Cell-Type-Specific Type I Interferon Antagonism Influences Organ Tropism of Murine Coronavirus[▽]

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Previous studies have demonstrated that mouse hepatitis virus (MHV) hepatotropism is determined largely by postentry events rather than by availability of the viral receptor. In addition, mutation of MHV nonstructural protein 2 (ns2) abrogates the ability of the virus to replicate in the liver and induce hepatitis but does not affect replication in the central nervous system (CNS). Here we show that replication of ns2 mutant viruses is attenuated in bone marrow-derived macrophages (BMM) generated from wild-type (wt) mice but not in L2 fibroblasts, primary astrocytes, or BMM generated from type I interferon receptor-deficient (IFNAR^{-/-}) mice. In addition, ns2 mutants are more sensitive than wt virus to pretreatment of BMM, but not L2 fibroblasts or primary astrocytes, with alpha/beta interferon (IFN- α/β). The ns2 mutants induced similar levels of IFN- α/β in wt and IFNAR^{-/-} BMM, indicating that ns2 expression has no effect on the induction of IFN but rather that it antagonizes a later step in IFN signaling. Consistent with these *in vitro* data, the virulence of ns2 mutants increased to near that of wt virus after depletion of macrophages *in vivo*. These data imply that the ability of MHV to replicate in macrophages is a prerequisite for replication in the liver and induction of hepatitis but not for replication or disease in the CNS, underscoring the importance of IFN signaling in macrophages *in vivo* for protection of the host from hepatitis. Our results further support the notion that viral tissue tropism is determined in part by postentry events, including the early type I interferon response.

Coronaviruses are positive-strand RNA viruses that induce a wide variety of diseases in both humans and animals. Five to 30 percent of common colds are caused by human coronaviruses (HCoVs) (44). The emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 resulted in over 8,000 documented cases of SARS before the outbreak was extinguished, with a mortality rate of 9.6%, highlighting the importance of understanding coronavirus-associated diseases. Mouse hepatitis virus (MHV), a murine coronavirus, infects several organ systems in laboratory mice, thereby providing models for virus-induced acute encephalitis, hepatitis, and pneumonitis in a natural host, as well as for chronic demyelinating diseases such as multiple sclerosis (8, 44). The MHV model has been used extensively for investigating coronavirus-host interactions, including elucidating the determinants of tissue tropism and virulence. Infections with a collection of recombinant viral strains and mutants that display different tropisms and virulence levels have demonstrated that the organ tropism and virulence of MHV depend on a combination of viral genes and host response factors (21, 23–26).

Comparison of the pathogenesis of wild-type (wt) MHV strains with different abilities to replicate in the liver and induce hepatitis, namely, the hepatotropic MHV A59 and non-hepatotropic JHM.SD strains, with recombinant A59/JHM chimeric viruses demonstrated, perhaps surprisingly, that the ability to infect the liver did not map to the spike gene, which

encodes the protein responsible for receptor interaction and viral entry, but rather to background genes, implying that MHV tropism is due at least in part to postentry mechanisms (23). One early and effective host defense mechanism against viral invasion is the type I interferon (IFN; primarily IFN- α/β) response. There are published data indicating that basal expression levels of interferon-stimulated genes (ISGs) vary among different cell types and organs and can play an important role in determining susceptibility to initial viral infection, thus affecting organ tropism (14, 48). Indeed, the importance of the type I IFN response for restriction of MHV infection is implied by the observation that mice deficient in type I IFN signaling (IFNAR^{-/-} mice) exhibit increased viral replication and spread within and outside the central nervous system (CNS), loss of organ tropism barriers, and rapid death when infected with neurovirulent strains of MHV (31).

Despite the important role of type I IFN signaling in protection from MHV *in vivo*, most strains of MHV are relatively insensitive to IFN- α/β treatment of cell lines *in vitro* (30, 32, 43, 47). In addition, we have observed that IFN-treated neurons are unable to restrict the replication of MHV (unpublished data). However, other primary cell types, such as bone marrow-derived macrophages (BMM) and microglia, respond to IFN treatment by restricting MHV replication (30; unpublished data). These observations suggest that MHV has evolved mechanisms to antagonize the IFN response in a cell-type-specific fashion, which may influence pathogenesis *in vivo*.

We have previously shown that the accessory protein non-structural protein 2 (ns2) expressed by the dual-tropic (hepatotropic and neurotropic) MHV A59 strain is a liver-specific virulence factor that is necessary for the induction of hepatitis but dispensable for pathogenesis in the CNS (33). The se-

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quence of ns2 places it in the eukaryotic-viral LigT-like family of 2H-phosphoesterases, proteins involved in splicing during tRNA processing. As such, ns2 contains two conserved His-X-Thr/Ser motifs in which the His residues are predicted to be catalytic; homologous proteins are encoded by other group IIa coronaviruses as well as by toroviruses and human rotavirus (20, 39). Such proteins are predicted to have cyclic nucleotide phosphodiesterase (CPD) and/or RNA ligase activity and thus could possibly process or modify host or viral RNAs. In support of this proposed mechanism, mutation of either of the two predicted catalytic His residues (H46A or H126R mutation) attenuates A59 replication in the liver and reduces hepatitis to a minimal level, while substitutions of other amino acids near the catalytic His residues have no detectable effect on pathogenesis (33), implicating the activity of CPD in pathogenesis. However, no enzymatic activity has been demonstrated for ns2. We show here that MHV ns2 H46A and H126R mutants have increased sensitivity to type I IFN signaling in BMM but not in other cell types, implying that ns2 confers a cell-type-specific ability to antagonize the type I IFN antiviral response in macrophages. We conclude that IFN signaling in macrophages blocks viral infection of the liver but not of the brain and infer that the ability of a virus to overcome type I IFN signaling in macrophages is a prerequisite for invasion of the liver parenchyma and subsequent induction of hepatitis.

