. Natl. Acad. Sci. U. S. A.* 104:2849–2854. <http://dx.doi.org/10.1073/pnas.0610944104>.
 39. Najjar I, Fagard R. 2010. STAT1 and pathogens, not a friendly relationship. *Biochimie* 92:425–444. <http://dx.doi.org/10.1016/j.biochi.2010.02.009>.
 40. Azim T, Zaki MH, Podder G, Sultana N, Salam MA, Rahman SM, Sefat-e-Khuda, Sack DA. 2003. Rotavirus-specific subclass antibody and cytokine responses in Bangladeshi children with rotavirus diarrhoea. *J. Med. Virol.* 69:286–295. <http://dx.doi.org/10.1002/jmv.10280>.
 41. Didsbury A, Wang C, Verdon D, Sewell MA, McIntosh JD, Taylor JA. 2011. Rotavirus NSP4 is secreted from infected cells as an oligomeric lipoprotein and binds to glycosaminoglycans on the surface of non-infected cells. *J. Virol.* 85:551. <http://dx.doi.org/10.1186/1743-422X-8-551>.
 42. Mitzel DN, Weisend CM, White MW, Hardy ME. 2003. Translational regulation of rotavirus gene expression. *J. Gen. Virol.* 84:383–391. <http://dx.doi.org/10.1099/vir.0.18558-0>.
 43. Pina-Vazquez C, De Nova-Ocampo M, Guzman-Leon S, Padilla-Noriega L. 2007. Post-translational regulation of rotavirus protein NSP1 expression in mammalian cells. *Arch. Virol.* 152:345–368. <http://dx.doi.org/10.1007/s00705-006-0850-8>.
 44. Pulendran B, Ahmed R. 2006. Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 124:849–863. <http://dx.doi.org/10.1016/j.cell.2006.02.019>.
 45. Kasturi SP, Skountzou I, Albrecht RA, Koutsouanos D, Hua T, Nakaya HI, Ravindran R, Stewart S, Alam M, Kwissa M, Villinger F, Murthy N, Steel J, Jacob J, Hogan RJ, Garcia-Sastre A, Compans R, Pulendran B. 2011. Programming the magnitude and persistence of antibody responses with innate immunity. *Nature* 470:543–547. <http://dx.doi.org/10.1038/nature09737>.