Induction and Inhibition of Type I Interferon Responses by Distinct Components of Lymphocytic Choriomeningitis Virus

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Type I interferons (IFNs) play a critical role in the host defense against viruses. Lymphocytic choriomeningitis virus (LCMV) infection induces robust type I IFN production in its natural host, the mouse. However, the mechanisms underlying the induction of type I IFNs in response to LCMV infection have not yet been clearly defined. In the present study, we demonstrate that IRF7 is required for both the early phase (day 1 postinfection) and the late phase (day 2 postinfection) of the type I IFN response to LCMV. We further demonstrate that LCMV genomic RNA itself (without other LCMV components) is able to induce type I IFN responses in various cell types by activation of the RNA helicases retinoic acid-inducible gene I (RIG-I) and MDA5. We also show that expression of the LCMV nucleoprotein (NP) inhibits the type I IFN response induced by LCMV RNA and other RIG-I/MDA5 ligands. These virus-host interactions may play important roles in the pathogenesis of LCMV and other human arenavirus diseases.

Type I interferons (IFNs), namely, alpha interferon (IFN-α) and IFN-β, are not only essential for host innate defense against viral pathogens but also critically modulate the development of virus-specific adaptive immune responses (6, 8, 28, 30, 36, 50, 61). The importance of type I IFNs in host defense has been demonstrated by studying mice deficient in the type I IFN receptor, which are highly susceptible to most viral pathogens (2, 47, 62).

Recent studies have suggested that the production of type I IFNs is controlled by different innate pattern recognition receptors (PRRs) (19, 32, 55, 60). There are three major classes of PRRs, including Toll-like receptors (TLRs) (3, 40), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (25, 48, 51), and nucleotide oligomerization domain (NOD)-like receptors (9, 22). TLRs are a group of transmembrane proteins expressed on either cell surfaces or endosomal compartments. RLRs localize in the cytosol. Both TLRs and RLRs are involved in detecting viral pathogens and controlling the production of type I IFNs (52, 60). In particular, the endosome-localized TLRs (TLR3, TLR7/8, and TLR9) play important roles in detecting virus-derived double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and DNA-containing unmethylated CpG motifs, respectively. In contrast, RIG-I detects virus-derived ssRNA with 5’-triphosphates (5’-PPPs) or short dsRNA (<1 kb), whereas melanoma differentiation-associated gene 5 (MDA5) is responsible for recognizing virus-derived long dsRNA as well as a synthetic mimic of viral dsRNA poly(I):poly(C) [poly(I · C)] (24, 60). Recognition of viral pathogen-associated molecular patterns (PAMPs) ultimately leads to the activation and nuclear translocation of interferon regulatory factors (IRFs) and nuclear factor κB (NF-κB), which, in turn, switches on a cascade of genes controlling the production of both type I IFNs and other proinflammatory cytokines (10, 11, 60).

Lymphocytic choriomeningitis virus (LCMV) infection in its natural host, the mouse, is an excellent system to study the impact of virus-host interactions on viral pathogenesis and to address important issues related to human viral diseases (1, 45, 49, 67). LCMV infection induces type I IFNs as well as other proinflammatory chemokines and cytokines (6, 41). Our previous studies have demonstrated that TLR2, TLR6, and CD14 are involved in LCMV-induced proinflammatory chemokines and cytokines (66). The mechanism by which LCMV induces type I IFN responses, however, has not been clearly defined (7, 8, 31, 44). The role of the helicase family members RIG-I and MDA5 in virus-induced type I IFN responses has been recently established. RIG-I has been found to be critical in controlling the production of type I IFN in response to a number of RNA viruses, including influenza virus, rabies virus, Hantaan virus, vesicular stomatitis virus (VSV), Sendai virus (SeV), etc. In contrast, MDA5 is required for responses to picornaviruses (15, 25, 63).

In the present study, we demonstrated that LCMV genomic RNA strongly activates type I IFNs through a RIG-I/MDA5-dependent signaling pathway. Our present study further demonstrated that the LCMV nucleoprotein (NP) blocks LCMV RNA- and other viral ligand-induced type I IFN responses.

MATERIALS AND METHODS

Virus, cells, mice, and plasmids. LCMV-Armstrong (LCMV-Arm) strain 53b was kindly provided by Raymond Welsh and Lisa K. Selin (University of Massachusetts Medical School, MA) and was propagated on BHK-21 cells (ATCC) at a multiplicity of infection (MOI) of 0.01. Viral titers were determined with an immunological focus assay using antibody against LCMV NP VL4, kindly provided by Demetrius Moskophidis, Medical College of Georgia (5). The VSV-
Indiana serotype was used (58). Virus stocks were prepared on BHK-21 cells infected at a MOI of 0.01. Viral titers were determined by plaque assay on Vero cells (56, 65, 66). MDA5 knockout (KO) and MAVS KO mice were kindly provided by M. Colonna (Washington University) (15) and Z. J. Chen (UT Southwestern Medical Center) (54), respectively. IRF7 KO (19) and IRF5 KO (53) mice were kindly provided by Tedagawa Taniguchi and Atsushi Yoshikai (Riken BioResource Center, Japan) and Michael David (University of California, San Diego), respectively. 3d mice were kindly provided by B. Beutler (The Scripps Research Institute) (59). All KO mice were backcrossed for more than six generations; MAVS KO, IRF7 KO, and IRF3 KO mice were backcrossed with C57BL/6 mice, and MDA5 KO mice were backcrossed with 129S1/SvImJ mice (WT mice were obtained from Jackson Laboratories). Mice were infected intraperitoneally (i.p.) with 2 × 10^6 PFU of LCMV-Arm clone 53b. Serum samples were collected at the indicated time points.

