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# NOD1 Participates in the Innate Immune Response Triggered by Hepatitis C Virus Polymerase

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## ABSTRACT

Hepatitis C virus (HCV) triggers innate immunity signaling in the infected cell. Replication of the viral genome is dispensable for this phenotype, and we along with others have recently shown that NS5B, the viral RNA-dependent RNA polymerase, synthesizes double-stranded RNA (dsRNA) from cellular templates, thus eliciting an inflammatory response, notably via activation of type I interferon and lymphotoxin  $\beta$ . Here, we investigated intracellular signal transduction pathways involved in this process. Using HepaRG cells, a model that largely recapitulates the *in vivo* complexities of the innate immunity receptor signaling, we have confirmed that NS5B triggered increased expression of the canonical pattern recognition receptors (PRRs) specific for dsRNA, namely, RIG-I, MDA5, and Toll-like receptor 3 (TLR3). Unexpectedly, intracellular dsRNA also led to accumulation of NOD1, a receptor classically involved in recognition of bacterial peptidoglycans. NOD1 activation, confirmed by analysis of its downstream targets, was likely due to its interaction with dsRNA and was independent of RIG-I and mitochondrial antiviral signaling protein (MAVS/IPS-1/Cardif/VISA) signaling. It is likely to have a functional significance in the cellular response in the context of HCV infection since interference with the NOD1 pathway severely reduced the inflammatory response elicited by NS5B.

## IMPORTANCE

In this study, we show that NOD1, a PRR that normally senses bacterial peptidoglycans, is activated by HCV viral polymerase, probably through an interaction with dsRNA, suggesting that NOD1 acts as an RNA ligand recognition receptor. In consequence, interference with NOD1-mediated signaling significantly weakens the inflammatory response to dsRNA. These results add a new level of complexity to the understanding of the cross talk between different classes of pattern recognition receptors and may be related to certain complications of chronic hepatitis C virus infection.

Hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus of the *Flaviviridae* family. Its genome encodes a polyprotein of ~3,010 amino acids, which is cleaved into three structural and seven nonstructural proteins. Persistent HCV infection, which is estimated to affect close to 150 million people worldwide (1), is the most common infectious cause of chronic liver disease in industrialized countries (2). In patients persistently infected with HCV, chronic inflammation resulting from the immune response against infected hepatocytes typically lasts many decades. It frequently leads to progressive liver disease that ranges from mild inflammation to severe fibrosis that can culminate in life-threatening disease, such as hepatic cirrhosis and hepatocellular carcinoma (HCC) (for a review, see reference 3). While the molecular mechanisms underlying HCV-induced liver cancer have not been fully elucidated, it is generally accepted that both the direct effects exerted by the virus on the infected cell and indirect effects, due to the inflammatory host response, participate in triggering and promoting malignant transformation (for a review, see reference 4). Indeed, the persistent inflammatory response observed in the liver of HCV-positive patients impacts many aspects of HCV-associated pathology, including HCC, by creating an environment rich in mediators perturbing tissue homeostasis, such as reactive oxygen species (5) and cytokines (6–8). It is thus of considerable interest to investigate what initiates and what modulates the inflammatory response to HCV. It has previously been reported that the HCV core and NS3 proteins induce expression of proinflammatory cytokines through as yet to be

identified mechanisms (9, 10). More recently, we along with others have shown that NS5B, the HCV RNA-dependent RNA polymerase, activates innate immune signaling through the production of small RNA species that are recognized by intracellular receptors and give rise to cytokine production, including type I interferons (11–13). Thus, expression of viral gene products fuels the persistence of inflammatory signaling.

The innate immune system is a major contributor to acute inflammation following microbial infection or tissue damage (14, 15). Viruses are detected by cellular pattern recognition receptors (PRRs) through the recognition of viral nucleic acids that act as pathogen-associated molecular patterns (PAMPs). PRRs include the membrane-bound Toll-like receptors (TLRs) that sense bacterial and viral PAMPs present outside the cell or within endosomes, RIG-like receptors (RLRs), which are cytoplasmic PRRs that sense intracellular viral RNA, and NOD-like receptors

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(NLRs), also cytoplasmic and classically involved in the antibacterial response (16). The NLRs constitute a subfamily of innate immunity proteins that comprises more than 20 members. Their cytosolic localization allows detection of intracellular PAMPs and of endogenous products of tissue injury (17, 18). NLR proteins trigger a number of signal transduction cascades, which include the proinflammatory nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling and the inflammasome pathways, as well as activation of autophagy and cell death pathways (for a review, see reference 19). The closely related NOD1 and NOD2 proteins are members of the NLR family. They share a leucine-rich repeat (LRR) domain, involved in PAMP recognition, and a nucleotide binding domain (NBD), important for self-oligomerization and activation, and differ in having either one (NOD1) or two (NOD2) caspase recruitment domain (CARD) motifs (20). NOD1 has a wide expression pattern, while NOD2 is mainly expressed in myelomonocytic, dendritic, and intestinal epithelial cells (21–23). The conventional downstream effector molecule for both NOD1 and NOD2 is RIPK2. It initiates downstream signaling toward a variety of pathways, leading to mitogen-activated protein kinase (MAPK) activation, type I interferon (IFN-I) production, and NF- $\kappa$ B-dependent signaling, associated with transcriptional activation of proinflammatory cytokines (24, 25). Until recently, NOD1 and NOD2 were thought to be activated specifically in response to bacterial infection through recognition of peptides derived from the degradation of constituents of the bacterial wall (20). Recent reports suggest that both receptors might also respond to viral infections, thus participating in the coordinated host defense against viruses (26–29). In the context of viral infections, activation of both NOD1 and NOD2 has been reported to depend on type I IFN, synthesized as a result of activation of other PRRs. In addition, NOD2, but not NOD1, appears to directly recognize single-stranded viral RNA (ssRNA) (28). Symmetrically, RIG-I, a classical antiviral PRR, has recently been shown to respond to *Listeria monocytogenes* RNA (30). These examples illustrate the existence of an extensive cross talk between different classes of innate response signaling pathways (reviewed in reference 16).

We now report a novel functional interaction between NLRs and RLRs. Our data suggest that in hepatocytes NOD1 is involved in recognition of dsRNA and that its presence is required for an efficient inflammatory response to this ligand. Our findings may have profound implications for the understanding of cellular consequences of the HCV viral infection.

## MATERIALS AND METHODS

**Animals and primary hepatocyte isolation.** Three-month-old FL-N/35 transgenic (31) male mice were used for this study. Briefly, livers from control and FL-N/35 mice were washed with liver perfusion medium (Invitrogen, Carlsbad, CA) and perfused with digestion medium (Dulbecco's modified Eagle medium [DMEM], low glucose, with  $1 \times$  penicillin-streptomycin [Pen-Strep], 15 mM HEPES, and 100 collagen digestion units [CDU]/ml of collagenase type VI [Sigma, St. Louis, MO, USA]). Livers were flushed through the portal vein at a rate of 8 ml/min. Cell viability, assessed by a trypan blue exclusion test, was higher than 80%. Hepatocytes were seeded at a density of  $2 \times 10^6$  cells in 60-mm petri dishes.

**Ethics statement.** Mice were bred and maintained according to French Ministry of Agriculture and European institutional guidelines (appendix A, European Treaty Series [ETS] number 123). Experiments were performed according to French regulations, and this study was specifically approved (approval identification number D34-172-16) by the regional ethics committee of Languedoc-Roussillon (Comité Régional d'Éthique).

**Cell culture.** HepaRG cells (32) were grown in William's E medium supplemented with 10% fetal calf serum, 5 mg/ml insulin,  $5 \times 10^{-5}$  M hydrocortisone hemisuccinate, 100 units/ml penicillin, and 100 mg/ml streptomycin. Huh7 and Nneo/3-5B replicon cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 ng/ml streptomycin, 100 U/ml penicillin, and 400  $\mu$ g/ml of G418 for cells harboring the Nneo/3-5B replicon. Cells were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

**Treatments.** A total of  $30 \times 10^4$  HepaRG cells were seeded in six-well plates 1 day before treatment. Poly(I-C) complexed with the transfection agent LyoVec [poly(I-C)/LyoVec; InvivoGen, CA, USA] was added directly to the medium for the times indicated on the figures. Doses of 0.1 to 1  $\mu$ g/ml poly(I-C) gave comparable results.  $\gamma$ Tri-DAP (L-Ala-gamma-D-Glu-mDAP [meso-diaminopimelic acid]; Cayla, France) was complexed with the transfection agent SAINT-PhD protein high delivery (Synvolux Therapeutics, Netherlands) according to the manufacturer's instructions and added to the medium for the times indicated on the figures.

