Role of Interferon Regulatory Factor 3 in Type I Interferon Responses in Rotavirus-Infected Dendritic Cells and Fibroblasts[▽]

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The main pathway for the induction of type I interferons (IFN) by viruses is through the recognition of viral RNA by cytosolic receptors and the subsequent activation of interferon regulatory factor 3 (IRF-3), which drives IFN- α/β transcription. In addition to their role in inducing an antiviral state, type I IFN also play a role in modulating adaptive immune responses, in part via their effects on dendritic cells (DCs). Many viruses have evolved mechanisms to interfere with type I IFN induction, and one recently reported strategy for achieving this is by targeting IRF-3 for degradation, as shown for rotavirus nonstructural protein 1 (NSP1). It was therefore of interest to investigate whether rotavirus-exposed DCs would produce type I IFN and/or mature in response to the virus. Our results demonstrate that IRF-3 was rapidly degraded in rotavirus-infected mouse embryonic fibroblasts (MEFs) and type I IFN was not detected in these cultures. In contrast, rotavirus induced type I IFN production in myeloid DCs (mDCs), resulting in their activation. Type I IFN induction in response to rotavirus was reduced in mDCs from IRF-3^{-/-} mice, indicating that IRF-3 was important for mediating the response. Exposure of mDCs to UV-treated rotavirus induced significantly higher type I IFN levels, suggesting that rotavirus-encoded functions also antagonized the response in DCs. However, in contrast to MEFs, this action was not sufficient to completely abrogate type I IFN induction, consistent with a role for DCs as sentinels for virus infection.

Rotaviruses, members of the *Reoviridae* family, are the most common causative agents of severe gastroenteritis in infants and young children, worldwide. Rotavirus infection is a major health and economic burden to many countries, and the availability of a prophylactic vaccine that provides protection against severe disease remains a high global priority (23). Two oral vaccine candidates have recently been licensed for humans, Rotarix and Rotateq (25), both of which are based on live attenuated viruses. Rotaviruses are nonenveloped viruses with segmented double-stranded RNA genomes that encode six structural proteins (VP1 to 6) and six nonstructural proteins (NSP1 to 6). Although the functions of several of the NSPs remain obscure, a function was recently ascribed to rotavirus NSP1. Using a yeast two-hybrid screen, it was shown that NSP1 interacts with interferon regulatory factor 3 (IRF-3) and that regions in the C-terminal domain of NSP1 were important for mediating this interaction (26). IRF-3 is required for the early production of type I interferons (IFN) in most cell types; thus, this finding suggested that NSP1 plays a role in suppressing the host innate immune response. More recent studies using rotavirus mutants with C-terminal truncations of NSP1 demonstrated that viruses encoding wild-type (wt) NSP1, but not viruses encoding C-terminally truncated NSP1, caused rapid and profound degradation of IRF-3 in MA104 cells (7). Inter-

ference with IRF-3 function has been reported for many other viruses (8, 18, 21, 22, 41, 57). However, none of the previously described IRF-3 inhibitory activities has been shown to involve proteasome-dependent degradation of IRF-3. Thus, the strategy employed by rotavirus represents a new mechanism by which viruses interfere with the host immune response.

During the early phase of an infection, the most important role of type I IFN is to signal the establishment of an antiviral state to uninfected cells, allowing the host to control viral spread. However, type I IFN also play a role in driving the generation of adaptive immune responses (45–49, 61, 65), a process during which dendritic cells (DCs) play a central role. Studies have shown that virus exposure stimulates DCs to upregulate the surface expression of costimulatory molecules (11, 29, 52, 70) and that type I IFN are important for mediating this effect (30, 31). Viruses that encode type I IFN antagonists may therefore induce suboptimal immune responses not only in the early phase of the infection but also in terms of long-term adaptive responses, as recently discussed for influenza virus and its type I IFN antagonist, nonstructural protein 1 (NS1) (19).

Two different pathways have been described for type I IFN induction in DCs. First, like all other cells, DCs are capable of responding to virus infection through the recognition of viral RNA by cytosolic receptors, such as retinoic acid-inducible gene I (RIG-I) products and melanoma differentiating associated factor 5 (Mda5) (36, 37, 77). RIG-I and Mda5 signal via the adaptor protein IFN- β promoter stimulator (IPS-1), also referred to as MAVS, Cardif, or VISA (38, 44, 58, 68, 76), to activate IRF-3 and nuclear factor κ B (NF- κ B), both of which

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are required for the transcription of early IFN subtypes IFN- α 4 and IFN- β . In the second pathway, DCs express Toll-like receptors (TLRs), which can mediate recognition of viruses and induce the production of type I IFN (14, 28, 43, 53, 54). In the human system, two main subtypes of DCs have been identified, myeloid DCs (mDCs), also referred to as conventional DCs, and plasmacytoid DCs (pDCs) (12). These DC subtypes express different repertoires of TLRs and as a consequence respond differently to pathogens and pathogen-associated structures. While mDCs have been shown to be highly efficient as antigen-presenting cells (5), pDCs are unique in their ability to produce high levels of type I IFN in response to virus (4, 12). Type I IFN production by the TLR7/8/9 pathway is independent of IRF-3 and is instead mediated by IRF-7, which is constitutively expressed in pDCs (34).

The role of different DC subsets for the control of rotavirus infection remains poorly characterized, although the extraintestinal spread of virus into the mesenteric lymph nodes has been shown (62). Furthermore, cells in the mesenteric lymph nodes of rotavirus-infected mice that express both rotavirus antigens and DC-specific markers have been reported, suggesting that DCs can support rotavirus protein synthesis (16). Nevertheless, as rotavirus infection occurs via the gastrointestinal route, it seems likely that DCs present in Peyer's patches are the first antigen-presenting cells to come in contact with the virus. Epithelial cells at the apex of the villi in the small intestine are the principal target cells for rotavirus infection, and these cells have been shown to contribute to innate immune responses to rotavirus infection (66). However, epithelial cells are unlikely to be the main antigen-presenting cells during the priming of antigen-specific immune responses due to the lack of costimulatory molecules on these cells. Viral antigens have been found in Peyer's patch-associated DCs in rotavirus-infected mice (13), but it remains unknown if this is due to productive infection of DCs or if antigen is acquired by DCs from dying infected epithelial cells in the intestine, as shown in mice infected with reoviruses (20). Since type I IFN are potent modulators of adaptive immunity, it is of interest to determine if DCs can produce type I IFN in response to rotavirus and the role of IRF-3 in such responses.

To address some of these questions, we exposed mouse embryonic fibroblasts (MEFs) and murine bone marrow-derived DCs to rhesus rotavirus (RRV). Consistent with previous studies with MA104 cells, we demonstrate that IRF-3 was rapidly degraded in RRV-infected MEFs and consequently there was no detectable type I IFN production from these cells. In contrast, RRV-exposed mDCs were capable of type I IFN production, which led to the upregulation of cell surface activation markers. By using mDCs from IRF-3^{-/-} mice, we show that type I IFN production in response to RRV was mediated via an IRF-3-dependent pathway. Furthermore, mDCs from MyD88^{-/-} and TLR3^{-/-} mice produced levels of type I IFN similar to those from control animals, indicating that the response was independent of TLR signaling. When we exposed mDCs to UV-treated RRV, we observed significantly higher type I IFN levels, suggesting that virus-encoded functions were suppressing the response. In contrast to MEFs, the suppression was not sufficient to completely abrogate the type I IFN production, consistent with an important role for DCs as sentinels for virus infection.