MATERIALS AND METHODS

Cell lines, plasmids, viruses, and mice. Murine L2 fibroblast cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), HEPES (10 mM), and 1% penicillin-streptomycin. Plaque assays were performed on L2 cells as described previously (13). Recombinant coronaviruses inf-MHV-A59 (referred to as wt A59 or A59 in this report), inf-ns2-H126R, and inf-ns2-H46A (referred to as ns2-H126R and ns2-H46A, respectively, in this report) have been described previously (33). MHV Wb1 (referred to as JHM.WUΔns2 in this report) and MHV Wb3 (referred to as JHM.WU in this report) have been described previously (36). Wb1 and Wb3 were both plaque purified from a tissue culture-passaged JHM stock; they are thus closely related but not isogenic. Both sets of viruses were provided by Stuart Siddell (University of Bristol, Bristol, United Kingdom). C57BL/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD). IFNAR^{-/-} mice (B6 background) were obtained from Michael Diamond (Washington University, St. Louis, MO) and were bred in our animal facility as described previously (32).

Infection of mice. Four- to 5-week-old B6 mice were anesthetized with isoflurane (IsoFlo; Abbott Laboratories) and inoculated intracerebrally (i.c.) in the left cerebral hemisphere with 10 PFU of JHM.WU or JHM.WU-Δns2 in 20 μl of phosphate-buffered saline (PBS) containing 0.75% bovine serum albumin (BSA) or intrahepatically (i.h.) with 500 PFU of A59 or ns2-H126R in 50 μl of PBS containing 0.75% BSA. At day 5 postinfection, the animals were sacrificed. Organs were harvested, and viral titers were determined by plaque assay on L2 cells (12, 33). Alternatively, organs were used for immunohistochemistry as described below. All experimental procedures were approved by the University of Pennsylvania IACUC. For macrophage depletion, clodronate-filled liposomes (200 μl) (provided by Nico Van Rooijen) were injected into the tail veins of 4- to 5-week-old C57BL/6 mice (42). PBS and empty liposomes (200 μl) were injected as controls. The next day, mice were anesthetized with isoflurane and inoculated i.h. with 500 PFU of A59 or ns2-H126R in 50 μl of PBS with 0.75% BSA.

Histology and immunohistochemistry. Brains and livers were removed and fixed overnight in 4% paraformaldehyde. Tissues were embedded in paraffin and sectioned, and sections were blocked with 10% normal donkey serum. The liver sections were stained with hematoxylin and eosin, and immunostaining was performed using a monoclonal antibody against MHV nucleocapsid (N) protein (1:20 dilution; kindly provided by Julian Leibowitz) or using F4/80 (1:50 dilution; Invitrogen). Staining was developed using an avidin-biotin-immunoperoxidase technique according to the manufacturer's directions (Vector Laboratories).

Astrocyte and microglia isolation. Primary mixed glial cultures were established as described previously (32). The brains of 1- to 3-day-old neonatal C57BL/6 mice were dissociated by repeated pipetting (approximately 20 times) and were passed through 75-μm nylon mesh. The cells were washed once in cold PBS and cultured in DMEM (high glucose) supplemented with 10% FBS and 1% penicillin-streptomycin. The medium was changed on days 3, 5, and 7 for astrocytes and on day 3 only for microglia. On day 10, flasks were shaken for 2 h at 260 rpm to remove nonadherent cells (microglia). The remaining adherent astrocytes were detached with trypsin-EDTA and replated. The microglia were plated, and the medium was changed 30 min after plating to remove any non-adherent cells. Astrocyte and microglial cultures were each >95% pure (1).

BMM isolation. Primary BMM were generated from the hind limbs of 6- to 8-week-old B6 or IFNAR^{-/-} mice as described previously (3). Macrophages were cultured in DMEM supplemented with 10% FBS, 20% L929 cell conditioned medium, 1 mM sodium pyruvate, 25 mM HEPES, and 1% penicillin-streptomycin and were harvested on day 6 after plating.

qRT-PCR. RNA was isolated with an RNeasy minikit (Qiagen, Valencia, CA). Real-time quantitative reverse transcriptase PCR (qRT-PCR) was performed as described previously (29, 32). Briefly, 350 ng of total RNA (either from cells or from tissue) was transcribed into cDNA by use of a Superscript III reverse transcription kit (Invitrogen), using a total reaction mix volume of 20 μl. Two microliters of cDNA was combined with 12.5 μl of iQ5 SYBR green mix (Bio-Rad, Hercules, CA), 6.5 μl diethyl pyrocarbonate (DEPC)-treated water, and 4 μl primer mix (5 μM concentration of each primer). DNA was amplified using an iQ5 iCycler (Bio-Rad), and cycle threshold (C_T) values were recorded. There is an inverse relationship between the C_T value and mRNA concentration, and each C_T unit represents a 2-fold difference in mRNA concentration. Basal ISG mRNA levels were expressed as ΔC_T values relative to actin mRNA [$\Delta C_T = C_{T(\text{ISG})} - C_{T(\beta\text{-actin})}$]. Induced ISG mRNA expression levels were expressed as fold changes relative to mock infection levels, using the variable $2^{-\Delta\Delta C_T}$. All primer sequences and thermocycle protocols for different primers are available upon request.

Western immunoblots. Cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Roche). Protein concentrations were measured using a DC protein assay kit (Bio-Rad). Equal quantities of protein were analyzed by 10% SDS-polyacrylamide gel electrophoresis. Following electrophoretic transfer, polyvinylidene difluoride (PVDF) membranes were blocked with 5% nonfat milk and then probed with polyclonal antibodies directed against MDA5 (Alexis), IFIT1, IFIT2 (Affinity Bioreagents), and tubulin (Abcam). The blots were visualized using ECL+ reagent (GE) and detected under an Intelligent dark box II (Fujifilm). ISG protein levels were normalized to tubulin levels by use of ImageJ software (NIH).