**Generation of cell lines.** NS5B, NS5A, core, NS3, and NS4A cDNA sequences from the genotype 1b isolate N HCV were subcloned downstream of a Myc tag into pMSCV-puromycin (where MSCV is mouse stem cell virus) and pMSCV-hygromycin retroviral vectors (Clontech, Palo Alto, CA, USA). Dominant negative (DN) NOD1 cDNAs were obtained from pDeNy-hNOD1 (InvivoGen, CA, USA) and cloned in an N-terminal Myc-tagged pMSCV retroviral vector (Clontech). A total of  $1.5 \cdot 10^6$  293T cells were transfected twice at 24-h intervals with 3  $\mu$ g of retroviral vector using jetPEI (Polyplus, Illkirch, France). Forty-eight hours after the second transfection, supernatants were collected, filtered (0.45-mm pore size filter; Millipore, Temecula, CA, USA), supplemented with 8  $\mu$ g/ml of Polybrene, and added to cells to be transduced for 16 h. Infected cells were selected after 48 h with puromycin (2  $\mu$ g/ml) or hygromycin (150  $\mu$ g/ml). The lentiviral pLKO.1-puro vectors containing a short hairpin RNA (shRNA) targeting mitochondrial antiviral signaling protein (MAVS) (TRCN0000236031) or a control shRNA (SHC002) were from Sigma (St. Louis, MO, USA).

CRISPR subgenomic RNA (sgRNA) sequences were as follows: NOD1, 5'-CACCGCGTAGGCATCTGCGAGTTGC-3' and 5'-AAACGC AACTCGCAGATGCCTACGC-3'; RIG-I, 5'-CACGGGGTCTTCC GGATATAATCC-3' and 5'-AAACGGATTATATCCGGAAGACCCC-3'; RIPK2, 5'-CACCGATATATATCGTGCCTTGATAC-3' and 5'-AAAC GTATCAAGCACGATATATC-3'; control, 5'-CACCGACGGAGGCT AAGCGTCGCAA-3' and 5'-AAACTGCGACGCTTAGCCTCCGTC-3'.

Synthetic double-stranded DNA (dsDNA) fragments were cloned in lentiCRISPR v2 vector (a gift from Feng Zhang; plasmid 52961 [Addgene]), as described in Sanjana et al. (33). Cells were plated in a six-well plate, infected with the lentivirus, treated with Polybrene for 1 h, and selected over 3 days with 5  $\mu$ g/ml puromycin. The efficiency of CRISPR-mediated deletion was assessed by Western blotting, and the whole population of deleted cells was used in experiments.

**In vitro mutagenesis.** (i) **NS5B catalytic site mutant.** The point mutation G317V (34) was introduced in the GDD motif of the NS5B protein as previously described (12).

(ii) **NOD1 deletion mutant.** To generate the NOD1 mutant lacking the LRR domain (amino acids [aa] 657 to 933), we used a PCR cloning strategy with the pET15b-Nod1 construct as a template with the primers 5'-GATCCTCGAGCAGCATCCAGATGAACGTGGGC-3' and 5'-GAT CCTCGAGAACCTGATAAAACCAGAGGAGGC-3' positioned, respectively, upstream and downstream of the LRR domain. Following amplification, the PCR product was gel purified, digested with XhoI, and ligated. The construct was fully sequenced before further use.

**HCV infection.** Primary human hepatocytes (Biopredic, Rennes, France) were isolated from healthy liver tissue obtained from adult patients undergoing hepatectomy for the therapy of metastases. Patients were seronegative for HCV, hepatitis B virus, and human immunodeficiency virus. For HCV infection, hepatocytes were inoculated 3 days post-

seeding with JFH1-derived chimeric JCI1 virus (35) at a multiplicity of infection (MOI) of 0.1, and samples were analyzed 2 weeks later.

**Immunoblotting.** Cells were lysed by boiling in SDS sample buffer, sonicated, and complemented with dithiothreitol (DTT). Protein concentrations were measured by a bicinchoninic acid (BCA) protein assay kit (Pierce, MA, USA). Equal amounts of protein from total cell lysates (10 to 30  $\mu$ g) were loaded on SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were blocked and incubated overnight at 4°C with primary antibodies and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham, Piscataway, NJ). The antibodies used were anti-phosphorylated extracellular signal-regulated kinase (ERK) (catalog number 9101; 1:1,000 dilution), anti-ERK (9108; 1:1,000 dilution), anti-NOD1 (3545; 1:1,000 dilution), and anti-RIG-I (3743; 1:1,000 dilution) from Cell Signaling (Danvers, MA, USA) and anti-NS5B (catalog number ab35586; 1:1,000 dilution), anti-TLR9 (ab52967; 1/250 dilution), and anti- $\beta$ -tubulin (ab6046; 1/5,000 dilution) from Abcam (Cambridge, United Kingdom). After membranes were washed and incubated with the HRP-conjugated secondary antibodies (Amersham, Piscataway, NJ) for 1 h, bands were visualized by autoradiography. The signal was quantified by scanning the membranes and determining the number of pixels per surface area (ImageJ software).

**RNA isolation and analysis.** Total RNA was extracted from cultured cells with an RNeasy minikit (Qiagen, Germantown, MD, USA). Total mRNA was reverse transcribed using a QuantiTech reverse transcription kit (Qiagen) according to the manufacturer's protocol. Transcript levels were measured with a LightCycler 480 SW 1.5 apparatus (Roche, Indianapolis, IN, USA) and normalized to the expression of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT). The level of transcripts in the control cells was arbitrarily set at 1.

**Sequences of oligonucleotides.** Human primers were as follows: NOD1, ACTGAAAAGCAATCGGGAACCTT (forward [fw]) and CACACACAATCTCCGCATCTT (reverse [rv]); NOD2, GGGCTTCCACCTCC TGGGCCAGC (fw) and GCTGTGGCCTGGGCTTCTTGGG (rv); IFN- $\alpha$ 1 to - $\alpha$ 13, TGGCTGTGAAGAAATACTTCCG (fw) and TGTTTT CATGTTGGACCAGATG (rv); IFN- $\beta$ , GTCTCCTCCAAATTGCTCTC (fw) and ACAGGAGCTTGTGACACTGA (rv); tumor necrosis factor alpha (TNF- $\alpha$ ), GCCGTCTCCTACCAGACCAAGGTCA (fw) and GACCCTCCAGATAGATGGGCTCA (rv); interleukin-8 (IL-8), GGCTCTC TTGGCAGCCTTCTT (fw) and TTTGGGGTGGAAAGGTTTGGG (rv); RIG-I, CTGGACCTACCTACATCTG (fw) and GGCATCCAAAAG CCACGG (rv); TLR3, TCAACTCAGAAGATTACCAGC (fw) and AGTT CAGTCAAATTCGTGCAG (rv); TLR7, TCCTTGGGGCTAGATGG TTCT (fw) and TCCAGATCACATGGTTCTT (rv); TLR9, CTGCTT CCTACCCTGTGAG (fw) and GGATGCGGTTGGAGGACAA (rv); MDA5, ATGTGCAATGGGTATTCCACA (fw) and GAAGCACGAGAT GAGATAGCG (rv); IL-6, TCCAAAGATGTAGCCGCCAC (fw) and TTCTGCCAGTGCCTCTTGTGCTG (rv); HPRT, TGAGACTGGCAAAA CAATGCA (fw) and GGTCCTTTTACCAGCAAGCT (rv).

Mouse primers were as follows: TNF- $\alpha$ , CCGAGTACAAGCCTG TAGC (fw) and GAAGGAGACGGTAAAGTTGTTGA (rv); IFN- $\beta$ , CTC ACCTACAGGGCGGACT (fw) and GGCAAAGGCAGTGAACCTT; IL-8, ATGGGCTCTGACTGGAATACT (fw) and CGTGTCTTACAT GCTATCAAGC (rv); HPRT, AGTCCCAGCGTCGTGATTAG (fw) and TTCCAAATCCTCGGCATAATGA (rv).

**Poly(I-C) pulldown assays.** HepaRG cells were transfected with 1  $\mu$ g of high-molecular-weight poly(I-C) or 1  $\mu$ g of high-molecular-weight poly(I-C)-biotin (InvivoGen, CA, USA) complexed with the transfection reagent LyoVec (InvivoGen, CA, USA). After 24 h, cells were lysed in HNTG buffer (20 mM HEPES, pH 7.9, 150 mM NaCl, 1% Triton, 10% glycerol, 1 mM MgCl<sub>2</sub>, 1 mM EGTA) with protease inhibitors. Cell lysates were incubated with 70  $\mu$ l of Dynabeads MyOne Streptavidin T1 (Invitrogen, Carlsbad, CA) for 30 min at 4°C. After samples were washed extensively in HNTG buffer, the precipitates were eluted by boiling in Laemmli sample buffer and analyzed by immunoblotting with the respective antibodies. For *in vitro* pulldown assays, human NOD1 and RIG-I cDNA

open reading frames (ORFs) were cloned downstream of the T7 promoter sequence in pET15b His Rfb (Novagen). Each protein marked with [<sup>35</sup>S]methionine (PerkinElmer) was synthesized in a separate reaction with a TNT coupled reticulocyte lysate kit (L4611; Promega) according to the manufacturer's instructions. Fifteen microliters of extracts was mixed with 50  $\mu$ g of poly(I-C) or 1  $\mu$ g of biotinylated poly(I-C) or both (50:1) in a final volume of 200  $\mu$ l of HNTG buffer and incubated overnight at 4°C on a rolling platform. A pulldown experiment was carried out as described above, and results were analyzed by SDS-PAGE, transfer, and autoradiography.