MATERIALS AND METHODS

Cells. African green monkey MA104 cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine, 1 mM sodium pyruvate, 0.02 M HEPES, and gentamicin (Sigma, St. Louis, MO). Primary MEFs, obtained from 12- to 13-day-old C57BL/6 fetuses, and L929 cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin. Murine mDCs were generated as previously described (29) from IRF-3^{-/-} mice (B6) (67), IFNAR1^{-/-} mice (Sv129) (63), TLR3^{-/-} mice (129/B6) (2), and MyD88^{-/-} mice (B6) (1) and their respective wt controls (B6, Sv129, or 129/B6). Animals were kept and bred under pathogen-free conditions at the animal facilities at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet. Briefly, bone marrow cells were isolated from femurs and tibiae, and red blood cells were lysed. Cells plated at 4×10^5 cells/ml in DC medium (RPMI 1640 [Sigma] containing 10% FCS, L-glutamine, penicillin, streptomycin, 1 mM sodium pyruvate, 0.02 M HEPES, and 50 μ M 2-mercaptoethanol) and supplemented with 100 ng/ml of murine recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, London, United Kingdom) were harvested at day 6. Murine bone marrow-derived pDCs were generated from cultures supplemented with 100 ng/ml of murine recombinant fms-like tyrosine kinase 3 ligand (Flt3-L) (R&D Systems) and were harvested at day 10 (24).

Viruses. Tissue culture-adapted RRV was grown in MA104 cells, and virus titers were determined as previously described (60, 72). Briefly, confluent monolayers of MA104 cells were infected with trypsin-activated RRV. After 1 h of adsorption at 37°C, the virus inoculum was replaced with serum-free DMEM containing 0.5 μ g/ml trypsin (Sigma). The cells were harvested 2 to 3 days postinfection and freeze-thawed three times. The virions were recovered from the cell lysate after treatment with trichlorotrifluoroethane, followed by two successive rounds of high-speed ultracentrifugation in sucrose-CsCl gradient to separate double-layered particles (DLP) and triple-layered particles (TLP) (71). The purity and identity of the purified DLPs and TLPs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by titration of MA104 cells. The crude supernatant from RRV-infected MA104 cells (hereafter referred to as RRV) contains both DLPs and TLPs. UV inactivation of RRV was performed using an Amersham UV cross-linker as previously described (29), using 2,000 μ J/cm² for 30 min to generate UV'-RRV or 20,000 μ J/cm² for 30 min to generate UV''-RRV. Infectious RRV titers and progeny virus production in DC supernatants were determined using serial dilutions on monolayers of MA104 cells grown in 96-well plates. Infectivity was determined by immunoperoxidase staining as described previously and expressed as peroxidase-forming units (60, 72). The UV-treated viruses showed a decrease in infectious titer by at least 10³-fold (UV'-RRV) and 10⁴-fold (UV''-RRV), respectively, compared side-by-side with RRV. Single-round infectious recombinant Semliki Forest virus (rSFV) encoding enhanced green fluorescent protein (rSFV-EGFP) was generated as previously described (29, 69), and the titers of the viral stocks were determined according to standard methods (35). Replication-competent full-length SFV4 for use in the bioassay for murine type I IFN (see below) was generated from the infectious SFV cDNA clone (51).

DC stimulations for flow cytometric analysis and type I IFN measurements. The DC cultures were set up with 1×10^6 cells in 500 μ l of DC medium for stimulation with CpG-ODN (1 μ M; Cybergene, Sweden), DLP, TLP, RRV, UV'-RRV, or UV''-RRV. Unless otherwise mentioned, TLP and RRV were used at a multiplicity of infection (MOI) of 5, and equivalent volumes corresponding to equivalent numbers of particles were used for the UV-treated RRV viruses. The noninfectious DLP preparation and the infectious TLP preparation were adjusted to similar viral protein concentrations and used at the same volume (Fig. 1A). At 24 h after stimulation, the cells were harvested and analyzed. The phenotype and activation status of the cells was determined by staining with monoclonal antibodies against CD11c, CD11b, CD45R/B220, CD86, and CD40 (PharMingen). Flow cytometric analysis was performed using a FACS-calibur instrument (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Inc.).

Measurements of type I IFN. A bioassay was employed to determine the total amount of active type I IFN (29). Briefly, flat-bottom 96-well plates were seeded with L929 cells, and the following day, twofold serially diluted samples or IFN- α/β standard (NIAID, Gu02-901-511) was added to the cells. After overnight incubation, the cells were infected with SFV4. Mitochondrial dehydrogenase activity was assayed at 2 days after infection using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) according to Sigma kit CGD-1. The absorbance was read at 570 nm and normalized against 630 nm in a microplate reader (Elx 800 UV; BIO-TEK Instruments, Winooski, VT). The data were

FIG. 1. Activation of dendritic cells by rotavirus. (A) Preparations of purified DLP and TLP were analyzed by SDS-PAGE and Coomassie blue staining. Positions of viral proteins are indicated on the right. (B) Bone marrow-derived mDCs generated in the presence of GM-CSF were stimulated with TLP, DLP, RRV, and CpG (1 μ M). TLP and RRV were used at an MOI of 5. The noninfectious DLP preparation and the infectious TLP preparation were adjusted to similar viral protein concentration as described in Materials and Methods. Surface expression of CD11c, CD11b, CD40, and CD86 was analyzed by flow cytometry after 24 h of incubation. CD40 and CD86 expression on CD11c⁺ CD11b⁺ cells is shown. Mock-stimulated cells are shown as dashed lines, and stimulated cells as solid lines. (C) Type I IFN production in the supernatants of mDCs harvested 6 or 24 h after treatment with CpG, DLP, TLP, or RRV (as described above) was measured by using a bioassay and expressed as U/ml. The figure shows data representative of at least three independent experiments.

converted to U/ml by comparing the samples to the muIFN- α/β standard (NIAID, NIH). IFN- α and IFN- β levels in DC supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits (PBL Biomedical Laboratories) according to the manufacturer's instructions.

Western blotting. For analysis of protein by Western blotting, cells were lysed on ice in lysis buffer (1% NP-40, 50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 2 mM EDTA), clarified by centrifugation at $6,000 \times g$ for 5 min in a microcentrifuge at 4°C, separated by SDS-PAGE, and transferred to Hybond-P (Amersham) membranes. Antibodies used for immunoblotting were polyclonal rabbit anti-RRV (K230 serum), rabbit anti-IRF-3 (Zymed), goat anti-IRF-3 (Santa Cruz), and goat antiactin (Santa Cruz). The secondary antibodies were horseradish perox-

idase-conjugated goat anti-rabbit serum (BD Biosciences) and rabbit anti-goat serum (DAKO). Chemiluminescence was detected using the ECL reagents (GE Healthcare). Membranes were stripped by washing in 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, for 30 min at 50°C, washed, blocked, and probed again as above.