IFN- α/β ELISA. The amounts of IFN- α/β protein in supernatants of MHV-infected BMM or liver homogenates were quantified by use of a commercial capture enzyme-linked immunosorbent assay (ELISA) kit (PBL Laboratories, Piscataway, NJ) according to the manufacturer's instructions.

Interferon sensitivity assay. L2 cells, astrocytes, and BMM were pretreated with 0, 1, 10, 100, or 1,000 IU/ml universal human IFN- α (PBL). After treatment for 24 h for L2 cells and astrocytes and for 6 h for BMM, cells were washed once with cold PBS and then infected with MHV at a multiplicity of infection (MOI) of 1. At 18 h postinfection (p.i.) for L2 cells, 48 h for astrocytes, and 14 h p.i. for BMM, cells and supernatants were combined and stored at -80°C . Cells were lysed by two freeze-thaw cycles, and combined cellular and released virus titers were determined by plaque assays on L2 cells.

Statistical analysis. An unpaired two-tailed t test was used to determine statistical significance. All data were analyzed with GraphPad Prism software (GraphPad Software, Inc., CA).

RESULTS

Disruption of the JHM.WU ns2 gene attenuates viral replication in the liver but not in the brain. We have previously shown that amino acid substitutions of the predicted catalytic residues (His46A and His126R mutations) of the A59 ns2 gene confer attenuation of viral replication in the liver but not in the brain in C57BL/6 (B6) mice (33). We subsequently obtained similar results in a comparison of a naturally occurring deletion mutant of ns2 (JHM.WU-Δns2) with its wt counterpart (JHM.WU) (obtained from Stuart

FIG. 1. Replication of JHM.WU and JHM.WU-Δns2 in the brain and liver after i.c. inoculation. (A) Four- to 5-week-old B6 mice were infected i.c. with 10 PFU of JHM.WU or JHM.WU-Δns2 ($n = 5$). At day 5 postinfection, mice were sacrificed and the viral loads in the brain and liver were determined by plaque assay on L2 cells. The error bars represent the standard errors of the means (SEM). (B) MHV antigen was detected by staining with anti-MHV N monoclonal antibody ($n = 3$). (C) B6 mice were infected i.h. with 500 PFU of JHM.WU, JHM.WU-Δns2, A59, or ns2-H126R ($n = 5$). At day 5 postinfection, mice were sacrificed and viral loads in the liver were determined by plaque assay on L2 cells. The dashed line represents the limit of detection, and the error bars represent the SEM. (D) Livers and brains were harvested from uninfected B6 mice ($n = 8$), and total RNA was isolated. Basal mRNA levels of ISGs were quantified by qRT-PCR and normalized to the actin mRNA level [$\Delta C_T = C_{T(\text{ISG})} - C_{T(\beta\text{-actin})}$]. The C_T values for β -actin were approximately the same in the brain and the liver. The line represents the limit of detection, and the error bars represent the SEM. Asterisks indicate that differences are statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Data shown were derived from one representative experiment of two.

Siddell; originally named MHV Wb1 and MHV Wb3, respectively [36]). The Δns2 mutant has a 318-base deletion of the 5' end of the ns2 open reading frame (ORF 2a), including the transcription regulatory sequence (TRS) required for transcription. The 50% lethal dose (LD_{50}) of JHM.WU-Δns2 after intranasal inoculation is approximately 60 to 80 PFU, with no significant difference in lethality between wild-type and Δns2 JHM.WU strains (S. Perlman and J. Leibowitz, personal communication; L. Zhao, unpublished data). As expected, the two isolates replicated similarly in the brain following i.c. infection (Fig. 1A). We quantified virus titer and antigen spread at day 5 postinfection, the peak of virus replication in both organs (21, 22). While JHM isolates, including the extremely neurovirulent JHM.SD strain used most often in our lab (referred to as rJHM), generally do not infect the liver even at very high doses (2, 7, 22), we were surprised to find that JHM.WU replicated efficiently in the liver following i.c. inoculation; as with the previously published A59 ns2 mutants, the viral titers were lower in the livers of mice infected with JHM.WU-Δns2 (approximately 32-fold; $P = 0.005$) than in those of mice infected with

wild-type JHM.WU (Fig. 1A). Consistent with these findings, there was no obvious difference in the amount of viral antigen staining, as assessed by staining with anti-MHV N monoclonal antibody, in brain sections from JHM.WU- and JHM.WU-Δns2-infected mice; however, infection with JHM.WU caused hepatocellular necrosis that colocalized with MHV antigen staining, while infection of JHM.WU-Δns2 induced no necrosis and only small, scattered foci of antigen staining (Fig. 1B).

We next compared the replication of each ns2 mutant with that of its respective wt virus following infection by the i.h. route, which typically produces very high levels of MHV A59 replication in the liver, peaking at day 5 postinfection (22). As with i.c. infection, JHM.WU-Δns2 titers in the liver were significantly lower (55-fold lower; $P = 0.001$) than those of wt JHM.WU, and ns2-H126R titers were much lower (1,520-fold lower; $P < 0.001$) than those of wt A59 (Fig. 1C). The viral load of JHM.WU was actually higher (about 14-fold; $P = 0.0038$) than that of MHV A59, demonstrating that in contrast to all other known JHM isolates, JHM.WU is highly hepatotropic as well as highly neurovirulent.

FIG. 2. Mutation of the ns2 gene in the A59 and JHM.WU backgrounds attenuates viral replication in BMM and microglia. L2 cells, primary astrocytes, microglia, or BMM (as indicated) were infected with A59, ns2-H126R, JHM.WU, or JHM.WU-Δns2 at an MOI of 1 PFU/cell. At the indicated time points postinfection, cells were lysed in their medium by freeze-thawing, and titers were determined by plaque assay on L2 cells. The data are from one representative experiment of at least two, each performed in triplicate.