A mutant RIG-I construct lacking the CARD domains as a dominant negative (DN) RIG-I (DN-RIG-C) was kindly provided by Takashi Fujita (Kyoto University, Kyoto, Japan). To construct plasmid-expressing V5/histidine-tagged full-length LCMV nucleoprotein (NP), reverse transcription-PCR (RT-PCR) was performed. Briefly, LCMV-Arm genomic RNA (LCMV RNA) was extracted using Trizol reagent (Invitrogen) as described below. First-strand cDNA was synthesized using the SuperScript III reverse transcriptase (Invitrogen) and primer GCCACAGGGTCTAGATGTCGACAC (corresponding to LCMV small RNA positions 1627 to 1647) and primer GGTTCAAGATTGTGACAATG-3 (EcoRV restriction site is underlined). PCR was performed using GeneAmp PCR system 9700 to amplify the full-length cDNA encoding LCMV NP. PCR products, digested with both BamHI and EcoRV, were ligated directly into the pcDNA3-V5/His vector (Invitrogen) similarly digested with both BamHI and EcoRV, generating plasmid pcDNA3-V5/His-NP, also confirmed as described previously by restriction analysis and DNA sequencing (Tufts University Core Facility).

To generate LCMV NP mutants (amino acid substitution for alanine) at amino acids D382 (D382A) and G385 (G385A), the parent plasmid encoding wild-type (WT) LCMV NP (pcDNA3-NP) and the QuikChange site-directed mutagenesis kit (Stratagene) were used by following the manufacturer’s recommendations. The mutations were verified by sequence analysis. The expression of both the LCMV WT and mutant NP was verified by flow cytometry using anti-LCMV NP antibody VL4 (5) and Western blotting using anti-V5 antibody (Invitrogen).

Immortalized wild-type (WT) murine embryonic fibroblasts (MEFs), primary MDA5 KO MEFs, and their WT control MEFs were gifts from Wen-Chen Yeh (University of Toronto, Toronto, Canada) and Shizuo Akira (Osaka University, Japan), respectively. Primary WT, TLR7, 3d, and MDA5 KO MEFs were generated as previously described (29), and low-passage MEFs (<8 passages) were used.

Preparation of GM-CSF or Flt3L-expanded bone marrow-derived DCs. Bone marrow-derived plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) were prepared as previously described (35). Briefly, to generate granulocyte-macrophage colony-stimulating factor (GM-CSF)-expanded cDCs, bone marrow cells were seeded at 2 × 10^6 cells per 100-mm dish in 10 ml of RPMI 1640 containing 100 U/ml recombinant mouse GM-CSF (rmGM-CSF; R&D Systems). Half of the medium was removed every 3 days of culture, and low-passage MEFs (<8 passages) were used.

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Preparation of viral RNAs. LCMV or VSV virions were concentrated from the supernatants collected from virus-infected BHK-21 cells by precipitation with NaCl and polyethylene glycol (PEG) (46). Briefly, PEG 35000 and NaCl were added into virus-containing supernatants at final concentrations of 4% and 6% (wt/vol), respectively, and stirred overnight at 4°C, and then virions were pelleted by centrifugation at 12,000 rpm for 1 h. The final virion pellets were resuspended in Trizol reagent (Invitrogen), and viral RNA was extracted and purified by following the manufacturer’s recommendations. RNA was dissolved in RNA storage solution (Ambion), and the concentration was determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. To verify the LCMV RNA, RT-PCR using primers specific for LCMV-Arm glycoprotein (GP) was conducted by following the same strategy described above. The primers were designed according to the sequence deposited in GenBank (accession no. M20869.1). Primer 5′-GGTACTAGTATGATGATGATGATG-3′ (corresponding to LCMV small RNA position 3376) and 5′-CCGAGATTCTTGCAGAAGATTG-3′ were used for cDNA synthesis. To amplify the full-length LCMV GP-encoding gene, the following primer sets were used: forward primer, 5′-GACAATGTTGATGTTGATGATGATG-3′; reverse primer, 5′-CTTGTATTTCTTCGTTTTCTCAG-3′. PCR products were cloned into the pcDNA3-V5/His vector by following the manufacturer’s recommendations (Invitrogen). Plasmid DNA containing full-length LCMV GP cDNAs was confirmed by both restriction enzyme digestion and sequencing (Tufts University Core Facility).

RNA transfection and luciferase assay. HEK293 cells in 96-well plates were transfected with an IFN-β promoter-driven luciferase reporter plasmid (IFN-β-luc) using GeneJuice transfection reagent (Novagen). A total of 18 h after transfection, cells were transfected with LCMV RNA, VSV RNA, or poly(I·C) (Corning) using lipofectamine 2000 (Invitrogen) or infected with SeV and incubated for an additional 18 h. Cell lysates were prepared, and the activities of the luciferase reporter were determined using the Steady-Glo luciferase assay system (Promega). Results were normalized to those of mock-treated cells and expressed as mean values of normalized data ± standard errors of the means (SEM).