Immunofluorescence. Antibodies used for immunofluorescence include mouse subgroup-specific monoclonal antibody 255 directed against trimeric VP6 (27), polyclonal rabbit anti-NSP4 antiserum (K3) (59, 73), goat anti-IRF-3 (Santa Cruz), and rabbit anti-NF- κ B p65 (Santa Cruz). Secondary antibodies were Cy2- and Cy3-conjugated donkey anti-goat sera, Cy3-conjugated donkey anti-mouse serum, and Texas Red-conjugated donkey anti-rabbit serum (all ML grade from Jackson ImmunoResearch). Staining was performed as previously described (55). Briefly, cells grown on coverslips were fixed by incubation in 4% paraformaldehyde (in phosphate-buffered saline) for 8 to 10 min at room temperature, followed by incubation in methanol for 8 to 10 min at -20°C . Coverslips were blocked, and primary antibodies were diluted in blocking buffer and incubated with the cells for 1 to 3 h. Secondary antibodies were diluted in blocking buffer containing 0.5 $\mu\text{g}/\text{ml}$ Hoechst 33258 (Molecular Probes) for identification of cell nuclei. Washed coverslips were then mounted in vinol mounting medium, and images were captured using a Leica DM RB fluorescent microscope with a Hamamatsu cooled charge-coupled device camera C4880. Images were processed and compiled using Adobe Photoshop software.

Statistical analysis. Where applicable, data are presented as mean values $\pm$ standard deviations (SD) of the means. Differences were tested for significance by the Student *t* test.

RESULTS

RRV infection stimulates DC activation and type I IFN production. To determine if rotavirus infection stimulates DC activation and type I IFN production, we first used three preparations of rotavirus: (i) crude supernatant from infected MA104 cells (RRV), (ii) purified DLPs, and (iii) purified TLPs. The RRV supernatant contains a mixture of DLP and TLP. The DLP lack the outer-capsid proteins VP4 and VP7 required for infectivity and, thus, they are noninfectious (entry-incompetent), while the TLP contain all structural proteins and thus are infectious (entry-competent) (71). The availability of the two purified preparations allowed us to determine if DCs responded differently to infectious and noninfectious virus. To confirm the protein composition of DLP and TLP, the same protein amount of each preparation was separated by SDS-PAGE and the viral proteins were visualized by Coomassie blue staining. As illustrated in Fig. 1A, VP2, VP5 (a trypsin cleavage product of VP4), VP6, and VP7 were detected in TLPs, while DLPs lacked the outer-capsid proteins.

We next treated mDC cultures overnight with the three virus preparations to determine if they stimulated DC activation. As a positive control, we included CpG-ODN, a TLR9 ligand previously shown to induce activation of murine mDC (32). RRV, TLP, and CpG stimulated mDCs to upregulate the surface expression of the costimulatory molecules CD86 and CD40, while treatment with DLP led to only a very minor upregulation of these molecules, suggesting that noninfectious rotavirus did not activate mDCs to a similar extent as infectious virus (Fig. 1B). Since one of the earliest responses to virus infection is the induction of type I IFN production, we next examined the levels of type I IFN in the supernatant of mDC cultures treated with RRV, DLP, TLP, or CpG. We found that mDCs produced type I IFN in response to RRV and TLP but not in response to noninfectious DLP (Fig. 1C). Type I IFN production in response to the virus was detectable only at the 24-hour time point, while CpG induced a high type I IFN response after 6 h, which had declined 24 h after stim-

FIG. 2. Analysis of IRF-3 and NF- κ B in RRV-infected cells. (A) MA104 cells were either mock infected or infected with RRV at an MOI of 1 and processed for immunofluorescence at 6 hpi with anti-RRV VP6 (green) and anti-IRF-3 (red). (B) Similarly, mock-infected (M) or infected (I) MA104 cells were lysed at 6 hpi and analyzed by Western blotting using anti-RRV serum (upper panel, positions of the viral proteins are indicated at the right), anti-IRF-3 (middle panel), and antiactin (lower panel). (C) MEF cells were either mock infected or infected with RRV or with rSFV-EGFP (both at an MOI of 1) and processed for immunofluorescence at 6 hpi with anti-RRV VP6 (green) or EGFP (green) and anti-IRF-3 (red). (D) Similarly, mock-infected (M) or infected (I) MEFs were lysed at 6 hpi and analyzed by Western blotting using anti-RRV (upper panel, positions of the viral proteins and a murine protein reacting nonspecifically with the serum [*]) are indicated at the right), anti-IRF-3 (middle panel), and antiactin (lower panel) sera. (E) MEF cells were either mock infected or infected with RRV or rSFV-EGFP at an MOI of 1 and processed for immunofluorescence at 6 hpi with anti-RRV VP6 (green) or EGFP (green) and anti-NF- κ B p65 (red). (F) Type I IFN production by MEFs and mDCs in response to RRV at 4, 10, and 24 hpi as determined by a bioassay is shown.

ulation (Fig. 1C). These data show that the ability of the different stimuli to activate mDCs paralleled their ability to induce type I IFN production, suggesting that these two events were linked.

Type I IFN production in RRV-infected MEFs is abrogated via degradation of IRF-3. Studies using RRV in the mouse model have been frequently described (9, 16, 17, 33). However, one possible explanation for why murine mDCs were capable of type I IFN production in response to rotavirus infection despite the reported effect of NSP1 on IRF-3 stability in MA104 (African green monkey) cells was that the interaction between NSP1 and IRF-3 was species-specific. NSP1 is the most highly variable of the rotavirus proteins (15, 40), and we therefore could not be certain that NSP1 would interact with murine IRF-3 and mediate its degradation as reported with MA104 cells. To investigate this, we set up parallel RRV infections of MA104 cells and MEFs using an MOI of 1. Viral protein synthesis and IRF-3 expression were analyzed by immunofluorescence at 6 h postinfection (hpi). The results dem-

onstrate that all IRF-3 was localized to the cytoplasm in mock-infected cells, while there was a complete and profound loss of IRF-3 in RRV-infected MA104 cells (Fig. 2A), as previously reported (7). Some IRF-3-positive cells were detected, but these were not infected, as shown by a lack of VP6 staining. A loss of IRF-3 was also observed when IRF-3 expression was examined by Western blotting analysis of lysates from cells harvested 6 hours after infection. The lysates were probed using a polyclonal antiserum against RRV to detect viral proteins and with an IRF-3-specific antibody (Fig. 2B). Similarly, we found no or very weak staining of IRF-3 in RRV-infected MEFs and there was no nuclear translocation of IRF-3 in any of the infected cells (Fig. 2C). As a control in the MEF experiment, we used rSFV-EGFP particles at an MOI of 1. SFV is a known inducer of type I IFN, and we have previously shown that rSFV-EGFP stimulates rapid translocation of IRF-3 into the nucleus of infected cells (29). We found that all rSFV-EGFP-positive cells exhibited a clear nuclear staining of IRF-3, despite low or not-yet-detectable levels of EGFP ex-

FIG. 3. Analysis of IRF-3 and viral protein synthesis in RRV-treated mDCs. (A) mDC cultures were either mock-infected (M) or inoculated with RRV at an MOI of 5, and lysates were prepared at 4, 24, and 48 hpi and analyzed by Western blotting using the anti-RRV K230 serum (upper panel). The positions of the viral proteins and a murine protein reacting nonspecifically with the serum (*) are indicated at the right. The lysates were also probed with an anti-IRF-3 (middle panel) and an antiactin (lower panel) antibody. (B) mDC cultures were either mock infected or treated with TLP at an MOI of 5 or treated with an equivalent amount of DLP. Cells were processed for immunofluorescence at 24 hpi using anti-RRV VP6 (green) and anti-NSP4 (red). (C) The presence of newly produced infectious virus in RRV-treated mDC cultures was determined by titrating the virus supernatant collected at 4, 10, and 24 h. The data are shown as peroxidase-forming units (PFU)/ml of supernatant over time.

pression in some individual cells at this early time point. At later time points (12 and 24 h), there were no viable cells in the RRV- or rSFV-infected cultures due to widespread virus-induced cell death in the culture (data not shown). When cell lysates from the RRV-infected MEFs were analyzed by Western blotting analysis, the expression of VP2 and VP6 was confirmed, but there was no detectable IRF-3 signal at 6 h postinfection (Fig. 2D). These data demonstrate that RRV infection efficiently degrades IRF-3 in MEFs.