Based on previous reports that basal expression levels of ISGs play an important role in host defense against viral invasion and may thus be partially responsible for determining susceptibility to viral infection (14, 48), we compared the basal mRNA expression levels in the brain and liver of a group of ISGs encoding both IFN signaling and antiviral molecules. Using qRT-PCR, we found that the basal mRNA expression levels of this group of ISGs were significantly higher (4- to 16-fold) in the liver than in the brain ($P < 0.05$), suggesting that the liver may be better prepared than the brain to mount an early innate antiviral response (Fig. 1D). This difference may contribute to the liver-specific attenuation of ns2 mutants.

ns2 mutations attenuate replication in wt but not IFNAR^{-/-} macrophages or microglia. The *in vitro* replication kinetics of each ns2 mutant (ns2-H126R and JHM.WU-Δns2) were similar to those of the respective wt viruses in murine 17Cl-1 and DBT fibroblast cell lines (33, 36). However, these cell types neither produce any detectable type I IFN in response to MHV infection nor restrict MHV infection when treated with exogenous IFN-β (32, 43, 47). In order to compare the abilities of ns2 mutants and wt viruses to replicate in cell types in which MHV does induce a type I IFN response, we assessed the replication kinetics of both sets of wt and ns2 mutant viruses in BMM and microglia and compared them to the respective replication patterns in a mouse fibroblast cell line (L2) and in primary mouse astrocytes, a cell type that produces minimal levels of IFN-β mRNA only late during MHV infection. In L2 fibroblasts and astrocytes, the replication kinetics of the ns2 mutant viruses were comparable to those of their respective wt counterparts. However, in BMM, the peak titers (at 12 h p.i.) of the ns2-H126R and JHM.WU-Δns2 viruses were approximately 115-fold and 20-fold lower, respectively, than those of the wt viruses (Fig. 2). Strikingly, the replication kinetics

of both ns2 mutant viruses were restored to wt levels in IFNAR^{-/-} BMM and microglia (Fig. 3) after infection at an MOI of either 1 PFU/cell (Fig. 3A, B, E, and F) or 0.01 PFU/cell (Fig. 3C and D); the latter condition would be expected to emphasize any replication defect in the mutants. In microglia, the resident macrophages of the CNS, ns2-H126R and JHM.WU-Δns2 had similar phenotypes to those observed in BMM, although the attenuation of replication in wt cells was not as severe (Fig. 2). In addition, replication of the ns2 mutants relative to their wt counterparts was decreased only slightly in BMM isolated from mice in which expression of MDA5, the major sensor for detection of MHV RNA in this cell type (31), had been ablated (MDA5^{-/-} mice) (data not shown). Taken together, these results suggest that the attenuation of ns2 mutants is specific to macrophages/microglia and is related to an increased sensitivity to IFN induction or signaling.

ns2 mutants induce type I IFN production to the same extent as wt virus. To investigate the effect of ns2 on IFN production, the levels of IFN-β mRNA and protein induced by MHV infection of several cell types were analyzed by qRT-PCR and ELISA, respectively. In L2 cells (Fig. 4A) and primary astrocytes (Fig. 4B), IFN-β mRNA was detected only at very low levels and late in infection, and IFN-β protein could not be detected after MHV infection (data not shown), consistent with previous studies with cell lines. In contrast, Sendai virus (SeV) induced much higher levels of IFN-β mRNA than those induced by MHV, demonstrating that this cell type is capable of expressing type I IFN (32, 47). Importantly, there was no significant difference ($P > 0.05$) between IFN-β mRNA levels induced by ns2 mutant viruses and their corresponding wt viruses. There were also no significant differences in induction of IFN-β or IFN-α4 mRNA between BMM infected with

FIG. 3. ns2 mutants replicate to the same extent as wt viruses in BMM and microglia from IFNAR^{-/-} mice. IFNAR^{-/-} BMM were infected with the indicated viruses at an MOI of 1 PFU/cell (A and B) or 0.01 PFU/cell (C and D), and IFNAR^{-/-} microglia were infected at an MOI of 1 PFU/cell (E and F). At the indicated times postinfection, titers of viruses in the cell lysates and supernatants were determined by plaque assay on L2 cells. The data are from one representative experiment of two, each performed in triplicate.

ns2 mutant viruses and those infected with their wt counterparts at 6 h p.i. (A59) (Fig. 4C) or 9 h p.i. (JHM.WU) (Fig. 4D), the time points after which wt and mutant growth curves diverged (Fig. 2). To compare type I IFN induction at later time points under conditions in which IFN expression was due only to the initial induction by virus infection rather than to type I interferon receptor (IFNAR)-dependent amplification of IFN- β expression, we repeated the experiment with IFNAR^{-/-} BMM. Again, there were no significant differences ($P > 0.05$) in IFN- β mRNA or protein induction between BMM infected with ns2 mutant and wt viruses at time points up to 12 h p.i. (Fig. 4F). Unexpectedly, both JHM.WU- $\Delta ns2$ and JHM.WU induced much lower levels of IFN- β mRNA, with no detectable IFN- β protein, than those induced by wt or ns2 mutant A59 (Fig. 4E). These data show that ns2 does not compromise the IFNAR-independent in-