To knock down the expression of RIG-I, a mutant RIG-I construct lacking a CARD domain (RIG-I mutant sequence for cDNA cloning vector, RIG-Arm-C) was kindly provided by Takashi Fujita, Kyoto University, Kyoto, Japan. HeLa cells were transfected with poly(I·C) and incubated for an additional 18 h. Cell lysates were prepared, and the activities of the luciferase reporter were determined. To determine the ability of LCMV RNA to induce the bioactivity of type I IFNs, WT MEFs were seeded into 96-well plates in triplicate and transfected with LCMV RNA, VSV RNA, or poly(I·C) or infected with SeV and incubated for an additional 18 h. Cell lysates were prepared, and the activities of the luciferase reporter were determined.

To determine the ability of LCMV RNA to induce the bioactivity of type I IFNs, cell cultures were transfected with LCMV RNA, VSV RNA, or poly(I·C) or infected with SeV and incubated for an additional 18 h. Cell lysates were prepared, and the activities of the luciferase reporter were determined.

Immunoprecipitation of MDA5/RNA and RIG/E-IRNA complexes from LCMV-infected cells. HEK293T cells were plated on 6-well plates at 2 × 10^5 cells/well and allowed to grow overnight. Cells were transfected with plasmids encoding either green fluorescent protein (GFP)-tagged RIG-I or Flag-tagged MDA5. After incubation for 24 h, cells were transfected with LCMV-Arm at an MOI of 0.5 and cultured for an additional 24 h. As a control, cells were transfected but left uninfected. Cell lysates were prepared using the passive lysis buffer (Promega) and subjected to an immunoprecipitation (IP) assay using anti-GFP antibody (Sigma) together with protein G plus agarose beads (Thermo Scientific) to pull down either RIG-I- or MDA5-containing complexes by following the manufacturer’s instructions. Rabbit IgG or mouse IgG isotype control antibodies were included in the IP assay to precipitate either RIG-I- or MDA5-containing complexes. After being washed thor-
oughly with PBS, the precipitates/agarose beads were subjected to RNA isolation using Trizol reagent (Invitrogen) as described above. The concentration of the extracted RNA was determined by measuring the A260 with a spectrophotometer. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and an oligo(dT) primer by following the manufacturer's recommendations. Full-length cDNA encoding LCMV GP was amplified as described above.

Anti-LCMV NP pulldown assay and Western blotting. HEK293T cells were plated in 6-well plates at 2 × 10^5 cells/well and allowed to grow overnight. Cells were cotransfected with plasmids encoding V5-tagged WT or mutant LCMV NP, GFP-tagged RIG-I, or Flag-tagged MDA5. The expression of GFP is routinely analyzed with anti-V5-conjugated agarose beads (Sigma) to pull down the NP-containing protein complex by following the manufacturer's instructions. After the final washing, 2× sodium dodecyl sulfate (SDS) sample buffer (Sigma) was added and incubated for 5 min at 100°C. Anti-V5 bead-precipitated proteins were separated by 4 to 12% SDS-polyacrylamide gel electrophoresis (PAGE) and Western-blotted antibody to either GFP (indication of RIG-I) (Abcam) or Flag (indication of MDA5) (M2 clone; Sigma). The same membrane was stripped with Restore Western blotting stripping buffer (Thermo Scientific) and reprobed sequentially with anti-β-actin (Abcam) and anti-V5 (Invitrogen) (indication of LCMV NP).

Statistical analysis. Data were evaluated using the two-tailed Student t test. Results were expressed as means ± standard deviations. P values of <0.05 were regarded as significant.

RESULTS

The RIG-I/MDA5-MAVS signaling pathway is involved in LCMV-induced type I IFNs in vivo. To determine whether the RLR-mediated signaling pathway is involved in LCMV-induced type I IFNs, we took advantage of recently developed knockout mice. MDA5 KO and MAVS KO mice were infected with LCMV-Arm. MAVS, also known as IPS-1/VISA/Cardif (27, 42, 64), is a crucial adaptor protein in the RIG-I/MDA5 signaling pathway. Serum levels of type I IFNs were measured using a type I IFN bioassay. Interestingly, we observed that both MDA5 KO (Fig. 1A) and MAVS KO (Fig. 1B) mice produced levels of type I IFNs at day 1 postinfection (p.i.; early phase of type I IFN responses) comparable to those of WT controls; however, type I IFNs in the sera of both MDA5 and MAVS KO mice dropped to basal levels at day 2 p.i. (second wave of type I IFN responses) as opposed to WT mice, which maintained high levels at day 2 p.i.

Recent studies have suggested that the production of type I IFNs is controlled by a group of transcriptional factors, including IRF3 and IRF7 (18, 19, 53). Both IRF3 and IRF7 are the downstream transcriptional factors important for both TLR- and RLR-mediated type I IFN production (60). The role of these IRFs in regulating LCMV-induced type I IFN responses remains largely unexplored. To determine whether IRF3 and IRF7 are involved in this response, mice deficient in IRF3 and IRF7 were infected with LCMV-Arm intraperitoneally (i.p.). Both WT control and IRF3 KO mice produced type I IFNs in the sera following LCMV infection (Fig. 1C and D). Strikingly, type I IFNs were absent in IRF7 KO mice following LCMV infection. Of note, when infected with herpes simplex virus type 1 (HSV-1), IRF7 KO mice generated levels of type I IFN comparable to those of WT mice (our unpublished results).