Type I IFN production also requires the activation and nuclear translocation of NF- κ B. To determine if RRV-infected MEFs were capable of responding to RRV infection, we analyzed the subcellular localization of NF- κ B p65 in RRV-infected cells by immunofluorescence. Each slide was also analyzed for VP6 expression. rSFV-EGFP-infected MEFs were analyzed for NF- κ B activation and EGFP expression in parallel slides (Fig. 2E). We observed nuclear translocation of NF- κ B in both RRV- and rSFV-infected cells, demonstrating there was no defect in the ability of murine cells to recognize RRV and to respond to the infection. Finally, we compared type I IFN production in RRV-infected MEFs and RRV-infected mDCs using comparable cell numbers and viral MOI (Fig. 2F). Consistent with the results shown in Fig. 1C, mDCs were capable of type I IFN production in response to RRV, but there was no detectable type I IFN in the supernatant of the MEFs.

Viral protein synthesis in RRV-exposed mDCs but no detectable degradation of IRF-3. Having demonstrated that RRV infection led to IRF-3 degradation in MEFs, we refocused our attention on mDCs. We first performed Western blotting analysis on lysates from mDC cultures at different time points after RRV infection to investigate if the cells were productively infected with RRV (Fig. 3A, upper panel). mDCs were exposed to RRV at an MOI of 5 for 1 hour, after which the cells were extensively washed to remove any unbound virus and were then placed in fresh medium. As shown using the poly-

clonal antiserum against rotavirus, there was an increase in viral protein synthesis over time, with both VP2 and VP6 expression increasing between 4 and 24 h after infection. In contrast to RRV infection of MEFs, there was no detectable loss of cell viability during this time period (data not shown). This is in agreement with results reported for human DCs, where a thorough analysis of the cell viability after RRV infection showed no cytopathic effect under the conditions tested (64). Interestingly, when we examined the expression of IRF-3 by Western blotting analysis (Fig. 3A, middle panel), the levels were unaffected even at the time points where viral protein production was detected.

Next, we analyzed the mDCs for expression of the viral proteins VP6 and NSP4 using immunofluorescence. Here, cells were either mock infected or treated with equivalent quantities of DLP or TLP and analyzed at 24 h postinfection. The results demonstrate that only the cells treated with infectious TLP showed the presence of viral proteins (Fig. 3B). VP6 was expressed at high levels, and its cellular localization was similar to that in infected MEFs and MA104 cells. Interestingly, only a small proportion of these cells expressed NSP4 at detectable levels, consistent with studies of RRV infection of immature human DCs (64). We did not detect any VP6 or NSP4 signal from mock-infected cells or from cells exposed to the noninfectious DLP preparation (Fig. 3B). As the DLP preparation contains amounts of VP6 equal to that of the TLP preparation (Fig. 1A), this supports our interpretation that the signal detected in TLP-treated cells derives from newly produced viral proteins. Taken together, the results in Fig. 3A and B show that viral protein synthesis can be detected in mDC cultures. To determine if RRV infection of mDCs was productive in terms of progeny virus formation, supernatants from mDCs exposed to RRV for 4 h, 10 h, or 24 h were analyzed for infectious virus. We found that the viral titers declined over time (Fig. 3C). Thus, there was no measurable release of newly synthesized infectious virus particles from the mDC culture,

despite viral protein production being detected by Western blotting and immunofluorescence analysis (Fig. 3A and B).

Type I IFN production in response to UV-treated RRV and in IRF-3^{-/-} mDCs. The data presented above suggested that although the majority of the mDCs were exposed to infectious virus at a high MOI, viral protein synthesis was limited in these cells and/or that the viral life cycle was aborted before particle assembly. This led us to take two independent approaches to investigate whether viral protein synthesis suppressed the production of type I IFN from RRV-treated mDCs and whether this was mediated by targeting IRF-3. First, we generated attenuated RRV preparations by exposing the virus to different doses of UV treatment (see Materials and Methods). The preparations, termed UV'-RRV and UV''-RRV, were reduced in their infectious titer by, respectively, 10³-fold and 10⁴-fold compared to RRV. To confirm that the UV treatment had abrogated the ability of the viruses to direct viral protein synthesis, we exposed mDCs to RRV (MOI, 5) and the equivalent volumes of UV'-RRV and UV''-RRV, and cell lysates were taken after 24 or 48 h. As in the previous experiment, we were able to detect VP2 synthesis by RRV, but only very low levels of VP2 were observed in the lysates from mDCs exposed to the UV-treated viruses, likely representing input virus (Fig. 4A, upper panel). When probing the lysates for IRF-3, we found levels in mock-treated cells that were similar to those in cells treated with RRV, UV'-RRV, and UV''-RRV at each time point (Fig. 4A, middle panel), consistent with the data shown in Fig. 3A. The expression of IRF-3 was lower in the samples taken at 48 hpi than in samples taken at 24 hpi; however, this difference was also seen in the mock-treated cells and is therefore not a virus-specific effect. Interestingly, when the supernatants were analyzed for type I IFN, we found that mDCs exposed to the UV'-RRV or UV''-RRV produced significantly higher levels of type I IFN than mDCs exposed to RRV (P , <0.01). UV''-RRV, which had the lowest infectious titer, induced the highest levels of type I IFN (Fig. 4B). When the expression of the DC activation marker CD86 was analyzed, we found that cells exposed to the UV-inactivated virus expressed significantly higher levels of CD86 than cells exposed to RRV (P , <0.01; and P , <0.05 for UV'-RRV and UV''-RRV, respectively) (Fig. 4C). Similar results were obtained when analyzing CD40 expression (data not shown).

In a second approach to investigate a potential role for IRF-3 in RRV-induced type I IFN in mDCs, we exposed mDCs from mice lacking a functional gene for IRF-3 (IRF-3^{-/-} mice) to RRV. We first determined that GM-CSF-differentiated bone marrow-derived mDCs from wt and IRF-3^{-/-} mice have similar phenotypes in terms of DC-specific cell surface markers CD11b and CD11c (Fig. 5A). We next exposed the cells to equivalent amounts of DLP, TLP, and RRV, and the supernatants were harvested at 24 h after infection and analyzed for type I IFN production. Consistent with our previous results, there was no detectable production of type I IFN in DLP-treated cultures, while both TLP- and RRV-treated culture supernatants contained type I IFN, as determined by bioassay analysis (Fig. 5B) and by using an IFN- β ELISA (Fig. 5C). The level of type I IFN was markedly reduced in IRF-3^{-/-} mDCs compared to that in wt mDCs, consistent with a substantial fraction of the type I IFN in RRV- and TLP-treated mDCs being produced via an IRF-3-dependent pathway. Al-

FIG. 4. Analysis of viral protein synthesis, type I IFN production, and DC activation by UV-treated RRV. (A) mDC cultures were either mock infected (M) or treated with RRV, UV'-RRV, or UV''-RRV (MOI 5 or the equivalent amount), and lysates were prepared at 24 or 48 hpi and analyzed by Western blotting using the anti-RRV K230 serum (upper panel). The positions of VP2 and a murine protein reacting nonspecifically with the serum (*) are indicated at the right. The lysates were also probed with an anti-IRF-3 (middle panel) and an antiactin (lower panel) antibody. (B) Type I IFN production in the supernatants of mDCs harvested 24 h after treatment with RRV, UV'-RRV or UV''-RRV (MOI 5 or the equivalent amount) was measured using a bioassay and expressed as U/ml. (C) Upregulation of the CD86 activation marker on mDCs exposed to RRV, UV'-RRV, and UV''-RRV compared to those of untreated controls is shown as the percentage of increase in mean fluorescence intensity (MFI). The figure shows mean values $\pm$ SD from mDCs from three individual mice.

though IRF-3 degradation could not be detected in bulk lysates from RRV-exposed mDC, inactivation of virus by UV treatment enhanced the type I IFN response, suggesting the possibility of some level of NSP1-mediated IRF-3 degradation.