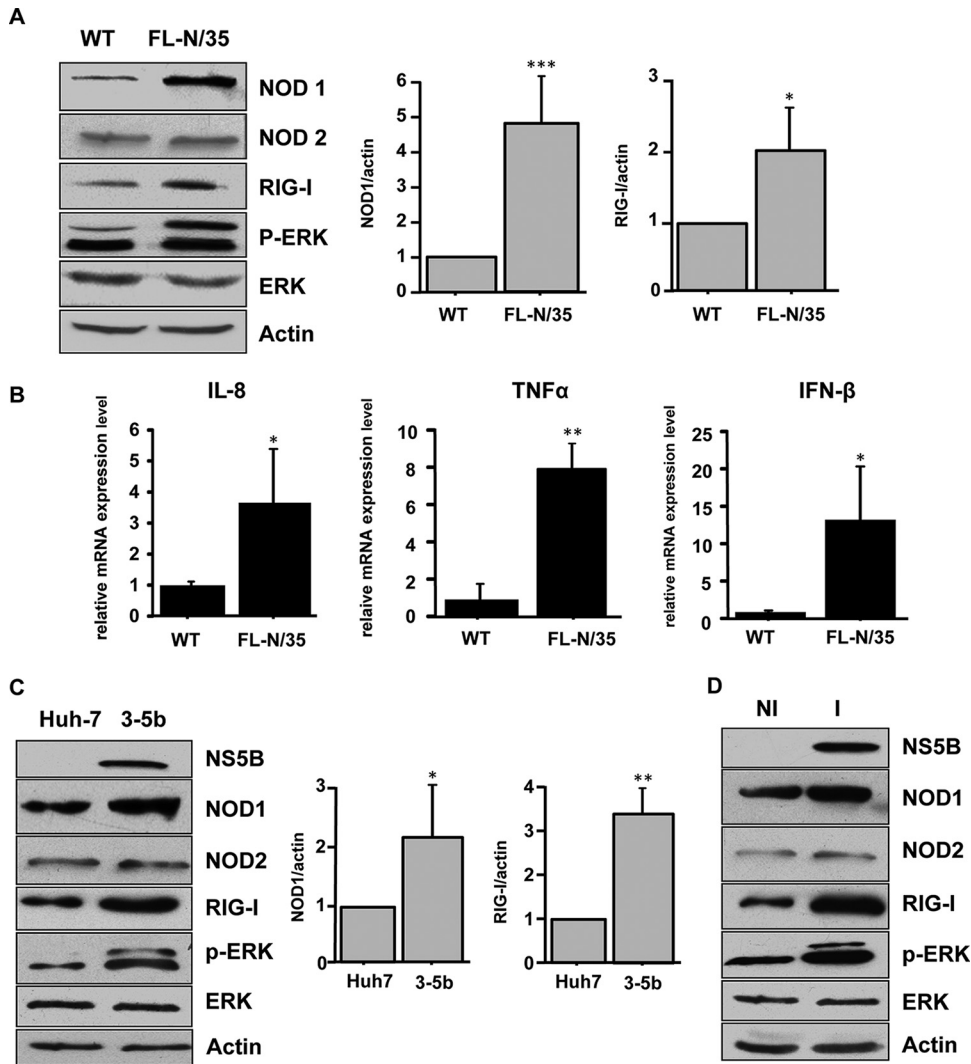
**Statistical analysis.** Experiments were performed at least three times. Data are presented either from a representative experiment or as means  $\pm$  standard errors of the means (SEM). Comparisons between groups were analyzed by Student's *t* test as indicated.

## RESULTS

**NS5B increases NOD1 expression and activates its downstream targets.** Long-term expression of the full complement of HCV proteins in livers of transgenic mice of the FL-N/35 lineage (31) gives rise to numerous physiopathological alterations, reminiscent of those encountered in human hepatitis C virus infection (31, 36–38). Despite lack of overt inflammation in this model of constitutive transgene expression, we have recently described the role of viral proteins and, more specifically, of the viral polymerase, in triggering inflammatory signaling during hepatocarcinogenesis driven by the HCV transgene (12). NS5B, the HCV RNA-dependent RNA polymerase, synthesizes double-stranded RNA of cellular origin that triggers innate immune responses in hepatocytes and leads to production of type I IFN and other inflammatory cytokines (12, 13, 39–42). However, pathways involved in this process have not been fully elucidated.

Expression levels of PRRs generally increase in concert to the mounting response to microbial infection, and such increase is considered to reflect pathway activation (43, 44). Accordingly, expression of RIG-I, an RNA sensor with a well-documented role in HCV infection, is increased in FL-N/35 primary hepatocytes compared to levels in cells isolated from the littermate controls (Fig. 1A). More unexpectedly, this is also the case for NOD1, a receptor classically activated by PAMPs derived from a bacterial wall (24), while the low-level expression of the related NOD2 protein is not altered in the HCV transgenic livers. Increased NOD1 expression is accompanied by activation of its downstream target, MAPK/ERK, as evidenced by increased levels of the phosphorylated form of the kinase (Fig. 1A). Expression levels of IL-8 and TNF- $\alpha$ , two cytokines classically induced by NOD signaling (24), are also increased in the livers of the FL-N/35 animals, as is the expression of IFN- $\beta$ . Furthermore, analysis of cells actively replicating viral RNA and of primary human hepatocytes (PHH) productively infected by HCV (35) showed a significant increase of NOD1 and phosphorylation of MAPK/ERK (Fig. 1B and C), indicating that activation of NOD signaling also occurs in the course of HCV infection.

To investigate the mechanisms of induction of inflammatory signaling by HCV proteins, we used HepaRG cells, which are human hepatocyte progenitors closely related to primary cells, notably with respect to their fully functional immune signaling network (32, 45, 46). Since we have previously shown that the viral polymerase triggers inflammatory signaling in hepatocytes (12), we now analyzed its effects on the level of expression of several cellular PRRs (i.e., RIG-I, MDA5, TLR3, TLR9, NOD1, and NOD2). As expected, we observed increased expression of RIG-I,



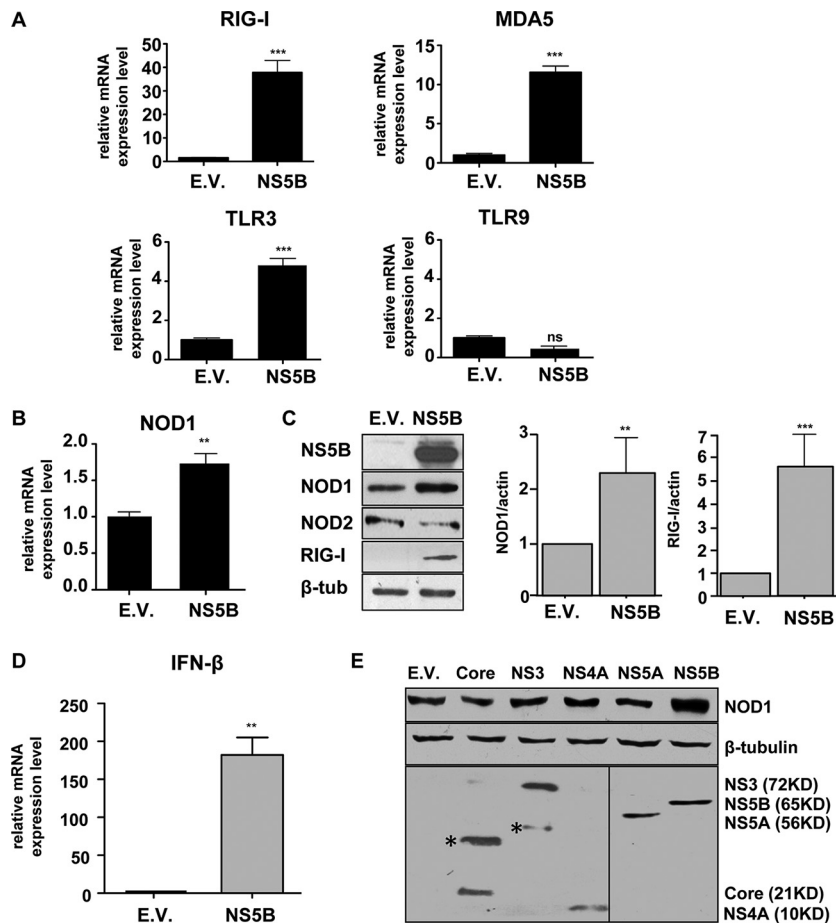
**FIG 1** HCV proteins increase NOD1 expression. Primary hepatocytes were purified from FL-N/35 and age- and sex-matched wild-type mice. (A) Representative Western blot (left panel) and quantifications from three independent experiments (right panels). The relative expression levels in the wild-type mice were arbitrarily set as 1. (B) Reverse transcription-quantitative PCR analysis of IL-8, TNF- $\alpha$ , and IFN- $\beta$  mRNAs in livers of wild-type and FL-N/35 transgenic animals. (C) Proteins were extracted from exponentially growing subgenomic (Nneo/3-5B) replicon and parental Huh-7 cells, analyzed by Western blotting, and quantified. The relative expression of proteins in the Huh7 parental cells was arbitrarily set at 1. (D) Primary human hepatocytes were infected with the HCV Jc1 chimeric virus. Proteins were extracted 2 weeks later and analyzed by Western blotting. NI, noninfected control cells; I, infected cells. Representative Western blots are shown; where appropriate, results are presented as means  $\pm$  SEM of at least three independent experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.001$ ). p-ERK, phospho-ERK.