To determine whether the endosomal TLRs (TLR3, -7/8, and -9) are involved in LCMV-induced type I IFN production, we utilized a mouse strain (3d mice) with a point mutation histidine-to-arginine substitution (H412R) in the polytopic membrane protein UNC-93B. UNC-93B is an endoplasmic reticulum protein involved in TLR3, TLR7/8, and TLR9 activation. It has been demonstrated that cells isolated from 3d mice had impaired cytokine production in response to TLR3, -7, -8, and -9 ligands (59). Our result demonstrated that TLR3/7/8/9 (3d) do not contribute to LCMV-induced type I IFN production in vivo (Fig. 1E). This is consistent with a recent report (21).

To determine whether the RIG-I/MDA5-MAVS signaling pathway affects the production of either IFN-α or -β, serum samples obtained from both MAVS KO and WT mice (day 1 p.i.) were pretreated with a neutralizing rat monoclonal antibody (MAb) against mouse IFN-α (RMMA-1 clone; PBL Biomedical Laboratories) for 1 h at 37°C before incubation with NCTC929 cells (65). Type I IFN activity in the sera of WT mice was completely blocked by treatment with neutralizing anti-IFN-α antibody (Fig. 1F). In contrast, treatment with neutralizing anti-IFN-α only partly blocked the activity of type I IFNs in sera from MAVS KO mice. These results suggest that the RNA helicase signaling pathway does not play a major role in the early phase of type I IFN production, which is predominately IFN-β (data not shown). These results are consistent with a recent report (21).

Taken together, these results demonstrate that the RLR-mediated signaling pathway plays a critical role in regulating the late phase (day 2 p.i.) of type I IFN responses to LCMV infection.

LCMV RNA induces a type I IFN response. The mechanisms underlying how LCMV infection induces type I IFNs in the mouse are poorly defined. In particular, it is unknown which LCMV component is responsible for activation of type I IFN responses. The helicases RIG-I and MDA5 are known to detect virus-derived dsRNA or ssRNA and activate type I IFN responses. We first determined whether LCMV RNA alone could induce type I IFN responses. We took advantage of an IFN-β promoter-driven luciferase reporter plasmid (IFN-β-luc). LCMV RNA was extracted from PEG-precipitated LCMV virions. When LCMV RNA was transfected into IFN-β-luc-expressing HEK cells, LCMV RNA induced IFN-β-luc activity (Fig. 2A). As controls, cells were also similarly transfected with either VSV RNA or poly(I·C) or infected with SeV (Fig. 2C). These three ligands have been demonstrated to induce a type I IFN response via either RIG-I (VSV RNA and SeV)- or MDA5 (poly[I·C])-dependent signaling pathways (25, 60). Consistent with the published results, negative-control cellular RNA extracted from BHK-21 cells did not induce a type I IFN response (Fig. 2A). The BHK-21 cell line is the parent cell line used to propagate LCMV. Interestingly, challenge with live LCMV did not induce a type I IFN response, in contrast to isolated LCMV RNA (Fig. 2A). These results demonstrate that LCMV RNA itself (without other viral components) is capable of inducing the type I IFN response but suggest that some other LCMV components must turn off the type I IFN response.

To further test how LCMV RNA induces type I IFNs, LCMV RNA was transfected into WT MEFs. LCMV RNA induced high levels of type I IFNs in MEFs (Fig. 2D). As positive controls, VSV RNA, SeV, and poly(I·C) all induced type I IFN production in WT MEFs (Fig. 2E). These studies...
demonstrate the ability of the isolated LCMV RNA to induce strong type I IFN production in multiple cell types.

To define the structural basis of the LCMV RNA responsible for the activation of the type I IFN response, LCMV RNA was treated with either the dsRNA-specific RNase III, which cleaves dsRNA, or shrimp alkaline phosphatase (SAP), which efficiently catalyzes the release of 5'- and 3'-triphosphate groups from RNA. Our results demonstrated that treatment with either RNase III or SAP ablated LCMV RNA-induced activation of the type I IFN response in either the IFN-α/luc reporter assay in HEK cells or the type I IFN bioassay in MEFs (Fig. 2B and D). Consistent with the published results (25, 51), treatment of VSV RNA with either RNase III or SAP abolished or significantly reduced its ability to induce type I IFN responses in either HEK293 cells or MEFs (Fig. 2C and E). Poly(I·C), a synthetic dsRNA resembling the RNA of infectious viruses, does not contain the 5'-triphosphate structures. Thus, treatment of poly(I·C) with SAP did not affect its ability to induce the type I IFN response in either HEK293 cells or MEFs, whereas treatment with RNase III completely abolished its ability to induce the type I IFN response (Fig. 2C and E). Thus, these results suggest that both dsRNA and 5'-triphosphate structures are involved in LCMV RNA-induced type I IFN responses.