FIG. 5. Type I IFN production in virus-treated wt and IRF-3^{-/-} mDC cultures. (A) CD11b and CD11c expression on mDCs generated from the bone marrow of wt and IRF-3^{-/-} mice was determined by flow cytometry. Percentages of wt and IRF-3^{-/-} mDCs in the cultures are shown in the upper right quadrant (96% and 94%, respectively). (B and C) Type I IFN production in mock-treated (M) or DLP-, TLP-, or RRV-treated wt and IRF-3^{-/-} mDCs was measured using a bioassay and (C) an IFN-β ELISA.

However, some IRF-3 remains active as the type I IFN response was reduced in IRF-3^{-/-} mDCs.

Type I IFN production and mDC activation in RRV-treated IFNAR1^{-/-} cultures. As described above, we found that exposure of mDC to RRV and TLP stimulated mDCs to upregulate costimulatory molecules and to induce type I IFN production (Fig. 1B and C) and that UV inactivation enhanced both the type I IFN response and the activation of mDCs (Fig. 4). We have previously shown that upregulation of costimulatory markers on mDC in response to rSFV is dependent on type I IFN signaling (30). However, since the type I IFN levels induced by RRV were relatively low, it was possible that other factors induced by RRV would be more important for DC activation than type I IFN. To investigate this, we stimulated mDC cultures from wt and IFNAR1^{-/-} mice with CpG, RRV, and rSFV. The results show that RRV-induced upregulation of CD40 was dependent on an intact type I IFN signaling pathway (Fig. 6). Similar results were obtained with CpG and rSFV, in agreement with previous reports (30, 32).

FIG. 6. Role of type I IFN signaling for mDC activation in response to RRV. The expression of the costimulatory molecule CD40 on wt and IFNAR1^{-/-} mDCs treated with CpG (1 μM), RRV (MOI, 5), or rSFV (MOI, 5) is shown. (A) Mock-treated cells are shown as dashed lines, and stimulated cells are shown as solid lines. (B) Percentage increase in mean fluorescence intensity (MFI) of CD40 from the same experiment as shown in panel A.

Induction of type I IFN in RRV-treated mDCs from MyD88^{-/-} and TLR3^{-/-} mice. To investigate the pathway by which RRV induces type I IFN, we exposed mDCs from wt, MyD88^{-/-}, and TLR3^{-/-} mice to RRV and to the UV-inactivated RRV preparations. The results demonstrate that similar type I IFN levels were induced in wt, MyD88^{-/-}, and TLR3^{-/-} mDCs in response to each of the virus preparations (Fig. 7), suggesting that type I IFN induction by mDCs in response to RRV was not induced via a TLR-dependent pathway.

FIG. 7. Type I IFN production in mDCs from MyD88^{-/-} and TLR3^{-/-} mice. Type I IFN production in the supernatants of mDCs from MyD88^{-/-} and TLR3^{-/-} mice and their respective wt controls (B6 for MyD88^{-/-} and 129/B6 for TLR3^{-/-} mice) harvested 24 h after treatment with RRV, UV'-RRV, or UV''-RRV (MOI 5 or the equivalent amount) was measured using a bioassay and expressed as U/ml. The figure shows mean values ± SD from three individual mice.

FIG. 8. Type I IFN production in rotavirus-treated DC cultures generated in the presence of Flt3-L. (A) Phenotypes of bone marrow-derived DCs generated in the presence of Flt3-L. CD11c⁺ CD11b[−] B220⁺ (pDCs) and CD11c⁺ CD11b⁺ B220[−] (mDCs) are shown in the panels to the right. (B) Comparison of type I IFN production measured with a bioassay, an IFN- α ELISA, or an IFN- β ELISA from DC cultures generated in the presence of GM-CSF (mDCs) or Flt3-L (mixed pDCs and mDCs) 24 h after RRV stimulation. (C) Type I IFN levels in Flt3-L culture supernatants 6 h after treatment with DLP, TLP, and RRV were measured using the bioassay. The figure shows data representative of two independent experiments.

Comparative analysis of type I IFN levels in Flt3-L and GM-CSF-differentiated cultures. Several studies have reported that pDCs are unique in their ability to produce high levels of type I IFN in response to viral infection and in response to synthetic ligands recognized by TLR7/8 and TLR9 (4, 12). One important reason for this is thought to be that pDCs express constitutive levels of IRF-7 (6), a transcription factor that other cells express only upon IFNAR signaling (50). Since IRF-7 can substitute for IRF-3 in pDCs, we were interested in determining if pDCs were capable of producing type I IFN in response to RRV. To investigate this, we performed experiments in Flt3-L-differentiated DC cultures in parallel with GM-CSF-differentiated cultures. Flow cytometric analysis of the Flt3-L cultures confirmed the presence of both CD11b negative/B220 high cells (pDCs) and CD11b high/B220 low cells (mDCs) (Fig. 8A). Since there are no pDCs in GM-CSF cultures (24), we compared Flt3-L- and GM-CSF-derived cultures side-by-side as a way to examine the type I IFN-inducing potential of pDCs in response to RRV (Fig. 8B). The levels of type I IFN in supernatants at 24 h after stimulation with RRV were measured using a bioassay (Fig. 8B, left panel), an IFN- α ELISA (Fig. 8B, middle panel), and an IFN- β ELISA (Fig. 8B, right panel). In all three assays, we found that the response to RRV was markedly higher in Flt3-L-derived cultures than in GM-CSF-derived cultures. The difference was greatest in the IFN- α ELISA, consistent with the constitutive expression of IRF-7 in pDCs and their ability to produce all IFN- α subtypes. To determine if this response required an infectious virus, Flt3-L cultures were exposed to DLP, TLP, and RRV, and supernatants from the virus-treated cultures were analyzed 6 h after stimulation. Both TLP and RRV, but not DLP, induced type I IFN production at this early time point (Fig. 8C), showing that infectious virus is required for type I IFN production also by Flt3-L cultures. Interestingly, the production of type I IFN was more rapid in Flt3-L cultures than in GM-CSF cultures, with measurable levels being detected as early as 6 h after virus stimulation. This is different from the response with GM-CSF cultures, which was detectable 24 h after stimulation but not 6 h after stimulation (Fig. 1C).