MDA5, and TLR3, three PRRs activated by viral dsRNA and previously suggested to be involved in IFN- $\beta$  induction mediated by NS5B activity (13, 39, 41, 42) (Fig. 2A). Accumulation of these RNA sensors was accompanied by a dramatic increase in IFN- $\beta$  expression (Fig. 2B), confirming previous results obtained in other cellular models (11, 13, 39, 41, 42, 47). In addition, NS5B gave rise to augmented expression of NOD1 (Fig. 2A). Of note, neither TLR9, which recognizes viral DNA (48), nor NOD2, which responds to a bacterial peptidoglycan (24) and to viral ssRNA (28), was upregulated following NS5B expression (Fig. 2A and data not shown).

The observed phenotype appears specific to NS5B since NOD1 expression was not altered by HCV core, NS3, NS4A, and NS5A proteins (Fig. 2C). Importantly, NS5B-driven activation of NOD1

was confirmed by increased expression of several canonical targets of this PRR (reviewed in reference 25). Specifically, in NS5B-expressing cells the levels of IL-8 and TNF- $\alpha$  mRNAs were comparable to those observed after treatment of parental cells with a peptide mimicking the bacterial ligand of NOD1 (Fig. 3A). MAP kinases ERK and Jun N-terminal protein kinase (JNK) were phosphorylated, as was I $\kappa$ B, the NF- $\kappa$ B inhibitor (Fig. 3B). As expected, I $\kappa$ B phosphorylation was accompanied by its destabilization, presumably leading to the activation of the conventional NF- $\kappa$ B signaling. Finally, in agreement with the fact that NS5B triggers RIG-I signaling leading to IFN- $\beta$  production (13) (Fig. 1 and 2), NS5B gave rise to activation of interferon regulatory factor 3 (IRF3).

To investigate if the enzymatic activity of NS5B was necessary



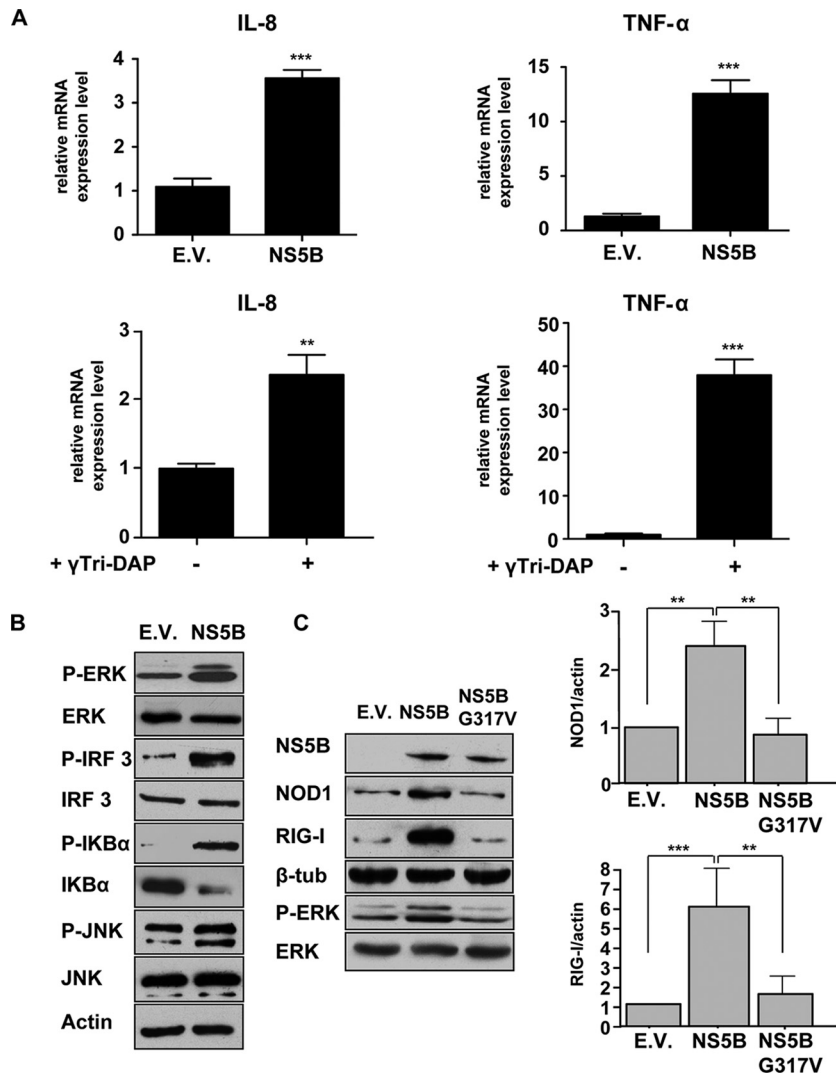
**FIG 2** NS5B activates innate immunity receptors. HepaRG cells were transduced with retroviruses encoding NS5B or with an empty vector (EV) as a control. (A) Reverse transcription-quantitative PCR analysis of RIG-I, MDA5, TLR3, and TLR9 mRNA. (B) Reverse transcription-quantitative PCR analysis of NOD1 mRNA. (C) Western blot analysis of NS5B, NOD1, NOD2, and RIG-I.  $\beta$ -Tubulin was used as a loading control. NOD1 and RIG-I protein levels were quantified in three independent experiments (right panel); the relative expression of proteins in the control cells was arbitrarily set as 1. (D) Reverse transcription-quantitative PCR analysis of IFN- $\beta$  expression in control (EV) and in NS5B-expressing cells. (E) HepaRG cells were transduced with retroviral vectors encoding the indicated Myc epitope-tagged HCV genotype 1b proteins. NOD1 expression was assayed by Western blotting with  $\beta$ -tubulin serving as a loading control. Viral protein expression was confirmed by probing a Western blot with an anti-Myc antibody. The asterisks indicate nonspecific bands. Where appropriate, results are presented as means  $\pm$  SEM of three separate experiments (\*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ ; ns, not significant).

for RIG-I and NOD1 induction and activation of their downstream targets, HepaRG cells were transduced with a vector encoding NS5B that is catalytically inactive because of a mutation in the GDD motif (34). NS5B G317V had no effect on either the level of RIG-I and NOD1 expression or ERK signaling (Fig. 3C), suggesting the importance of the enzymatic activity in NS5B-induced inflammatory signaling.

**Synthetic dsRNA stimulates NOD1 signaling.** It was previously reported that RNAs produced by NS5B are double-stranded species of cellular origin (13). In order to confirm that dsRNA is instrumental in activation of NOD1 in HepaRG cells, we used a synthetic dsRNA, the poly(I-C), that mimics viral infection in terms of eliciting the host's immune responses by triggering specific PRRs such as TLR3 (49), RIG-I, and MDA5 (50). In order to target intracellular PRRs, we used poly(I-C) complexed with a transfection reagent, LyoVec, thus stimulating RIG-I and MDA5, which are cytoplasmic receptors specialized in viral RNA recognition (51). As expected, the RIG-I pathway was activated upon treatment of HepaRG cells with poly(I-C)/LyoVec, as shown by

increased expression of RIG-I and IFN- $\beta$  (Fig. 4A and B) (28, 52). Interestingly, expression of NOD1 and its downstream target, phospho-ERK, was also increased (Fig. 4A). Of note, NOD1 protein expression preceded RIG-I activation, arguing that the early steps of NOD1 activation in this model are unlikely to be due to RIG-I signaling. Similar to stimulation by NS5B, synthetic dsRNA led to increased levels of TNF- $\alpha$  and IL-8 (data not shown). These results indicate that a synthetic dsRNA, in addition to activating RIG-I, also switches on the NOD1 pathway.