To further explore the mechanism by which LCMV RNA induces type I IFN responses, we asked whether LCMV RNA associates directly with either MDA5 or RIG-I. We took advantage of the overexpression system to transfect HEK293T cells with plasmids expressing either Flag-tagged MDA5 or GFP-tagged RIG-I, followed by challenge with LCMV-Arm.
MDA5- or RIG-I-containing protein complexes were precipitated using protein G-conjugated agarose beads. Total RNA was isolated from these precipitated protein complexes. The presence of LCMV RNA in both MDA5- and RIG-I-containing complexes was confirmed by RT-PCR to amplify the LCMV glycoprotein (GP) gene (1.5 kb). PCR products were analyzed in a 1% agarose gel (with ethidium bromide staining).

FIG. 2. LCMV RNA activates the type I IFN response and is inactivated by both RNase III and SAP. (A) HEK293 cells were transfected with IFN-β-luc. At 18 h after transfection, cells were transfected with Lipo as a control, LCMV RNA, or cellular RNA isolated from BHK-21 cells or challenged with LCMV-Arm. Cells were incubated for an additional 18 h. Cell lysates were prepared, and luciferase activity was measured. (B) LCMV RNA extracted from LCMV-Arm was treated with either RNase III or SAP. HEK293 cells were transfected with IFN-β-luc for 18 h. Cells were then transfected with untreated (−) or treated LCMV RNA. Cell lysates were prepared, and luciferase activity was measured. (C) HEK293 cells were similarly cotransfected with IFN-β-luc and untreated or treated VSV RNA or poly(I:C) (poly IC). Cells were also infected with SeV, and luciferase activity was measured 18 h later. (D) WT MEFs (immortalized) were transfected with untreated or treated LCMV RNA. At 18 h posttransfection, the bioactivity of type I IFNs in the supernatants was determined by type I IFN bioassay. (E) WT MEFs were transfected with untreated or treated VSV RNA or poly(I:C). Cells were infected with SeV at 18 h posttransfection/infection, and the bioactivity of type I IFNs in the supernatants was determined by type I IFN bioassay. (F) HEK293T cells in 6-well plates were transfected with plasmids expressing either Flag-tagged MDA5 or GFP-tagged RIG-I. After 24 h, cells were challenged with LCMV-Arm (MOI of 0.5). After culture for an additional 24 h, cell lysates were prepared. MDA5- or RIG-I-containing protein complexes were precipitated using either anti-Flag antibody (MDA5) or anti-GFP antibody (RIG-I) together with the protein G agarose beads. Total RNA were extracted from these precipitated protein complexes. The presence of LCMV RNA in both MDA5- and RIG-I-containing complexes was confirmed by RT-PCR to amplify the LCMV GP gene (1.5 kb). PCR products were analyzed in a 1% agarose gel (with ethidium bromide staining). *, P < 0.05.
interaction provides the molecular basis to trigger type I IFN responses through both the MDA5- and RIG-I-mediated signaling pathways.

**LCMV RNA activates the RIG-I/MDA5-MAVS-dependent signaling pathway to induce type I IFN responses.** To determine whether the RIG-I-mediated signaling pathway is involved in recognizing LCMV RNA and triggering IFN production, we used a mutant RIG-I lacking CARD domain function as a dominant negative (DN) RIG-I (DN-RIG-C). DN-RIG-C is known to be able to specifically block RIG-I-mediated type I IFN responses. HEK293 cells were first cotransfected with IFN-β-luc-encoding HEK293 cells in 96-well plates and DN-RIG-C or control plasmid pcDNA3 and incubated for 18 h. The next day, cells were transfected with LCMV RNA or other controls. After incubation for an additional 18 h, cell lysates were prepared, and the luciferase reporter activities were measured. Transfection of the dominant negative construct DN-RIG-C significantly reduced the LCMV RNA-induced type I IFN response (Fig. 3A). Consistent with published data, DN-RIG-C also significantly inhibited both VSV RNA- and SeV-induced activation of type I IFN responses but did not affect poly(I·C)-induced IFN responses (Fig. 3A), because poly(I·C) activates type I IFN responses through MDA5 rather than through RIG-I. These results suggest that the RIG-I-mediated signaling pathway is involved in LCMV RNA-induced IFN responses.

To define a role for other RLR proteins in the LCMV RNA-induced type I IFN response, experiments with MDA5 KO MEFs were carried out. Levels of type I IFNs in the supernatants were measured using a bioassay. LCMV RNA induced significantly lower levels of type I IFNs in MDA5 KO MEFs than in WT MEFs (Fig. 3B). These results are consistent with the patterns of LCMV-induced type I IFN responses in mice deficient in MDA5 (Fig. 1C). Taken together, these results demonstrate that neither the endosomal TLR signaling pathway nor MyD88 play a significant role in the LCMV RNA-induced type I IFN response. In contrast, the RIG-I/MDA5/MAVS-mediated signaling pathway is essential for the LCMV RNA-induced type I IFN response. Although our *in vivo* studies of mice and *in vitro* results using the HEK cell system have suggested that a TLR-MyD88-independent signaling pathway is involved in LCMV RNA-induced type I IFN responses, we wished to further determine whether the endosomal TLRs (TLR3, -7/8, and -9) are involved in LCMV RNA-induced type I IFN production. We utilized MEFs generated from a mouse strain with a point mutation histidine-to-arginine substitution (H412R) in the polytopic membrane protein UNC-93B (3d mice). 3d, TLR7, MyD88 KO, and WT MEFs were similarly plated and transfected with LCMV RNA or controls. LCMV RNA induced levels of type I IFN in MEFs deficient in TLR7, MyD88, or 3d comparable to those of WT MEFs (Fig. 3C). These results are consistent with the patterns of LCMV-induced type I IFN responses in mice deficient in 3d, TLR7, and MyD88 (Fig. 1E) (66). Taken together, these results demonstrate that neither the endosomal TLR signaling pathway nor MyD88 play a significant role in the LCMV RNA-induced type I IFN response. In contrast, the RIG-I/MDA5/MAVS-mediated signaling pathway is essential for the LCMV RNA-induced type I IFN response.