FIG. 4. ns2 does not antagonize IFN production. (A and B) L2 cells and primary astrocytes were infected with A59, ns2-H126R, JHM.WU, or JHM.WU- $\Delta ns2$ at an MOI of 1 PFU/cell. At 24 h p.i., IFN- β mRNA was quantified by qRT-PCR. (C and D) BMM were infected with A59, ns2-H126R, JHM.WU, or JHM.WU- $\Delta ns2$ at an MOI of 1. At 6 h p.i. for A59 and ns2-H126R and 9 h p.i. for JHM.WU and JHM.WU- $\Delta ns2$, IFN- β and IFN- $\alpha 4$ mRNAs were quantified by qRT-PCR. (E) At 12 h p.i., IFN- β protein in the medium above infected BMM was quantified by ELISA. (F) IFNAR^{-/-} BMM were infected with A59, ns2-H126R, JHM.WU, or JHM.WU- $\Delta ns2$ at an MOI of 1 PFU/cell. The kinetics of IFN- β mRNA expression were quantified by qRT-PCR. The error bars represent the SEM ($n = 4$). ND, not detectable.

duction of IFN- β and, in addition, that the highly virulent JHM.WU isolate is a very weak inducer of type I IFN.

ns2 mutants exhibit increased sensitivity to IFN signaling in microphages/microglia but not in L2 cells or astrocytes. To investigate whether ns2 is able to antagonize the antiviral effects of IFN signaling, L2 cells, primary astrocytes, and BMM were treated with increasing concentrations of IFN- α to induce ISGs and an antiviral state and were then infected with wt or ns2 mutant MHV. As shown in Fig. 5A and B, ns2 mutants (on both A59 and JHM.WU backgrounds) displayed similar sensitivities to IFN- α to those of their wt counterparts in both L2 cells and primary astrocytes. However, ns2-H126R showed en-

FIG. 5. Mutation of ns2 confers increased sensitivity to IFN in BMM. Murine L2 fibroblasts (A), astrocytes (B), or BMM (C) were pretreated with increasing doses of IFN- α . Twenty-four hours after IFN- α treatment for L2 and astrocytes or 6 h after treatment for BMM, cells were infected with the indicated viruses at an MOI of 1 PFU/cell. At 18 h p.i. for L2, 48 h for astrocytes, and 14 h p.i. for BMM, virus titers (cell-associated and released titers combined) were determined by plaque assay on L2 cells. (D) Relative changes in virus titers in BMM. (E) Microglia were pretreated with 10 IU/ml of IFN- α or mock treated for 6 h and then infected with A59 or ns2-H126R at an MOI of 1 PFU/cell. At the indicated time points postinfection, virus titers (cell-associated and released titers combined) were determined by plaque assay on L2 cells. The data are from one representative experiment of two, performed in triplicate.

hanced sensitivity to IFN in BMM, especially after low-dose IFN- α pretreatment (Fig. 5C and D). Pretreatment of BMM with 1 IU/ml and 10 IU/ml IFN- α produced 20- and 120-fold inhibition, respectively, of ns2-H126R, compared with 2-fold and 4-fold inhibition of wt virus. Similarly, pretreatment of microglia with 10 IU/ml IFN- α produced a 3,980-fold inhibition of ns2-H126R, compared with a 161-fold inhibition of wt virus at 24 h p.i. (Fig. 5E). These differences suggest that A59 ns2 confers the ability to antagonize IFN-induced antiviral responses in macrophages and microglia. In contrast, JHM.WU- Δ ns2 displayed similar or only slightly decreased sensitivity to IFN pretreatment in BMM compared with wt JHM.WU (Fig. 5C and D).

Interestingly, JHM.WU was less sensitive than A59 to the effects of IFN- α pretreatment of both BMM and primary astrocytes (Fig. 5B and C). In primary astrocytes, pretreatment with 1,000 IU/ml IFN- α produced a 1,210-fold inhibition for A59, compared with a 70-fold inhibition for JHM.WU. In BMM, the same treatment produced an 80-fold inhibition for A59, compared with a 20-fold inhibition for JHM.WU. The relative resistance of the JHM.WU isolate to IFN signaling in primary cells (astrocytes and BMM) may contribute to its very high virulence. The only minor sensitivity of JHM.WU to IFN pretreatment of BMM suggests that genes other than the ns2 gene confer type I IFN resistance to both JHM.WU and JHM.WU- Δ ns2, which could also explain the relatively small difference in resistance to IFN signaling between wt and ns2 mutant JHM.WU- Δ ns2 in BMM, as well as the relatively efficient replication of JHM.WU and JHM.WU- Δ ns2 in the liver compared to that of A59 and A59-ns2H126R (Fig. 1).

Macrophages and microglia express high basal levels of ISGs relative to L2 fibroblasts and astrocytes. To further elucidate the mechanisms responsible for differential restriction of MHV replication by IFN- α treatment in different cell types (Fig. 5), we compared the basal mRNA expression levels of a group of ISGs in macrophages and microglia with those in L2 fibroblasts and primary astrocytes. Not unexpectedly, the basal mRNA expression levels for several ISG proteins involved in viral detection and IFN signaling (MDA5, STAT1, IRF7, and IFNAR1), as well as those for several genes encoding antiviral activities (IFIT1, IFIT2, and ISG15), were higher in macrophages and microglia than in L2 cells and astrocytes, explaining at least in part the differences in response to IFN pretreatment (Fig. 6A to G). The expression level of the mRNA encoding suppressor of cytokine signaling protein 3 (SOCS3) showed the opposite pattern, with lower expression in BMM than in other cell types (Fig. 6H). The low-level expression of the SOCS3 mRNA may also contribute to enhancing the response of BMM to IFN treatment (34). Consistent with the mRNA levels, the protein levels of MDA5, IFIT1, and IFIT2 were highest in macrophages and lowest, even undetectable, in L2 cells (Fig. 6I). The basal level of MDA5 expression is probably an important determinant of restriction of MHV infection, as it is the only known pattern recognition receptor identified in the cell types being considered here.

ns2 suppresses expression of multiple mRNAs encoding ISG proteins with antiviral activities in BMM. To investigate whether ns2 expression inhibits ISG mRNA induction by IFN, BMM were infected with wt A59 or ns2-H126R and treated with IFN- α to enhance the induction of ISGs at 6 h p.i. (at