To gain a mechanistic insight into activation of NOD1 by dsRNA, we investigated their possible interaction. To ensure the physiological relevance of this approach, we chose to perform a pull-down assay following an *in vivo* exposure of receptors to biotinylated poly(I-C). Poly(I-C) or biotin-tagged poly(I-C) was complexed with the transfection agent LyoVec and introduced into cells. The two ligands gave rise to comparable activation levels of RIG-I and NOD1 (data not shown). At 24 h posttransfection, cells were lysed, and a pull-down assay was performed with streptavidin-bound magnetic beads. Strikingly, we observed that a signifi-



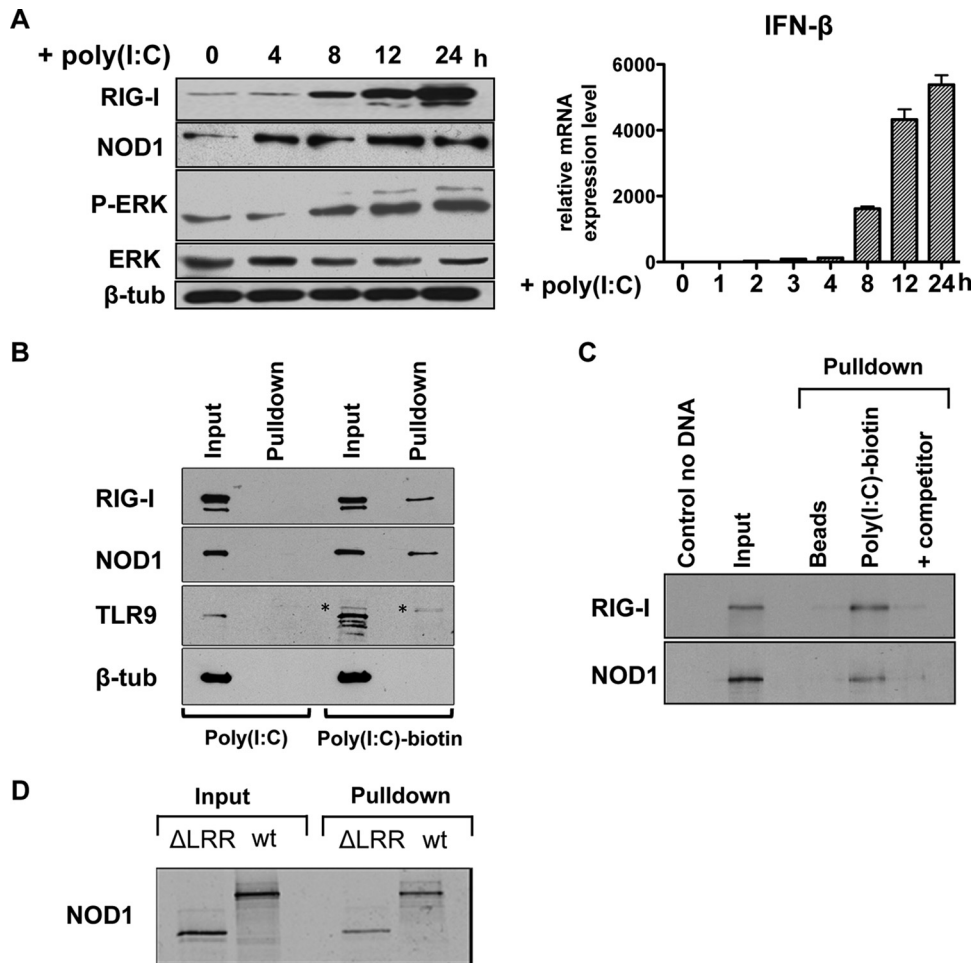
**FIG 3** NS5B activates NOD1 signaling. (A) Reverse transcription-quantitative PCR analysis of IL-8 and TNF- $\alpha$  mRNA expression. Total RNA was extracted from HepaRG cells transduced with either an empty vector (EV) or an NS5B-encoding (NS5B) retroviral vector (upper panel) or treated for 2 h with  $\gamma$ Tri-DAP at 10  $\mu$ g/ml (lower panel) and analyzed by reverse transcription-quantitative PCR. (B) Proteins were extracted from HepaRG cells transduced with an empty (EV) or an NS5B-encoding (NS5B) retroviral vector and analyzed by Western blotting. (C) HepaRG cells were transduced with an empty retroviral vector (E.V.) or a vector encoding a catalytically active (NS5B) or inactive (NS5B G317V) NS5B. Proteins were extracted and analyzed by Western blotting, and NOD1 and RIG-I expression levels were quantified relative to the level of  $\beta$ -tubulin. The relative expression levels of proteins in the control cells was arbitrarily set at 1. Where appropriate, results are presented as means  $\pm$  SEM of at least three independent experiments (\*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ ).

cant fraction of cellular NOD1 was bound to the synthetic dsRNA ligand, as was RIG-I, used as a positive control, but not TLR9, specific to dsDNA and used here as a negative control (Fig. 4B). No signal was detected with lysates from cells treated with untagged poly(I-C), demonstrating that the binding was not due to nonspecific retention of proteins by the magnetic beads. To confirm the specificity of the interaction with dsRNA, we next performed pulldown assays with *in vitro*-translated NOD1 or RIG-I proteins. In this experimental setup, both PRRs bind to synthetic dsRNA, and the interaction is inhibited by an excess of competitor RNA (Fig. 4C). Thus, NOD1 forms a complex with dsRNA.

Like all NLR family members, NOD1 is composed of three functional domains, with the carboxyl-terminal LRR motif serving as a ligand recognition domain (17). Since dsRNA is structurally unrelated to the conventional peptidoglycan activator of

NOD1 signaling, we asked if the LRR domain was also involved in this novel ligand recognition. This does not appear to be the case since *in vitro*-synthesized NOD1 with a deletion of the LRR (NOD1 <sup>$\Delta$ LRR</sup>) bound double-stranded synthetic RNA as efficiently as the wild-type form of the protein (Fig. 4D).

**NOD signaling participates in the inflammatory response elicited by dsRNA.** To investigate the role of NOD1 in the overall inflammatory response to double-stranded RNA, we employed a dominant negative mutant of this receptor. DN-NOD1 lacks a part of the NBD (amino acids 127 to 518) and interferes with signal transduction upon forming a dimer with a wild-type form of the receptor (53). Ectopic expression of Myc-tagged DN-NOD1 in HepaRG cells (Fig. 5A) compromises NOD1 signaling upon stimulation by a conventional NOD1 activator, the synthetic peptidoglycan  $\gamma$ Tri-DAP (Fig. 5B). Control cells, infected with the