DISCUSSION

The roles of type I IFN for the early control of rotavirus infection and for the generation of rotavirus-specific adaptive immune responses are not fully understood. Studies of IFNAR1^{−/−} mice have shown that mice lacking a functional type I IFN signaling pathway do not show more severe signs of disease or increased rotavirus shedding than wt control mice, suggesting that type I IFN play a minor role in the immediate control of the infection (3). In contrast, adult mice lacking the functional signal transducer and activator of transcription 1 (Stat1^{−/−} mice) shed more than 100-fold more virus than wt control mice when infected with murine rotavirus or with RRV (74). Independent of the role of type I IFN in providing immediate control of viral replication, it is of interest to know if rotavirus-exposed DCs are capable of type I IFN production, since early type I IFN responses may contribute to shaping long-lived adaptive immune responses. Several recent reports (7, 26, 39) prompted us to investigate the ability of RRV to induce type I IFN and to activate DCs in vitro. These studies show that RRV encodes a protein, NSP1, which interacts with IRF-3 and mediates its degradation in infected MA104 cells (7). Hence, one of the most central pathways for type I IFN induction is rendered defective by the virus, leading to a suppressed type I IFN response.

Here, we have investigated the ability of infectious and non-infectious rotavirus preparations to activate DCs and to induce type I IFN. We show that MEFs respond to RRV infection by rapid NF- κ B activation, suggesting that RRV does not interfere with the ability of cytosolic double-stranded RNA receptors or adaptor proteins (38) to recognize incoming viral genomes and to transmit the signal leading to the activation of transcription factors that regulate type I IFN production. Our results show that IRF-3 is degraded in RRV-infected MEFs and that these cells are unable to produce detectable levels of type I IFN. This is consistent with the report of NSP1-mediated IRF-3 degradation in MA104 cells (7). In contrast, RRV-treated DC cultures were capable of type I IFN production. The levels were relatively low but sufficient to induce upregu-

lation of costimulatory molecules. Despite viral protein synthesis in RRV-treated mDCs, as demonstrated by NSP4 and VP6 expression, there was no production of new infectious virus particles in the supernatant from these cells and there was no detectable virus-induced cytopathic effect in the culture (data not shown). This suggests that the viral life cycle is aborted at a stage prior to viral assembly or release, possibly because of a higher constitutive antiviral state in DCs than in other cells (29). Interestingly, when mDCs were exposed to UV-treated RRV, significantly higher levels of type I IFN were measured. This result is consistent with early reports of type I IFN induction by rotavirus, which showed that increasing doses of UV treatment enhanced the ability of the virus to induce type I IFN in MA104 cells (56, 75). It is also consistent with a role of virally encoded proteins in suppressing the type I IFN response, as UV treatment abolishes the ability of the viral RNA to replicate and to act as a template for protein production. We, and others, have previously shown that productive viral infection is not required for DC activation and type I IFN induction, since these can occur even in response to entry-competent UV-inactivated virus but not in response to entry-deficient virus (29, 70). The results obtained with the DLP-, TLP-, and UV-treated RRV preparations in this study are consistent with these results. It remains possible that UV treatment has additional effects on the virus that contribute to its enhanced ability to induce type I IFN, so this should also be taken into consideration.

When IRF-3 expression was examined in RRV-treated mDCs, there was no detectable loss of expression over time in repeated experiments. This could be because only a small fraction of the cells expressed NSP1 and degradation of IRF-3 in a small fraction of cells would not be detectable in bulk cell lysates. Additionally, DCs may restrict viral protein synthesis such that sufficient NSP1 levels needed to degrade all IRF-3 would not be accumulated. The effect of virally encoded type I IFN antagonists may therefore be less profound in DCs than in other cells, which could explain the differences in type I IFN production we observed between DCs and MEFs. Our results are in agreement with a recent study examining the interaction of rotavirus with human DCs, in which only a low percentage of immature DCs expressed nonstructural protein 4 (64). We were unable to analyze the expression of NSP1 in mDCs since an NSP1-specific antibody was not available to us. Instead, we used mDCs from IRF-3^{-/-} mice to investigate the role of IRF-3 for type I IFN production in response to DLP, TLP, and RRV. Our results show that a substantial part of the type I IFN response in RRV-treated mDCs is mediated by an IRF-3-dependent pathway, suggesting that complete inactivation of IRF-3 is not achieved. It is possible that other IRFs, such as IRF-7, could substitute for IRF-3 to drive the remaining response. Examination of RRV-treated IRF7^{-/-} DCs would shed light on this issue. Collectively these data suggest that mDCs are susceptible to RRV-mediated IRF-3 degradation but that not all IRF-3 in the responding cells is degraded. Furthermore, the entry-incompetent DLP preparation was consistently negative for type I IFN production, which served as a valuable control for nonspecific type I IFN responses in these experiments. Our results using mDCs from MyD88^{-/-} and TLR3^{-/-} mice also show that signaling through TLRs was not required for type I IFN induction, which suggests that

cytosolic recognition events mediated type I IFN induction in response to RRV. We do not rule out the possibility that viral components released from dying cells during productive viral infection *in vivo* may activate TLR-dependent pathways that could contribute to type I IFN induction. This stimulates interesting questions as to which pathway(s) is responsible for type I IFN induction following rotavirus infection.

Despite the relatively low levels of type I IFN production resulting from mDCs exposed to RRV, we found that these levels were sufficient to induce DC activation and that this response was dependent on type I IFN receptor signaling. This is similar to our previous work where we used single-round infectious rSFV to stimulate mDCs and the upregulation of costimulatory molecules was shown to be type I IFN dependent (30). Future studies are required to determine if rotavirus mutants lacking a functional NSP1 would induce higher type I IFN levels in DC cultures and *in vivo* and if such viruses would generate quantitatively and/or qualitatively distinct rotavirus-specific adaptive immune responses. Influenza viruses with different abilities to induce activation of DCs were recently examined (19). It was shown that viruses lacking a functional NS1 were more potent in inducing type I IFN production and in activating DCs. Attenuation of viruses by deleting type I IFN antagonists may therefore be interesting in the context of vaccine development, both because removal of virulence genes may create safer vaccines and because type I IFN have been shown to provide an adjuvant effect for both systemic and mucosal immune responses (10, 30). Serial passage of RRV at a high MOI has been shown to select for virus that has lost a functional NSP1 product (39); hence, this could be one way to generate RRV preparations lacking NSP1. Targeted manipulation of the rotavirus genome has so far been hindered by the lack of a reverse genetics system. However, the recent success in establishing such a system (42) may open up new avenues for rotavirus-based vaccine design and pathogenesis studies.

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REFERENCES

- Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9:143–150.
- Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413:732–738.
- Angel, J., M. A. Franco, H. B. Greenberg, and D. Bass. 1999. Lack of a role for type I and type II interferons in the resolution of rotavirus-induced diarrhea and infection in mice. *J. Interferon Cytokine Res.* 19:655–659.
- Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Briere, and G. Trinchieri. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat. Immunol.* 2:1144–1150.
- Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245–252.
- Barchet, W., M. Cella, B. Odermatt, C. Asselin-Paturel, M. Colonna, and U. Kalinke. 2002. Virus-induced interferon alpha production by a dendritic cell subset in the absence of feedback signaling *in vivo*. *J. Exp. Med.* 195:507–516.
- Barro, M., and J. T. Patton. 2005. Rotavirus nonstructural protein 1 subverts