**LCMV RNA induces type I IFNs in both pDCs and cDCs, and the MAVS-dependent signaling pathway is essential for type I IFN production from cDCs.** Although pDCs have been demonstrated to be the major type I IFN producer in response to selected viral infections, the role of pDCs during LCMV infection is unclear. To define whether LCMV RNA could induce type I IFN production from DCs, bone marrow-derived...
pDCs and cDCs were prepared. pDCs were defined as CD11c<sup>lo</sup> B220<sup>+</sup> Gr-1<sup>−</sup> CD11b<sup>−</sup>, and cDCs were defined as CD11c<sup>hi</sup> B220<sup>−</sup> Gr-1<sup>−</sup> CD11b<sup>+</sup>. The purity of both pDCs and cDCs was about 70% based on fluorescence-activated cell sorter (FACS) staining (data not shown). Interestingly, LCMV RNA induced comparable levels of type I IFNs in WT, MyD88 KO, and MAVS KO pDCs (Fig. 4A). As a control, CpG induced type I IFNs in both WT and MAVS KO pDCs but not in MyD88 KO pDCs. In contrast, LCMV RNA did not induce type I IFNs in MAVS KO cDCs but did induce similar high levels of type I IFNs in both WT and MyD88 KO cDCs (Fig. 4B). As a control, transfected poly(I·C) induced levels of type I IFNs in MAVS KO cDCs that were significantly lower than those in WT cDCs (Fig. 4B). Collectively, these results suggest that a MAVS-mediated signaling pathway is essential for the production of type I IFNs in response to LCMV RNA in cDCs. However, it seems that a signaling pathway independent of both MyD88 and MAVS is involved in the production of type I IFNs in pDCs.

**LCMV NP targets both RIG-I and MDA5 to inhibit the type I IFN response.** We have observed that live LCMV (LCMV-Arm strain) inhibits LCMV RNA-induced activation of type I IFN (data not shown). Additionally, studies from other groups have shown that LCMV NP inhibits the type I IFN response through targeting the transcriptional factor IRF3, although the details and molecular mechanisms of this inhibition have not been fully defined (37–39). IRF3 is a common downstream transcriptional factor in both the TLR- and RLR-mediated signaling pathways. Since we have demonstrated that LCMV RNA is responsible for triggering a RIG-I/MDA5/MAVS-dependent type I IFN response, we wanted to define whether LCMV NP could target both the RIG-I and MDA5 signaling pathways to inhibit the type I IFN response. Martinez-Sobrido et al. have recently demonstrated that a region in LCMV NP from residues 382 to 386 is critical for LCMV NP-mediated inhibition of type I IFN responses (37). To further study the molecular mechanism by which LCMV NP inhibits type I IFN responses, we generated plasmids encoding LCMV NP mutants carrying either the D382A or G385A mutation. These two mutations did not affect the expression of LCMV NP in HEK cells (data not shown). Interestingly, when cotransfected into HEK cells with IFN-β-luc

**FIG. 4.** LCMV RNA can induce type I IFN in both pDCs and cDCs, and LCMV RNA-induced type I IFN in cDCs is MAVS dependent. Bone marrow-derived pDCs (A) or cDCs (B) were plated in 96-well plates and transfected with purified LCMV RNA or control poly(I·C) or stimulated with CpG1826. After incubation for 18 h, the bioactivities of the type I IFNs in the supernatants were measured using a type I IFN bioassay. Data are representative of results from two experiments. *, P < 0.05.

**FIG. 5.** LCMV NP expression dramatically inhibits the LCMV RNA-induced type I IFN response as well as both the SeV- and poly(I·C)-induced type I IFN responses. HEK293T cells in 96-well plates were transfected with plasmids encoding either WT NP, D382A mutant NP, or G385A mutant NP together with IFN-β-luc for 18 h, followed by transfection with LCMV RNA (A), infection with SeV (B), or transfection with poly(I·C) (C) for an additional 18 h. The activity of luciferase was measured. Data are representative of results from at least four experiments. *, P < 0.05.
and challenged with either LCMV RNA, SeV, or poly(I·C), both mutants failed to inhibit activation of IFN-β-luc induced by LCMV RNA (Fig. 5A), SeV (Fig. 5B), or poly(I·C) (Fig. 5C), despite the marked inhibition by wild-type LCMV NP. These results are consistent with those of Martinez-Sobrido et al. (38), demonstrate that LCMV NP inhibits both RIG-I and MDA5 pathways, and confirm that both the D382 and G385 residues are critically involved in blocking LCMV RNA- and other virus ligand-induced activation of IFN-β.