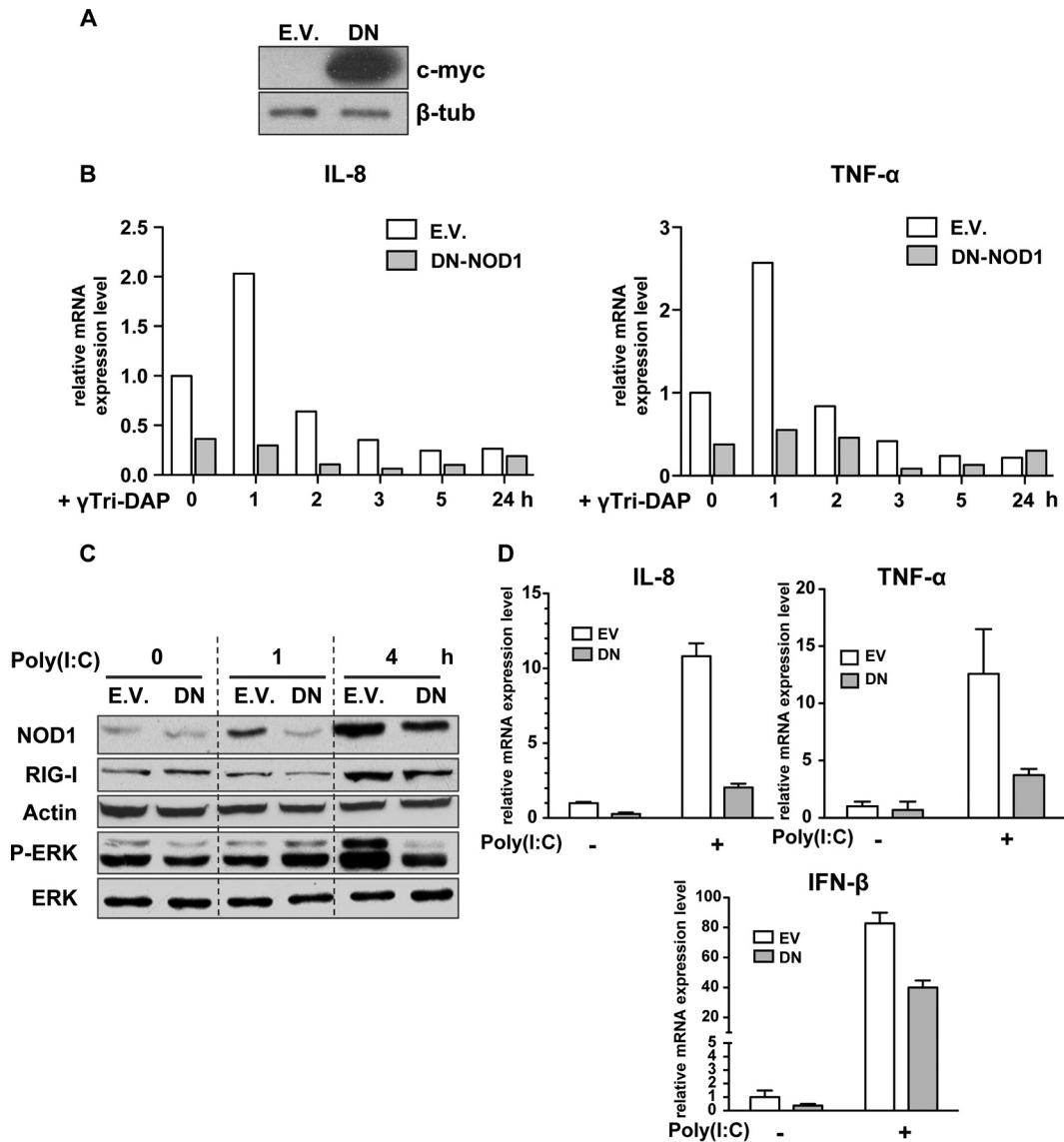


**FIG 4** Synthetic dsRNA binds and activates NOD1. (A) HepaRG cells were treated with poly(I:C)/LyoVec (1  $\mu$ g/ml) for the indicated times, and proteins and RNA were analyzed by Western blotting and reverse transcription-quantitative PCR, respectively. Western blot analysis was performed of RIG-I, NOD1, phospho-ERK (p-ERK), and ERK (left panel). Tubulin served as a loading control. Reverse transcription-quantitative PCR analysis was performed of IFN- $\beta$  mRNA (right panel). (B) HepaRG cells were transfected with poly(I:C)/LyoVec or poly(I:C)-biotin/LyoVec. Cellular lysates were incubated with magnetic beads coupled to streptavidin, and retained proteins were analyzed by Western blotting. The input corresponds to 1% of the cell lysate used in the experiment; for pull-down assays, 25% of the eluted products was loaded on the gel. TLR9 and tubulin were used as negative controls. Asterisks indicate nonspecific bands recognized by the anti-TLR9 antibody. (C) *In vitro*-synthesized [ $^{35}$ S]methionine-labeled human NOD1 and RIG-I were incubated with biotinylated poly(I:C) in the presence or absence of 50 $\times$  excess of unlabeled poly(I:C) competitor, as indicated. Samples were exposed to streptavidin-coupled magnetic beads, and the retained proteins were analyzed by autoradiography following SDS-PAGE. The no-DNA control corresponds to a sample where no plasmid was included in the *in vitro* synthesis step. The input corresponds to 1% of the lysate used in the experiment. For pull-down assays, 50% of the eluted products was loaded. The lane labeled "Beads" corresponds to the experiment performed without biotinylated poly(I:C). (D) The experiment was performed as described for panel C using either the full-length construct encoding wild-type NOD1 or a deletion mutant lacking the C-terminal LRR domain, as indicated.

empty retroviral vector (EV) and treated with poly(I:C), showed increased NOD1 and RIG-I expression, ERK phosphorylation, and activation of IFN- $\beta$ , TNF- $\alpha$ , and IL-8 synthesis (Fig. 5C and D). In contrast, the same treatment of their counterparts with compromised NOD1 signaling gave rise to impairment of NOD1 and RIG-I accumulation and ERK phosphorylation (Fig. 5C) as well as to severely diminished production of IL-8, TNF- $\alpha$ , and, to a lesser extent, of IFN- $\beta$  (Fig. 5D), the last being particularly striking since it is a classical outcome of RIG-I activation. It would thus appear that functional NOD1 signaling is required for the full activation of several downstream effectors of poly(I:C) stimulation.

To examine the impact of NOD1 on the cellular response to endogenously produced dsRNA in a context relevant to a viral infection, we next interfered with NOD1 function in cells consti-

tively expressing NS5B. Compromised NOD1 signaling fully abrogated the increase of expression of NOD1 as well as activation of the conventional NOD1 targets: MAPK/ERK, IL-8, and TNF- $\alpha$  (Fig. 6A and B). Moreover, NS5B-triggered production of IFN- $\beta$  was also severely impaired in the absence of functional NOD1 (Fig. 6B). To eliminate the possibility that the dominant negative form of NOD1 had artifactual effects unrelated to NOD1 inhibition, we next interfered with NOD1 using gene editing by CRISPR/Cas9 technology. Despite several attempts, we were not successful in fully abrogating NOD1 expression in HepaRG cells. Nevertheless, even incomplete inhibition of this PRR (Fig. 6C) gave rise to a strong phenotype. In perfect concordance with results from the DN mutant, gene editing of NOD1 desensitized the cells to NS5B. Indeed, cells with compromised NOD1 showed impaired ERK activation and a strong decrease of IL-8, TNF- $\alpha$ ,

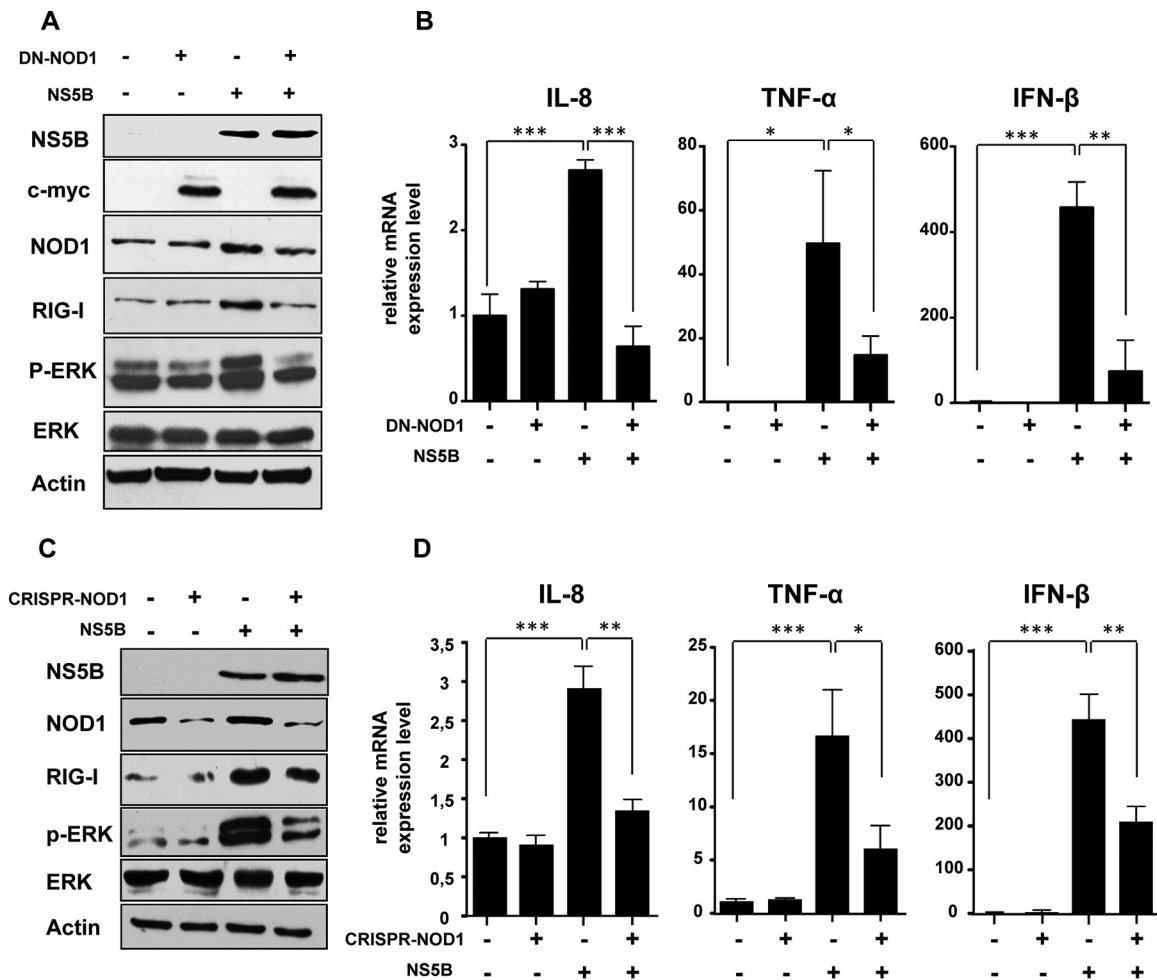


**FIG 5** Interference with NOD1 function inhibits RIG-I signaling. HepaRG cells were transduced with retroviral vectors, either an empty vector (EV) or a vector encoding a dominant negative (DN) form of the NOD1 receptor tagged with a Myc epitope. (A) Expression of the transgene was confirmed by probing a Western blot with an anti-Myc antibody. (B) Reverse transcription-quantitative PCR analysis of IL-8 and TNF- $\alpha$  mRNA expression in HepaRG and HepaRG-DN cells treated with  $\gamma$ Tri-DAP (10  $\mu$ g/ml) for the times indicated. (C and D) HepaRG and HepaRG-DN cells were treated with poly(I:C)/LyoVec (0.1  $\mu$ g/ml) for the indicated times, and protein (C) and mRNA (D) were analyzed by Western blotting and reverse transcription-quantitative PCR, respectively. Representative experiments out of three are shown in panels B and C.

and IFN- $\beta$  production upon stimulation with NS5B (Fig. 6D). Altogether these data highlight a novel activity of NOD1, likely to play a role in viral infection through its cooperation with RIG-I in the inflammatory response to double-stranded cytoplasmic RNA.