- innate immune response by inducing degradation of IFN regulatory factor 3. *Proc. Natl. Acad. Sci. USA* **102**:4114–4119.
8. Basler, C. F., A. Mikulasova, L. Martinez-Sobrido, J. Paragas, E. Mühlberger, M. Bray, H.-D. Klenk, P. Palese, and A. García-Sastre. 2003. The Ebola virus VP30 protein inhibits activation of interferon regulatory factor 3. *J. Virol.* **77**:7945–7956.
 9. Blutt, S. E., M. Fenaux, K. L. Warfield, H. B. Greenberg, and M. E. Conner. 2006. Active viremia in rotavirus-infected mice. *J. Virol.* **80**:6702–6705.
 10. Bracci, L., I. Canini, S. Puzelli, P. Sestili, M. Venditti, M. Spada, I. Donatelli, F. Belardelli, and E. Proietti. 2005. Type I IFN is a powerful mucosal adjuvant for a selective intranasal vaccination against influenza virus in mice and affects antigen capture at mucosal level. *Vaccine* **23**:2994–3004.
 11. Brimmes, M. K., L. Bonifaz, R. M. Steinman, and T. M. Moran. 2003. Influenza virus-induced dendritic cell maturation is associated with the induction of strong T cell immunity to a coadministered, normally nonimmunogenic protein. *J. Exp. Med.* **198**:133–144.
 12. Colonna, M., A. Krug, and M. Cella. 2002. Interferon-producing cells: on the front line in immune responses against pathogens. *Curr. Opin. Immunol.* **14**:373–379.
 13. Dharakul, T., M. Riepenhoff-Talty, B. Albin, and P. L. Ogra. 1988. Distribution of rotavirus antigen in intestinal lymphoid tissues: potential role in development of the mucosal immune response to rotavirus. *Clin. Exp. Immunol.* **74**:14–19.
 14. Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and E. S. C. Reis. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**:1529–1531.
 15. Dunn, S. J., T. L. Cross, and H. B. Greenberg. 1994. Comparison of the rotavirus nonstructural protein NSP1 (NS53) from different species by sequence analysis and northern blot hybridization. *Virology* **203**:178–183.
 16. Fenaux, M., M. A. Cuadras, N. Feng, M. Jaimes, and H. B. Greenberg. 2006. Extraintestinal spread and replication of a homologous EC rotavirus strain and a heterologous rhesus rotavirus in BALB/c mice. *J. Virol.* **80**:5219–5232.
 17. Feng, N., J. W. Burns, L. Bracy, and H. B. Greenberg. 1994. Comparison of mucosal and systemic humoral immune responses and subsequent protection in mice orally inoculated with a homologous or a heterologous rotavirus. *J. Virol.* **68**:7766–7773.
 18. Fensterl, V., D. Grotheer, I. Berk, S. Schlemminger, A. Vallbracht, and A. Dotzauer. 2005. Hepatitis A virus suppresses RIG-I-mediated IRF-3 activation to block induction of beta interferon. *J. Virol.* **79**:10968–10977.
 19. Fernandez-Sesma, A., S. Marukian, B. J. Ebersole, D. Kaminski, M. S. Park, T. Yuen, S. C. Sealton, A. Garcia-Sastre, and T. M. Moran. 2006. Influenza virus evades innate and adaptive immunity via the NS1 protein. *J. Virol.* **80**:6295–6304.
 20. Fleeton, M. N., N. Contractor, F. Leon, J. D. Wetzel, T. S. Dermody, and B. L. Kelsall. 2004. Peyer's patch dendritic cells process viral antigen from apoptotic epithelial cells in the intestine of reovirus-infected mice. *J. Exp. Med.* **200**:235–245.
 21. Foy, E., K. Li, R. Sumpter, Jr., Y. M. Loo, C. L. Johnson, C. Wang, P. M. Fish, M. Yoneyama, T. Fujita, S. M. Lemon, and M. Gale, Jr. 2005. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-1 signaling. *Proc. Natl. Acad. Sci. USA* **102**:2986–2991.
 22. Foy, E., K. Li, C. Wang, R. Sumpter, Jr., M. Ikeda, S. M. Lemon, and M. Gale, Jr. 2003. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* **300**:1145–1148.
 23. Franco, M. A., J. Angel, and H. B. Greenberg. 2006. Immunity and correlates of protection for rotavirus vaccines. *Vaccine* **24**:2718–2731.
 24. Gilliet, M., A. Boonstra, C. Paturel, S. Antonenko, X. L. Xu, G. Trinchieri, A. O'Garra, and Y. J. Liu. 2002. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **195**:953–958.
 25. Glass, R. I., J. Bresee, B. Jiang, U. Parashar, E. Yee, and J. Gentsch. 2006. Rotavirus and rotavirus vaccines. *Adv. Exp. Med. Biol.* **582**:45–54.
 26. Graff, J. W., D. N. Mitzel, C. M. Weisend, M. L. Flenniken, and M. E. Hardy. 2002. Interferon regulatory factor 3 is a cellular partner of rotavirus NSP1. *J. Virol.* **76**:9545–9550.
 27. Greenberg, H., V. McAuliffe, J. Valdesuso, R. Wyatt, J. Flores, A. Kalica, Y. Hoshino, and N. Singh. 1983. Serological analysis of the subgroup protein of rotavirus, using monoclonal antibodies. *Infect. Immun.* **39**:91–99.
 28. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **303**:1526–1529.
 29. Hidmark, Å. S., G. M. McInerney, E. K. L. Nordström, 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.
 30. Hidmark, Å. S., E. K. Nordström, P. Dosenovic, M. N. E. Forsell, P. Liljestrom, and G. B. Karlsson Hedestam. 2006. Humoral responses against coimmunized protein antigen but not against alphavirus-encoded antigens require alpha/beta interferon signaling. *J. Virol.* **80**:7100–7110.
 31. Honda, K., S. Sakaguchi, C. Nakajima, A. Watanabe, H. Yanai, M. Matsumoto, T. Ohteki, T. Kaisho, A. Takaoka, S. Akira, T. Seya, and T. Taniguchi. 2003. Selective contribution of IFN-alpha/beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. *Proc. Natl. Acad. Sci. USA* **100**:10872–10877.
 32. Hoshino, K., T. Kaisho, T. Iwabe, O. Takeuchi, and S. Akira. 2002. Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. *Int. Immunol.* **14**:1225–1231.
 33. Ishida, S.-I., N. Feng, B. Tang, J. M. Gilbert, and H. B. Greenberg. 1996. Quantification of systemic and local immune responses to individual rotavirus proteins during rotavirus infection in mice. *J. Clin. Microbiol.* **34**:1694–1700.
 34. Izaguirre, A., B. J. Barnes, S. Amrute, W. S. Yeow, N. Megjugorac, J. Dai, D. Feng, E. Chung, P. M. Pitha, and P. Fitzgerald-Bocarsly. 2003. Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells. *J. Leukoc. Biol.* **74**:1125–1138.
 35. Karlsson, G. B., and P. Liljestrom. 2003. Live viral vectors: Semliki Forest virus. *Methods Mol. Med.* **87**:69–82.
 36. Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi, and S. Akira. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* **23**:19–28.
 37. Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K. J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C. S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, and S. Akira. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**:101–105.
 38. Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* **6**:981–988.
 39. Kearney, K., D. Chen, Z. F. Taraporewala, P. Vende, Y. Hoshino, M. A. Torrici, M. Barro, and J. T. Patton. 2004. Cell-line-induced mutation of the rotavirus genome alters expression of an IRF3-interacting protein. *EMBO J.* **23**:4072–4081.
 40. Kojima, K., K. Taniguchi, and N. Kobayashi. 1996. Species-specific and interspecies relatedness of NSP1 sequences in human, porcine, bovine, feline, and equine rotavirus strains. *Arch. Virol.* **141**:1–12.
 41. Komatsu, T., K. Takeuchi, J. Yokoo, and B. Gotoh. 2004. C and V proteins of Sendai virus target signaling pathways leading to IRF-3 activation for the negative regulation of interferon-beta production. *Virology* **325**:137–148.
 42. Komoto, S., J. Sasaki, and K. Taniguchi. 2006. Reverse genetics system for introduction of site-specific mutations into the double-stranded RNA genome of infectious rotavirus. *Proc. Natl. Acad. Sci. USA* **103**:4646–4651.
 43. Krug, A., G. D. Luker, W. Barchet, D. A. Leib, S. Akira, and M. Colonna. 2003. Herpes simplex virus type 1 (HSV-1) activates murine natural interferon-producing cells through toll-like receptor 9. *Blood* **103**:1433–1437.
 44. Kumar, H., T. Kawai, H. Kato, S. Sato, K. Takahashi, C. Coban, M. Yamamoto, S. Uematsu, K. J. Ishii, O. Takeuchi, and S. Akira. 2006. Essential role of IPS-1 in innate immune responses against RNA viruses. *J. Exp. Med.* **203**:1795–1803.
 45. Le Bon, A., V. Durand, E. Kamphuis, C. Thompson, S. Bulfone-Paus, C. Rossmann, U. Kalinke, and D. F. Tough. 2006. Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming. *J. Immunol.* **176**:4682–4689.
 46. Le Bon, A., N. Etchart, C. Rossmann, M. Ashton, S. Hou, D. Gewert, P. Borrow, and D. F. Tough. 2003. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nat. Immunol.* **4**:1009–1015.
 47. Le Bon, A., G. Schiavoni, G. D'Agostino, I. Gresser, F. Belardelli, and D. F. Tough. 2001. Type I interferons potentially enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity* **14**:461–470.
 48. Le Bon, A., C. Thompson, E. Kamphuis, V. Durand, C. Rossmann, U. Kalinke, and D. F. Tough. 2006. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *J. Immunol.* **176**:2074–2078.
 49. Le Bon, A., and D. F. Tough. 2002. Links between innate and adaptive immunity via type I interferon. *Curr. Opin. Immunol.* **14**:432–436.
 50. Levy, D. E., I. Marie, E. Smith, and A. Prakash. 2002. Enhancement and diversification of IFN induction by IRF-7-mediated positive feedback. *J. Interferon Cytokine Res.* **22**:87–93.
 51. Liljestrom, P., S. Lusa, D. Huylebroeck, and H. Garoff. 1991. In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *J. Virol.* **65**:4107–4113.
 52. Lopez, C. B., B. Moltedo, L. Alexopoulou, L. Bonifaz, R. A. Flavell, and T. M. Moran. 2004. TLR-independent induction of dendritic cell maturation and adaptive immunity by negative-strand RNA viruses. *J. Immunol.* **173**:6882–6889.
 53. Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki. 2003. Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells. *J. Exp. Med.* **198**:513–520.
 54. Lund, J. M., L. Alexopoulou, A. Sato, M. Karow, N. C. Adams, N. W. Gale, A. Iwasaki, and R. A. Flavell. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* **101**:5598–5603.