**LCMV NP physically associates with both RIG-I and MDA5.**

To define the molecular mechanisms involved in LCMV NP inhibition of the type I IFN response, we asked whether LCMV NP could associate with either RIG-I or MDA5 signaling molecules. Cell lysates prepared from untransfected and GFP–RIG-I/V5-NP (wild-type) plasmid-cotransfected HEK293T cells were subjected to a pulldown assay with an anti-V5 antibody (LCMV NP). LCMV NP-associated protein complexes were analyzed by SDS-PAGE and immunoblotted with anti-GFP antibody. Our results demonstrated that an anti-V5 antibody could pull down RIG-I (Fig. 6A). These data indicate that LCMV NP physically associates with RIG-I. The same approach was utilized to define whether LCMV NP also interacts with MDA5. Cell lysates were prepared from Flag-MDA5- and V5-NP-cotransfected HEK293T cells. Anti-V5 antibody pulled down MDA5 (Fig. 6B), demonstrating that LCMV NP also associates with MDA5.

We further asked whether the association of LCMV NP with either RIG-I or MDA5 explained the inhibitory effect of LCMV NP on IFN induction. Using a similar experimental design, HEK293T cells were transfected with either GFP–RIG-I or Flag-MDA5 together with NP mutants. Interestingly, these two mutations (D382A and G385A in the LCMV NP gene) did not affect the interaction of LCMV NP with either RIG-I or MDA5 (Fig. 6A and B), although changing these two amino acids in LCMV NP eliminated the ability of this protein to inhibit type I IFN production.

**DISCUSSION**

Production of type I IFNs is a critical part of the innate immune response to viral pathogens. Induction of type I IFNs following virus infection is largely controlled by the innate PRRs, which are proteins expressed in cells of the innate immune system. LCMV infection in the mouse, a commonly used model for studying host responses to viruses, has been extensively characterized. However, the molecular mechanisms underlying how LCMV infection induces type I IFNs are poorly defined. In the present study, we have examined the role of LCMV RNA and NP in the activation of the type I IFN response. We demonstrated that LCMV RNA is capable of activating the type I IFN response. Moreover, both dsRNA and 5' triphosphated ssRNA derived from LCMV genomic RNA are responsible for type I IFN production. Second, the RIG-I/MDA5-MAVS-mediated signaling pathway is essential for the LCMV RNA-induced type I IFN responses both in vitro and in vivo. We provided evidence that LCMV RNA is physically associated with both MDA5 and RIG-I. Third, using bone marrow-derived DCs, we demonstrated that both pDCs and cDCs are able to produce type I IFNs in response to LCMV RNA. Finally, we demonstrated that LCMV NP blocks type I IFN induction.

In the present study, we characterized the innate immune recognition mechanisms involved in detecting LCMV RNA and triggering the type I IFN response. In LCMV-infected cells, LCMV RNA is associated with both MDA5 and RIG-I (Fig. 2F). This association could account for the activation of
type I IFN responses. Rehwinkel et al. recently demonstrated that RNAs bearing 5’-PPP derived from single-stranded RNA viruses, like influenza virus, trigger type I IFN induction through association with RIG-I (31a).

The cellular source of type I IFNs during LCMV infection is not clear (7, 8, 31). Multiple types of cells might be involved in LCMV-induced type I IFN responses, including spleen marginal zone (MZ) macrophages (34) and DCs (cDCs and pDCs) (7, 8, 31, 44). Depletion of MZ macrophages with clodronate liposomes prior to infection with LCMV in the mouse resulted in dramatic loss of type I IFN production (34). DCs, particularly pDCs, have been demonstrated to play a critical role in producing type I IFN against viral pathogens. Recent studies have also demonstrated that LCMV infection rapidly induces the activation of DCs in the spleen, and this activation is dependent on type I IFN signaling, suggesting a role for both cDCs and pDCs in type I IFN production during LCMV infection. To determine whether both pDCs and cDCs are capable of producing type I IFNs in response to LCMV, we took advantage of bone marrow-derived DCs.

It has been demonstrated that cDCs and pDCs utilize distinctive mechanisms to detect viral RNA and to generate type I IFN production. cDCs and other types of cells predominantly use the MAVS-mediated signaling pathway to generate type I IFNs (23, 25, 57). In contrast, expression of TLR7 and TLR9 in pDCs is responsible for ssRNA- and unmethylated CpG motif-induced type I IFN production, respectively (26). In the present study, we demonstrated that LCMV RNA-induced type I IFNs in cDCs are dependent on the MAVS signaling pathway. This is consistent with previous studies. The signaling pathway operating in pDCs responsible for LCMV RNA-induced type I IFN production remains undefined.