Given that the kinetics of NOD1 accumulation in response to dsRNA signaling apparently precedes that of RIG-I accumulation, we considered it unlikely that NOD1 is a downstream effector of RIG-I in our experimental setup. To further ascertain this point, we investigated NOD1 activity in the absence of RIG-I signaling. RIG-I expression was abrogated through CRISPR/Cas9-mediated gene editing. This did not interfere with either NS5B-induced NOD1 accumulation or MAPK/ERK phosphorylation or TNF- $\alpha$  and IL-8 production (Fig. 7A and B). In contrast, synthesis of

IFN- $\beta$ , while not abrogated, was diminished by about 50% in RIG-I-deficient cells. Similarly, RIG-I deletion did not interfere with NOD1 accumulation and ERK activation following intracellular stimulation with synthetic dsRNA (Fig. 7C), further strengthening the argument that NS5B's effect is mediated by the RNA products it synthesizes. Moreover, the same phenotype was observed in cells depleted of MAVS, a downstream effector of RIG-I and MDA5 that has been reported to be also essential in the alternative NOD2 pathway initiated by viral ssRNA recognition (28). Silencing MAVS in cells constitutively expressing NS5B decreased RIG-I accumulation but had no significant effect on either NOD1 expression or activation of its targets, the MAPK/ERK, IL-8, and TNF- $\alpha$  (Fig. 8A and B). In contrast, abrogation of RIPK2, a down-



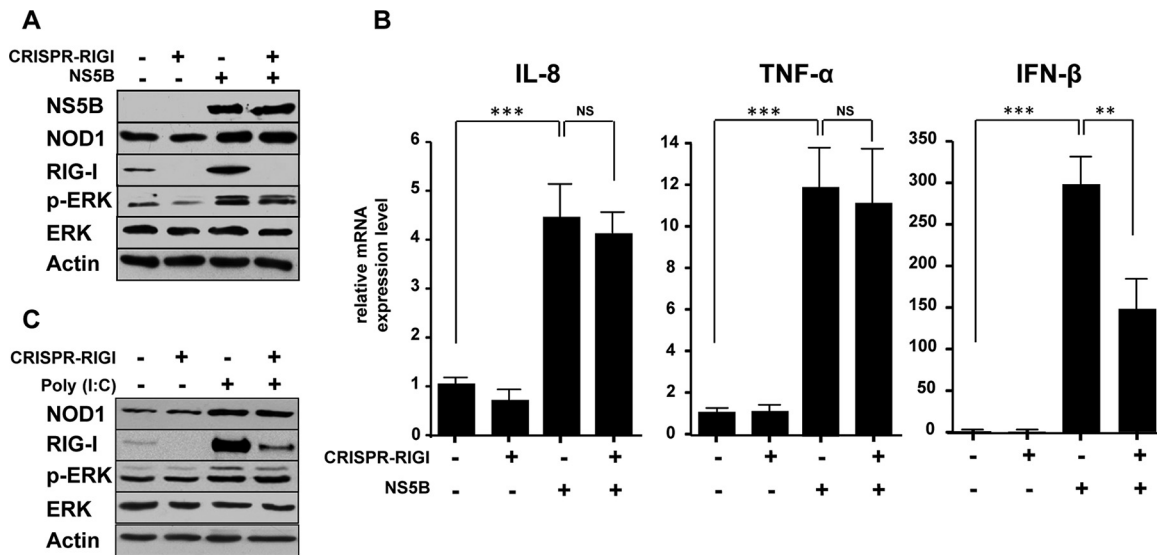
**FIG 6** Compromised NOD1 signaling inhibits NS5B-induced innate immune response. HepaRG and HepaRG-DN-NOD1 cells were transduced with a retroviral vector encoding NS5B or an empty vector as a control. (A) Western blot analysis of NOD1, RIG-I, ERK, and phospho-ERK in HepaRG cells expressing Myc-tagged dominant negative NOD1 and NS5B, as indicated. (B) mRNA expression of IL-8, IFN- $\beta$ , and TNF- $\alpha$  as a function of NS5B and DN-NOD1 expression, as indicated. (C and D) HepaRG and HepaRG-NS5B cells were transduced with a lentiviral vector encoding CRISPR/Cas9 directed against NOD1 or a scrambled sequence as a negative control. Proteins (C) and RNA (D) were analyzed by Western blotting and reverse transcription-quantitative PCR, respectively. Where appropriate, results are presented as means  $\pm$  SEM of three independent experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ ).

stream effector of NOD (24), phenocopied NOD1 inhibition or removal (Fig. 6). Indeed, in RIPK2-deficient cells activation of MAPK/ERK and synthesis of IL-8 and TNF- $\alpha$  were severely diminished while IFN- $\beta$  production was again decreased by about 50% (Fig. 8C and D). Altogether our data indicate that NOD1 signaling is triggered by dsRNA, either synthetic or produced by the viral polymerase, independently of the RIG-I/MAVS pathway.

## DISCUSSION

Innate immunity is mandatory for clearance of viral infections (54). When clearance is inefficient, the ongoing innate response contributes to pathologies related to a chronic state of inflammation. This is typically the outcome of HCV infections, where chronic inflammation is associated with many adverse effects, notably, liver fibrosis, cirrhosis, and HCC. We along with others have previously reported that NS5B, the HCV RNA-dependent RNA polymerase, induces expression of proinflammatory cytokines (12, 13). Double-stranded RNA, generated by viral RNA polymerases during replication and transcription of viral genomes, is a

common PAMP recognized by the immune system during infection by RNA viruses. In addition, NS5B exhibits little specificity toward template RNA and thus can activate innate immune signaling through dsRNAs that are transcribed from nonspecific cellular templates (13, 55–58). Both TLR3 and RIG-I innate immunity receptors have been described to trigger cellular responses to RNA produced by NS5B (11, 13, 39, 41, 42). Here, we show that NS5B, in addition to activating TLR3, RIG-I, and MDA5, also induces and activates NOD1, a receptor classically associated with a response to bacterial infection. Similar to activation of RIG-I and TLR3, the enzymatic activity of NS5B, rather than the protein *per se*, is required for NOD1 induction, suggesting that signaling is initiated by dsRNA. Moreover, the concomitant increase in expression of phospho-ERK, IL-8, and TNF- $\alpha$ , all of which are downstream targets of NOD1, strongly suggests that NS5B not only augments NOD1 expression but also activates the pathway. Of note, while the extensive cross talk between signal transduction pathways triggered by pattern recognition receptors (48) makes it difficult to define targets universally fully specific for a given PRR,



**FIG 7** Abrogation of RIG-I has little effect on NS5B- and poly(I-C)-induced NOD1 signaling. HepaRG and HepaRG-NS5B cells were transduced with a lentiviral vector encoding CRISPR/Cas9 directed against RIG-I or a scrambled sequence as a control. Proteins (A) and RNA (B) were analyzed by Western blotting and reverse transcription-quantitative PCR, respectively. Where appropriate, results are presented as means  $\pm$  SEM of three separate experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ ). (C) HepaRG cells were treated with poly(I-C)/LyoVec (1  $\mu$ g/ml) for the indicated times, and RIG-I, NOD1, p-ERK and ERK protein levels were analyzed by Western blotting.

we show that in the experimental model used in this work, ERK, IL-8, and TNF- $\alpha$  are in fact largely specific to the NOD1 pathway. Indeed, their activation by dsRNA is strongly inhibited by abrogation of NOD1 signaling, while shutting down either TLR3 or RIG-I signaling has little effect.

Several NOD-like receptors have recently been reported to participate in inflammatory responses to viral infection. For example, the NLRP3 inflammasome can mediate the response to influenza virus (59, 60) and is activated upon infection with adenovirus and Sendai virus (61), whereas NLRX1 interacts with MAVS to negatively regulate the interferon pathway (62), leading to attenuation of the inflammatory response to influenza virus infection (63).

While NOD1 is not classically associated with RNA recognition, our data are concordant with the recently reported increase in NOD1 and NOD2 signaling initiated by either poly(I-C) or infection with RNA viruses, for example, viral hemorrhagic septicemia virus (VHSV) as well as vesicular stomatitis virus (VSV) and murine norovirus-1 (MNV-1) (26, 27). In the study by Park et al. (27), activation of NOD1 and -2 in macrophages was primed by type I IFN, which was synthesized through the classical antiviral innate immunity pathways. In contrast to our results, NOD activation by RNA was only revealed following exposure to bacterial PAMPs. Moreover, in macrophages the NOD receptors did not contribute to interferon production. This is in striking contrast to the results we report regarding the role of NOD1 in the inflammatory response in hepatocytes exposed to dsRNA. Specifically, we show that NOD1 responds to dsRNA in the absence of bacterial antigens and does so in cells deficient in TLR3 as well as in cells depleted of either RIG-I or MAVS. While this is very different from the NOD response to RNA previously described in macrophages, it remains possible that indirect activation via type I IFN may participate in the long-term maintenance of NOD1 signaling in hepatocytes.