FIG. 6. Basal ISG mRNA and protein expression in different cell types. RNAs were purified from uninfected BMM, microglia, and astrocytes derived from B6 mice. The basal levels of ISG mRNA expression, normalized to β -actin mRNA levels, were quantified by qRT-PCR. (A) MDA5; (B) IRF-7; (C) STAT1; (D) IFNAR1; (E) IFIT1; (F) IFIT2; (G) ISG15; (H) SOCS3. Data were derived from one representative experiment of two; the error bars represent the SEM ($n = 4$). (I) Basal protein expression levels of MDA5, IFIT1, IFIT2, and tubulin were assessed by Western immunoblotting. ISG protein levels were normalized to the tubulin level in each cell type; the ratio of ISG to tubulin is indicated below each band.

which time the titers of both viruses were approximately the same) (18, 27). At several times post-IFN treatment, expression of mRNAs for a group of ISGs was quantified. As shown in Fig. 7, mRNAs encoding antiviral activities, i.e., IFIT1, IFIT2, and ISG15, were slightly enhanced at 0.5 h (Fig. 7A) and induced at significantly higher ($P < 0.05$) levels at 1 h (Fig. 7B) post-IFN treatment by ns2-H126R than by wt A59, while there were no significant differences in induction of mRNAs encoding MDA5, STAT1, or IRF7 between cells infected by these two viruses. By 1.5 h posttreatment, the replication of ns2 was significantly inhibited compared to that for wt rA59 (Fig. 2), and there were no differences observed in ISG expression (data not shown). This indicates that ns2 inhibits neither in-

duction of the canonical JAK-STAT pathway nor expression of the several signaling ISGs assessed but rather suppresses or delays the expression of specific antiviral ISGs, consistent with the finding that even wt A59 replication is restricted to a significant extent in BMM (Fig. 6C).

Replication of the ns2-H126R mutant virus in the liver is enhanced in macrophage-depleted mice. Based on the phenotype of ns2 mutant viruses relative to wt viruses in BMM *in vitro* and the greatly reduced ability of these mutants to replicate in the liver *in vivo*, we hypothesized that IFN signaling in macrophages presents a barrier to replication in the liver and therefore predicted that the ns2 mutants would regain the ability to replicate in the liver after *in vivo* depletion of mac-

FIG. 7. ns2 suppresses specific ISG mRNA expression in BMM. BMM were infected with A59 or ns2-H126R at an MOI of 1. At 6 h p.i., cells were treated with 1,000 IU/ml IFN- α . The cells were harvested at 0.5 (A) and 1 (B) h post-IFN- α treatment, and RNA was extracted. ISG mRNA expression was quantified by qRT-PCR ($n = 4$) and expressed as the fold change compared to untreated cells. The data were derived from one representative experiment of two. The error bars represent the SEM.

FIG. 8. Macrophage depletion restores the virulence of ns2-H126R. Four-week-old B6 mice were injected intravenously in the tail vein with clodronate-liposomes. Control mice were similarly treated with PBS or empty liposomes. The next day, mice were injected intrahepatically with 500 PFU A59 or ns2-H126R or with PBS. (A) At 5 days postinfection, livers from mock-infected mice were harvested and stained for F4/80 ($n = 3$). (B) Liver sections were stained with hematoxylin and eosin to detect pathological changes and with anti-MHV N monoclonal antibody to detect viral antigen expression ($n = 3$). (C) Virus titers in A59- or ns2-H126R-infected livers were determined by plaque assay. The dashed line represents the limit of detection, and the error bars represent SEM ($n = 5$). (D) Fold change of virus titer of A59 or ns2-H126R in livers from mice treated with clodronate-liposomes versus livers from those treated with empty liposomes or PBS. IFN-β (E) and IFN-α (F) protein levels in A59- or ns2-H126R-infected livers were quantified by ELISA. The error bars represent SEM ($n = 5$).

rophages. To test this hypothesis, we used the A59 background pair of viruses because the difference in wt A59 and ns2-H126R replication was quite dramatic and the ns2 mutant caused only minimal hepatitis, while JHM.WU-Δns2, although attenuated compared to JHM.WU, still replicated to significant titers in the liver. Thus, clodronate-filled liposomes were injected intravenously into mice to deplete all macrophages (37, 42), including the Kupffer cells, resident macrophages found in the liver sinusoids that comprise ~8% of liver cells (28, 38). PBS and liposomes lacking clodronate were injected as negative controls. Twenty-four hours after liposome transfer, mice were infected i.h. with 500 PFU of A59 or ns2-H126R and were sacrificed at 5 days postinfection (dpi), at which time the livers were harvested. Efficient depletion of macrophages in mice receiving clodronate-filled liposomes was demonstrated by staining liver sections from uninfected mice with a monoclonal antibody against F4/80, a mouse macrophage-specific marker (Fig. 8A). Hepatitis was evaluated by staining with hematoxylin and eosin. Following ns2-H126R infection, there were necrotic foci and parenchymal inflammation in liver sec-

tions from clodronate-liposome-treated mice; this was in contrast to the sections from PBS-treated mice, which exhibited no obvious pathological changes. Staining of sections from infected mice with anti-MHV N monoclonal antibody demonstrated a dramatic increase in the amount of ns2-H126R antigen localized to necrotic foci in liver sections from clodronate-liposome-treated mice compared with sections from PBS-treated mice (Fig. 8B), while more modest increases in antigen were observed in sections from A59-infected mice. These observations were confirmed by quantifying viral titers in the liver. The viral titers of A59 and ns2-H126R in livers from mice treated with control liposomes were comparable with those from mice treated with PBS, indicating that the liposomes had no effect on viral replication. After clodronate-liposome treatment, the viral loads in the liver were increased 553-fold ($P < 0.0001$) for the ns2-H126R mutant-infected mice compared with those from ns2-H126R-infected mice treated with control liposomes. In contrast, there was only a 10-fold change ($P = 0.0012$) for clodronate-liposome-treated A59-infected mice compared with control liposome-treated A59-infected mice (Fig. 8C and D).