The TLR family plays a critical role in regulating the type I IFN response as well as other inflammatory cytokine and chemokine responses to viral pathogens. We have previously observed that TLR2 KO mice had a defective type I IFN response during an acute LCMV infection. The mechanism has not been defined. In the present study, we demonstrated that following LCMV infection, wild-type mice had a greater increase in the mRNA levels of genes involved in type I IFN production, including IFN-α (IFN-α4, non-IFN-α4), IFN-β, and MDA5, than did TLR2 knockout mice (data not shown). Given that the expression of MDA5 and RIG-I is type I IFN inducible, it is conceivable that TLR2 and other unknown molecules could regulate the expression of MDA5 and RIG-I in certain types of cells through either activation of NF-κB signals or other unidentified mechanisms. Of note, the role of TLR2 in virus-induced type I IFN production in certain types of cells has also recently been observed by another group (4).

The MDA5/RIG-I-MAVS signaling pathway in vivo is critical for the LCMV-induced late phase (day 2 p.i.) of type I IFN responses, while the MDA5/RIG-I-MAVS signaling pathway is dispensable for the LCMV-induced early wave (day 1 p.i.) of type I IFN responses. Mice deficient in either MDA5 or MAVS generated comparable levels of type I IFN in the sera at day 1 p.i., while the type I IFN responses in these mice collapsed on day 2 p.i. It has been reported that MDA5 KO mice are capable of generating type I IFN responses against a number of viruses, including influenza virus (NS1 mutant), SeV, Newcastle disease virus (NDV), and Japanese encephalitis virus (JEV) (25). MAVS KO mice have also been demonstrated to be able to produce levels of type I IFN comparable to those of wild-type mice in response to VSV, although these mice are sensitive to VSV infection (57). Interestingly, a recent report demonstrated that in response to SeV infection, while MDA5 KO mice generated comparable levels of type I IFN mRNA at day 2 p.i., the levels of type I IFN at day 5 p.i. were dramatically decreased in MDA5 KO mice (16).

Like other viruses, LCMV has also evolved countermechanisms to modulate PRRs and other innate signaling molecules to block type I IFN induction. It has been demonstrated that LCMV NP blocks the type I IFN response by targeting IRF3 (39). IRF3 and IRF7 are the common downstream transcriptional factors involved in both the TLR- and RLR-mediated signaling pathways, and IRF3 has been demonstrated to be a potent transcription factor for activation of the IFN-β promoter (19, 60). In the present study, we confirmed and extended these findings by demonstrating that LCMV NP blocks type I IFN production through targeting both the MDA5- and RIG-I mediated signaling pathways.

Our experiments demonstrate that LCMV NP physically associates with both RIG-I and MDA5 (Fig. 6). It is not clear whether this association is responsible for the inhibitory effect of NP on the activation of type I IFN responses. The fact that mutant NPs that do not suppress the IFN response still associated with RIG-I and MDA5 indicates that simple association does not lead directly to inhibition. It is possible that the site of association is important or that the interaction of the two proteins leads to changes in the tertiary structure of these proteins or their interactions with additional proteins in the pathway. Other possible mechanisms responsible for the inhibitory effect of NP might include the ability of LCMV NP to bind and sequester viral RNA (14, 17, 43, 51), to degrade the host signaling molecules involved in detection of viral RNA (12, 33, 42), or to inhibit the positive regulatory loop of the RIG-I/MAVS signaling pathway (13). Our data do suggest that these two residues (D382 and G385) in LCMV NP are involved in the inhibitory effect of NP but are not required for the interaction with either RIG-I or MDA5.

The fact that LCMV NP-expressing cells are still able to respond to poly(I·C) suggests that different ligands may interact with the RIG-I/MDA5 pathways in different ways. Further experiments involving more specific localization of bind-
ing and analysis of multiple protein complexes may be necessary to define the mechanism of the inhibitory action of NP. Interestingly, Lee et al. have recently observed a similar phenomenon. They noted that mice chronically infected with LCMV had defective type I IFN responses to SeV but were capable of producing type I IFNs in response to other stimuli, including poly(I·C), albeit in a reduced manner compared to that of uninfected mice (31).

In summary, our current studies define a mechanism by which LCMV infection regulates the type I IFN response. Our current understanding of the recognition and regulation of the type I IFN response to LCMV indicates that this response is dependent upon IRF7 but independent of IRF3. There is no role for the RNA-sensing TLRs (neither TLR3 nor TLR7 seems to be important in the response to LCMV). While the “second wave” (day 2 p.i.) of the IFN response to LCMV is dependent on both MAVS and MDA5, the earliest response (day 1 p.i.) is independent of the RIG-I/MDA5 MAVS pathway, and both are independent of the endosomal TLRs (TLR3, -7, and -9). Our current model indicates that expression of the LCMV NP turns off the induction of type I IFN that is initiated by LCMV RNA interaction with RIG-I and MDA5 (Fig. 7).

We believe that there are dynamic interactions between LCMV RNA, LCMV NP, and the host type I IFN modulators, including MDA5 and RIG-I. These interactions could determine the pattern of type I IFN responses as well as viral pathogenesis during LCMV infection. These results will aid in understanding LCMV viral pathogenesis and the development of new strategies to treat human arenavirus infections as well as other viral diseases.

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REFERENCES


The remaining references are not shown here due to limited character space. The full list can be found in the original article. The references are numbered from 9 to 434, covering a range of studies from 1956 to 2008. The references are pertinent to the study of interferon responses, viral recognition, and viral pathogenesis.