NOD2, but not NOD1, can recognize and respond to single-stranded viral RNAs in a variety of cell types (28). In this context, NOD2 does not signal through its conventional effector RIPK2 but, rather, uses the alternative adaptor MAVS to give rise to activation of IRF3 and the consequent synthesis of type I IFN. This is not the case for the NOD1 response to dsRNA. Indeed, we show that in this scenario, inflammatory signaling requires NOD's conventional effector RIPK2, while MAVS has no discernible effect on NOD1 activation or signaling. This result is coherent with the report showing that, in contrast to NOD2, NOD1 failed to interact with MAVS (28). The interaction between NOD2 and MAVS, which requires the NBD and LRR domain of NOD2, seems to be an essential event that links recognition of ssRNA by this receptor to activation of IRF3 (28), whereas the conventional signal transmission through RIPK2 relies on the caspase recruitment domain (CARD). In contrast, LRR is believed to function in ligand recognition by the NLR (reviewed in reference 17). However, this does not appear to be true for the recognition by NOD1 of a dsRNA ligand since a mutant lacking the entire LRR domain is as efficient in dsRNA binding as the wild-type protein. Thus, while NOD1 and NOD2 receptors respond to, respectively, double-stranded and single-stranded RNA ligands, the mechanisms employed in the subsequent signal transduction appear to be quite different.

The apparent lack of involvement of MAVS in the NOD1-mediated response is of particular interest in the context of MAVS' well-established essential role in relaying signals from the RNA helicase RIG-I to induce type I IFNs during viral infection or following cytosolic delivery of dsRNA (for a review, see reference 64). It subsequently activates I $\kappa$ B kinases (IKKs), required for phosphorylation of IFN-regulatory factors 3 (IRF3) and 7 (IRF7) and thus transcriptional activation of type I IFNs (16). Interestingly, activation of RIG-I/MAVS and NOD1/RIPK2 by NS5B can be uncoupled, thus demonstrating that the latter is not a consequence of the former.

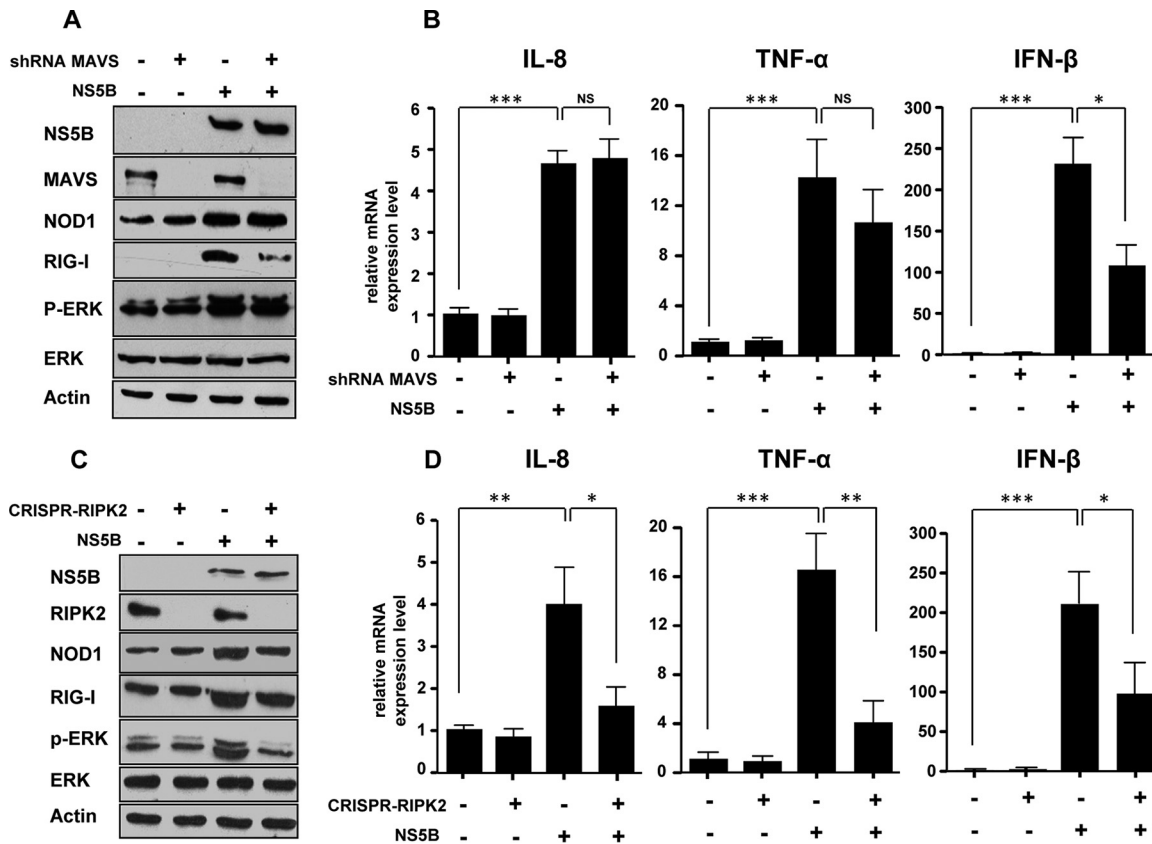


FIG 8 MAVS and RIPK2 pathways contribute to the innate immune response triggered by NS5B. (A and B) HepaRG and HepaRG-NS5B cells were transduced with a lentiviral vector encoding shRNA directed against MAVS or a control shRNA. Proteins (A) and RNA (B) were analyzed by Western blotting and reverse transcription-quantitative PCR, respectively. (C and D) HepaRG and HepaRG-NS5B cells were transduced with a lentiviral vector encoding CRISPR/Cas9 directed against RIPK2 or a scrambled sequence as a negative control. Protein extracts were analyzed by Western blotting (C), and RNA was analyzed by reverse transcription-quantitative PCR (D). Where appropriate, results are presented as means  $\pm$  SEM of three separate experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ ).

Expression of additional PRRs, namely, TLR3 and MDA5, is also increased in the presence of NS5B. It is unlikely that activation of these receptors accounts for the observed NOD1-mediated phenotype. Indeed, shRNA-mediated knockdown of MAVS expression (Fig. 8) would be expected to inhibit both RIG-I and MDA5 signaling, and we show that it has little effect on NOD1 downstream targets. Thus, MDA5 is unlikely to participate in NOD1 activation or signaling. In contrast to RLR and NOD receptors, TLR3 is in contact with the external milieu. While we cannot exclude the presence of low levels of extracellular dsRNA due, for example, to cell death in culture, the use of liposomes to introduce dsRNA into cells and the intracellular dsRNA production by NS5B make it unlikely that TLR3 stimulation constituted the initial signaling event in our experimental setup.

To the best of our knowledge, direct NOD1 activation by dsRNA has not so far been reported. We show that NOD1 forms a complex with dsRNA, both *in vivo* and *in vitro*. The complex does not require any additional hepatocyte-specific factors since *in vitro*-synthesized protein is also efficiently bound to RNA. The notion that NOD1-mediated recognition of dsRNA is indeed physiologically relevant is strengthened by our demonstration of severely perturbed cytokine production in response to dsRNA when NOD1 signaling is abrogated by its dominant negative isoform or by gene editing. We conclude that NOD1 is a significant

participant of the inflammatory response of hepatocytes to double-stranded RNA.

Thus, NOD1 may play a role in the innate immune reaction against viral infection both through its intrinsic activity and through cross talk with other PRRs (65–67). Importantly, NOD1 expression is augmented in hepatocytes of hepatitis C virus patients (68). We now report that, in addition to the acute response to a synthetic ligand and to ectopic expression of the viral polymerase, long-term expression of low levels of all viral proteins in mouse hepatocytes as well as bona fide infection of primary human hepatocytes with HCV also induce NOD1 expression. Altogether these data highlight a novel activity of NOD1, likely to play a role in HCV infection through its cooperation with RIG-I in the inflammatory response to double-stranded cytoplasmic RNA.

In addition to the novel role of NOD1 in the context of virus pathology, its increased expression may play a role in secondary bacterial infections, which can complicate chronic hepatitis C virus infection (69–71). This is reminiscent of the reported increase in mortality induced by secondary bacterial infection in virally infected mice, which is mediated by NOD1 and NOD2 (26), and of the critical role of NOD2 in severe liver injury (72). In the context of HCV infection, NS5B, in parallel to directly inducing inflammatory cytokines, might sensitize patients to bacterial in-

fection and thus further contribute to the inflammatory process and liver damage.

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