55. **McInerney, G. M., N. L. Kedersha, R. J. Kaufman, P. Anderson, and P. Liljestrom.** 2005. Importance of eIF2alpha phosphorylation and stress granule assembly in alphavirus translation regulation. *Mol. Biol. Cell* **16**:3753–3763.
56. **McKimm-Breschkin, J. L., and I. H. Holmes.** 1982. Conditions required for induction of interferon by rotaviruses and for their sensitivity to its action. *Infect. Immun.* **36**:857–863.
57. **Melroe, G. T., N. A. DeLuca, and D. M. Knipe.** 2004. Herpes simplex virus 1 has multiple mechanisms for blocking virus-induced interferon production. *J. Virol.* **78**:8411–8420.
58. **Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp.** 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **437**:1167–1172.
59. **Mirazimi, A., M. Nilsson, and L. Svensson.** 1998. The molecular chaperone calnexin interacts with the NSP4 enterotoxin of rotavirus in vivo and in vitro. *J. Virol.* **72**:8705–8709.
60. **Mirazimi, A., and L. Svensson.** 2000. ATP is required for correct folding and disulfide bond formation of rotavirus VP7. *J. Virol.* **74**:8048–8052.
61. **Montoya, M., G. Schiavoni, F. Mattei, I. Gresser, F. Belardelli, P. Borrow, and D. F. Tough.** 2002. Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* **99**:3263–3271.
62. **Mossel, E. C., and R. F. Ramig.** 2003. A lymphatic mechanism of rotavirus extraintestinal spread in the neonatal mouse. *J. Virol.* **77**:12352–12356.
63. **Muller, U., U. Steinhoff, L. F. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet.** 1994. Functional role of type I and type II interferons in antiviral defense. *Science* **264**:1918–1921.
64. **Narvaez, C. F., J. Angel, and M. A. Franco.** 2005. Interaction of rotavirus with human myeloid dendritic cells. *J. Virol.* **79**:14526–14535.
65. **Proietti, E., L. Bracci, S. Puzelli, T. Di Pucchio, P. Sestili, E. De Vincenzi, M. Venditti, I. Capone, I. Seif, E. De Maeyer, D. Tough, I. Donatelli, and F. Belardelli.** 2002. Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model. *J. Immunol.* **169**:375–383.
66. **Rollo, E. E., K. P. Kumar, N. C. Reich, J. Cohen, J. Angel, H. B. Greenberg, R. Sheth, J. Anderson, B. Oh, S. J. Hempson, E. R. Mackow, and R. D. Shaw.** 1999. The epithelial cell response to rotavirus infection. *J. Immunol.* **163**:4442–4452.
67. **Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T. Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, and T. Taniguchi.** 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity* **13**:539–548.
68. **Seth, R. B., L. Sun, C. K. Ea, and Z. J. Chen.** 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122**:669–682.
69. **Smerdou, C., and P. Liljestrom.** 1999. Two-helper RNA system for production of recombinant Semliki Forest virus particles. *J. Virol.* **73**:1092–1098.
70. **Spiegel, M., K. Schneider, F. Weber, M. Weidmann, and F. T. Hufert.** 2006. Interaction of severe acute respiratory syndrome-associated coronavirus with dendritic cells. *J. Gen. Virol.* **87**:1953–1960.
71. **Svensson, L.** 1984. Identification of an outer capsid glycoprotein of human rotavirus by concanavalin A. *J. Gen. Virol.* **65**:2183–2190.
72. **Svensson, L., P. R. Dormitzer, C. H. von Bonsdorff, L. Maunula, and H. B. Greenberg.** 1994. Intracellular manipulation of disulfide bond formation in rotavirus proteins during assembly. *J. Virol.* **68**:5204–5215.
73. **Tafazoli, F., C. Q. Zeng, M. K. Estes, K. E. Magnusson, and L. Svensson.** 2001. NSP4 enterotoxin of rotavirus induces paracellular leakage in polarized epithelial cells. *J. Virol.* **75**:1540–1546.
74. **VanCott, J. L., M. M. McNeal, J. Flint, S. A. Bailey, A. H. Choi, and R. L. Ward.** 2001. Role for T cell-independent B cell activity in the resolution of primary rotavirus infection in mice. *Eur. J. Immunol.* **31**:3380–3387.
75. **Welch, A. B., and M. J. Twiehaus.** 1973. Cell culture studies of a neonatal calf diarrhea virus. *Can. J. Comp. Med.* **37**:287–294.
76. **Xu, L. G., Y. Y. Wang, K. J. Han, L. Y. Li, Z. Zhai, and H. B. Shu.** 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol. Cell* **19**:727–740.
77. **Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita.** 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* **5**:730–737.