The IFN- α/β protein levels induced in mouse livers by the ns2-H126A mutant and wt A59 were quantified by ELISA. After clodronate-liposome treatment, the levels of IFN- β in wt and ns2 mutant virus-infected mouse livers were significantly lower than those in control liposome-treated infected mice ($P = 0.0158$ for A59 and $P = 0.0032$ for ns2-H126R) (Fig. 8E). This indicated that macrophages were the main source of IFN- β in the mouse liver after MHV infection. Interestingly, after clodronate-liposome treatment, the levels of IFN- α in A59- and ns2-H126R-infected mouse livers were higher than those in infected mice treated with control liposomes ($P = 0.0049$ for A59 and $P = 0.005$ for ns2-H126R) (Fig. 8F). This was most likely due to increased viral replication in plasmacytoid dendritic cells (pDCs), reported to be the main source of IFN- α expression after MHV infection (5). Nevertheless, the increased levels of IFN- α and the residual IFN- β in the liver were not sufficient to prevent efficient replication in the macrophage-depleted livers.

DISCUSSION

We show here, by comparison of wt and ns2 mutant MHV, that the abilities of MHV to replicate efficiently in BMM and to resist IFN treatment of BMM *in vitro* correlate with the ability to replicate efficiently in the liver and induce hepatitis. The observation that ns2 mutant viruses were able to replicate to the same extent as wt viruses in IFNAR^{-/-} BMM indicates that ns2 confers the ability to resist type I IFN signaling. These results, together with the finding that depletion of macrophages *in vivo* allows ns2-H126R to replicate in the liver and to induce hepatitis, led us to speculate that MHV must replicate successfully in Kupffer cells (liver macrophages) within the sinusoids of the liver in order to enter the liver parenchyma, infect hepatocytes, and induce hepatitis. Indeed, both hepatotropic and nonhepatotropic MHV strains can replicate in primary hepatocytes *in vitro* (31), supporting the notion that it is replication in a nonhepatocyte cell type that determines the ability to infect the liver and induce hepatitis (31). However, it is not possible to distinguish the effects of the loss of Kupffer cells from those of the loss of other macrophages, and it is possible that replication in macrophages other than Kupffer cells may also contribute to host defense from hepatitis. These data are consistent with a previous study utilizing mice with a cell-type-specific IFNAR ablation that concluded that macrophages and, to a lesser extent, DCs were the most important cell type for IFN signaling-mediated restriction of MHV-induced hepatitis (4). In addition, a study utilizing a lymphocytic choriomeningitis virus (LCMV) mouse model of hepatitis concluded that IFN signaling in tissue-resident macrophages provides a barrier to infection of the liver parenchyma (19).

Interestingly, in mice depleted of macrophages, the level of IFN- β in the liver was significantly lower than that in control mice, despite the fact that the viral loads in the livers of depleted mice were higher; this suggests that macrophages are a major source of IFN- β in the mouse liver during MHV infection. In contrast, the level of IFN- α was significantly increased in the livers of macrophage-depleted mice, most likely due to an increased viral load in the liver; this is consistent with a previous report that pDCs are a major source of IFN- α in MHV-infected mice (5). The residual level of IFN- β and in-

creased amount of IFN- α were unable to protect the host in the absence of macrophages, again supporting the hypothesis that IFN signaling in macrophages is an important component of host protection from MHV.

While wild-type MHV is susceptible to the antiviral effects of IFN- α/β , we have shown that MHV is able to partially resist IFN- α -induced signaling in 293T cells (29) and now in several other cell types, including BMM (Fig. 8). This suggests that MHV may be able to reduce the expression and/or resist the antiviral effects of one or more of the many ISG proteins that have direct antiviral activity (9). In BMM, MHV did partially inhibit the induction by IFN of the mRNAs encoding ISG15, IFIT2, and IFIT1, three ISG proteins with antiviral activity, but not the induction of the mRNAs encoding MDA5, STAT1, and IRF7, three molecules involved in the detection of viral RNA or in the canonical type I IFN signaling pathway. This indicates that ns2 is not able to inhibit the STAT-dependent pathway of ISG induction and suggests that there are differences in the pathways leading to induction of subgroups of ISGs (Fig. 7). In our earlier studies, we also observed that MHV infection differentially regulated the induction of different ISGs in 293T cells (29). There are several reports that type I IFN signaling may be activated by noncanonical signaling pathways in a cell-type-dependent manner (6, 35, 41). For example, comparison of ISG expression in wt and IKK ϵ -deficient cells demonstrated that transcription of some but not all ISGs required IKK ϵ expression; these IKK ϵ -dependent genes included the IRF-7 and STAT1 genes but not several other ISGs encoding proteins with direct antiviral activities (40). These groupings are consistent with our observation that MHV expressing wt ns2 inhibited the transcription of antiviral ISGs but not that of STAT1 and IRF7. Together, these results support the hypothesis that different activation pathways result in expression of different subgroups of ISGs.

Little is known about which type I IFN-induced antiviral effectors are most important for host restriction of MHV. We previously found that MHV is relatively resistant to IFN treatment of fibroblast cell lines (as in Fig. 5A), while similar IFN treatment completely restricts Newcastle disease virus (NDV) replication (32). Furthermore, during coinfections with MHV and either SeV or NDV, pretreatment of mouse fibroblasts with IFN- β is still unable to restrict MHV infection, while both of the other viruses fail to replicate even in the presence of MHV (29, 32). This suggests that either different antiviral activities are responsible for restricting each virus or MHV has a mechanism for avoiding antiviral activity that does not protect the coinfecting virus. Comparison of the data presented for ns2 mutant and wt viruses suggests that the expression or antiviral activities of ISGs may be cell type specific, as ns2 confers increased IFN resistance in BMM and microglia but not in astrocytes, in which wt and ns2 mutant viruses are equally resistant to IFN signaling (Fig. 5B). Recently, a methyltransferase mutant of MHV (nsp16-D130A) was shown to replicate less well than wt A59 in BMM from B6 mice but equally well in BMM derived from IFIT1^{-/-} mice (49), suggesting that RNA methylation may allow MHV to avoid the antiviral effect of IFIT1 in macrophages. However, IFIT1 did not preferentially inhibit nsp16-D130A replication when it was overexpressed in murine fibroblasts. This underscores the cell-

type-specific differences in virus interaction with the host type I IFN response.

Since most JHM isolates, including the JHM.SD strain used most often by our lab, are highly neurotropic but replicate minimally, if at all, in the liver (2), we were surprised to find that the JHM.WU strain used in these studies (originally called MHV Wb3 [36]), which was isolated after multiple passages in tissue culture, replicated to high titers in the liver, and induced severe hepatitis. Interestingly, JHM.WU exhibited less induction of IFN- β in BMM (Fig. 4) and much higher resistance to IFN pretreatment of primary cells (Fig. 5) than did A59. In contrast to the ns2 mutant and wt A59 viruses, JHM.WU- Δ ns2 and JHM.WU displayed similar resistances to IFN- α in BMM. Compared with wt JHM.WU, JHM.WU- Δ ns2 did exhibit decreased replication in the mouse liver, although this attenuation was also much less than that displayed by ns2-H126R compared with the wild-type parent A59. The data suggest that the ability of JHM.WU to inhibit IFN production and its high resistance to IFN treatment together contribute to its high hepatovirulence and that this evasion of the type I IFN response is much less dependent on ns2. We infer that one or more additional proteins encoded by the JHM.WU genome may strongly suppress type I IFN production and/or signaling.

Several MHV-encoded proteins have been reported to antagonize type I IFN signaling in various cell types. The nucleocapsid (N) protein of A59 expressed by a recombinant vaccinia virus was reported to inhibit RNase L activity in 17Cl-1 and HeLa cells, likely due to its RNA-binding properties (45). The papain-like protease (PLP), encoded within the coronavirus replicase locus and expressed as part of nonstructural protein 3 (nsp3), has deubiquitinating as well as protease activity. While SARS PLP interferes with the IRF3 and NF- κ B pathways (46), it not clear if the corresponding MHV protein has these two activities (11). Coronavirus nsp1 was reported to antagonize the effects of IFN- α in macrophages (50), and mutation of nsp1 conferred attenuation of replication in the liver and spleen; however, it is difficult to determine whether the effect on type I IFN signaling is a direct or indirect effect of the ability of nsp1 to degrade host cell mRNA (11, 15). The ns5a protein of A59 was recently reported to have type I IFN antagonist activity (17), but ns5a mutants, in contrast to ns2 mutants, were sensitive to IFN in astrocytes and murine fibroblasts as well as in BMM, and their replication was attenuated in the CNS as well as in the liver (data not shown). It is still not clear which of these proteins play major roles in MHV pathogenesis *in vivo*. Studies are under way to use reverse genetics to map the gene(s) of JHM.WU responsible for its ability to antagonize type I IFN and for its unusually strong virulence in the liver.

Our results suggest that the interaction of MHV with the early type I IFN response is an important determinant of cellular and organ tropism. More specifically, the basal level of ISG expression is an important factor in permissiveness to viral infection. Here we found that BMM have high basal expression levels of mRNAs encoding ISG proteins, including MDA5, STAT1, and other molecules crucial for recognizing viral invasion and producing an antiviral environment, and a lower basal expression level of SOCS3 mRNA. Indeed, while BMM expressed high basal levels of MDA5, IFIT1, and IFIT2 proteins, these ISG proteins were undetectable in uninfected L2

cells. BMM from IFNAR^{-/-} mice have lower basal and induced levels of ISGs (our unpublished data), consistent with the robust replication of ns2 mutants in such BMM. In addition, liver tissue has higher basal levels of these ISGs than brain tissue, consistent with the organ specificity of ns2 mutant-associated attenuation. Interestingly, CEACAM1a, the only known receptor for MHV, is expressed at a significantly higher level in the liver than in the brain, where it is barely detectable (1), while MHV A59 replicates robustly in both organs. Therefore, we suggest that the difference in ISG levels may compensate for the very different levels of receptor. We believe that this relationship between receptor level and the preparedness of a cell type for defense against viral invasion will be important in determining viral tropism and should be investigated further.

The mechanism by which ns2 antagonizes the type I IFN response is not known. As a member of the LigT-like family II of the superfamily of 2H-phosphoesterases, ns2 is predicted to have 2',3'- and/or 1'',2''-cyclophosphodiesterase or ligase activities and may be involved in modification of viral or host RNA (20). While no enzymatic activity has yet been reported for ns2, the abilities to act as a virulence factor in the liver and to antagonize IFN signaling both map to the predicted catalytic His residues (33). Among the members of the 2H-phosphoesterase superfamily, ns2 is most closely related to the human CGI18 and rat AKAP18 proteins, which, like ns2, have not yet been shown to have enzymatic activities but have important nonenzymatic activities as anchoring proteins that may potentially regulate multiple signaling pathways (10, 16, 20). Efforts are under way in our lab to determine if ns2 does indeed have a phosphodiesterase activity. Once this is determined, we can design experiments to begin to uncover the mechanisms underlying its ability to antagonize the IFN response as well as its role in pathogenesis in the liver.

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