

# Critical Role for Interferon Regulatory Factor 3 (IRF-3) and IRF-7 in Type I Interferon-Mediated Control of Murine Norovirus Replication

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**Human noroviruses (HuNoV) are the major cause of epidemic, nonbacterial gastroenteritis in the world. The short course of HuNoV-induced symptoms has implicated innate immunity in control of norovirus (NoV) infection. Studies using murine norovirus (MNV) confirm the importance of innate immune responses during NoV infection. Type I alpha and beta interferons (IFN- $\alpha/\beta$ ) limit HuNoV replicon function, restrict MNV replication in cultured cells, and control MNV replication *in vivo*. Therefore, the cell types and transcription factors involved in antiviral immune responses and IFN- $\alpha/\beta$ -mediated control of NoV infection are important to define. We used mice with floxed alleles of the IFNAR1 chain of the IFN- $\alpha/\beta$  receptor to identify cells expressing lysozyme M or CD11c as cells that respond to IFN- $\alpha/\beta$  to restrict MNV replication *in vivo*. Furthermore, we show that the transcription factors IRF-3 and IRF-7 work in concert to initiate unique and overlapping antiviral responses to restrict MNV replication *in vivo*. IRF-3 and IRF-7 restrict MNV replication in both cultured macrophages and dendritic cells, are required for induction of IFN- $\alpha/\beta$  in macrophages but not dendritic cells, and are dispensable for the antiviral effects of IFN- $\alpha/\beta$  that block MNV replication. These studies suggest that expression of the IFN- $\alpha/\beta$  receptor on macrophages/neutrophils and dendritic cells, as well as of IRF-3 and IRF-7, is critical for innate immune responses to NoV infection.**

Viruses within the genus *Norovirus* of the family *Caliciviridae* are the major etiologic agents of epidemic, nonbacterial gastroenteritis, causing ~90% of viral gastroenteritis and ~50% of all-cause outbreaks worldwide (22, 52). Human noroviruses (HuNoV) are also an important cause of sporadic gastroenteritis (53). HuNoV infection has been associated with necrotizing enterocolitis in infants and postinfectious irritable bowel syndrome in adults (44, 67, 71). Murine noroviruses (MNVs) are enteric viruses that share many molecular and biological properties with HuNoV (reviewed in references 25 and 77). MNV infection has been reported to trigger Crohn's disease-like intestinal pathology (7, 8) and atherosclerosis progression (51) and to exacerbate enteric bacterial infection (32, 37). The capacity of MNVs to grow in culture and infect mice permits the dissection of immune responses during NoV infection *in vitro* and *in vivo*.

The short course of HuNoV disease (often only 2 to 3 days) suggests that innate immune responses play a key role in control of HuNoV infection. Elevated interferon (IFN) levels are observed in *ex vivo*-stimulated peripheral blood mononuclear cells and in serum from human volunteers and gnotobiotic pigs inoculated with HuNoV (39, 63). Studies using MNV provide strong support for the role of innate immune responses, including both type I and type II IFN (IFN- $\alpha/\beta$  and IFN- $\gamma$ ), in controlling norovirus (NoV) replication both *in vivo* and *in vitro* (13, 28, 31, 32, 49, 76). The relevance of these observations is supported by studies showing a reduction of HuNoV RNA and protein expression in cells expressing replicons after IFN treatment (11, 12). IFN- $\alpha/\beta$  responses during NoV infection are likely initiated by the pattern recognition receptors melanoma differentiation-associated protein 5 (MDA-5) and Toll-like receptor 3 (TLR-3), since mice lacking MDA-5 or TLR-3 have elevated MNV replication compared to wild-type mice (46). However, the molecular pathways downstream of virus recognition which induce IFN- $\alpha/\beta$  responses during

NoV infection are unknown. In addition, the role of specific cells and tissues in mediating IFN- $\alpha/\beta$  responses during NoV infection has not been defined.

Despite the importance of noroviruses with regard to public health, no drug or vaccine is currently available to treat or prevent HuNoV infection. The potential for long-term homologous protection against NoV infection has been shown by previous studies using the murine and chimpanzee model systems (5, 9). Importantly, a recent study showed that it is possible to vaccinate against homologous challenge with a HuNoV strain, providing support for the idea that vaccination of the population may be possible (2, 21). Several recent studies show that IFN- $\alpha/\beta$  is also important for generating effective antiviral CD8 T-cell responses (41, 54, 70, 72–74). Therefore, it is likely that IFN- $\alpha/\beta$  responses participate in both innate and adaptive immune responses to NoV infection.

IFN regulatory factor 3 (IRF-3) and IRF-7 are key mediators of transcriptional responses upon virus infection and are therefore likely candidates for initiating IFN- $\alpha/\beta$  responses to NoV infection. IRF-3 is constitutively expressed in most cell types and activated by phosphorylation upon virus infection (60, 78). IRF-7 expression is induced by IFN in most cell types; IRF-7 is constitutively expressed in plasmacytoid dendritic cells and also activated by phosphorylation upon virus infection (4, 29, 56). Mice lacking IRF-3 (IRF-3<sup>-/-</sup>) are susceptible to lethal infection with encephalomyocarditis virus (EMCV) and West Nile virus (WNV), but their systemic IFN- $\alpha/\beta$  response upon virus infection remains relatively intact (17, 57). In

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contrast, the systemic IFN- $\alpha$  response upon virus infection is decreased in mice lacking IRF-7 (IRF-7<sup>-/-</sup>), and these mice are more susceptible than IRF-3<sup>-/-</sup> mice to infection with EMCV, WNV, and herpes simplex virus-1 (18, 26).

Here, we used mice with a targeted knockout (Ifnar1<sup>tm1Agt</sup>) or floxed allele (Ifnar1<sup>tm1Uka</sup>) of the IFNAR1 chain of the IFN- $\alpha/\beta$  receptor (IFN- $\alpha/\beta$ R) to show that signaling through the IFN- $\alpha/\beta$ R and cells expressing lysozyme M (macrophages/neutrophils) or CD11c (dendritic cells) restrict MNV replication *in vivo*. Further, we found that IRF-3 and IRF-7 are key initiators of antiviral immune responses that restrict MNV replication in multiple tissues and in primary macrophages and dendritic cells. Lastly, we found that IRF-3 and IRF-7 are essential for induction of IFN- $\alpha/\beta$  in primary macrophages, but not primary dendritic cells, and are not required for IFN- $\alpha/\beta$ -dependent generation of an antiviral state. These data suggest a key role for macrophages/neutrophils, dendritic cells, IRF-3, and IRF-7 in innate immunity to NoV infection.

## MATERIALS AND METHODS

**Mice and infections.** The original Ifnar1<sup>tm1Uka</sup> (herein called IFN- $\alpha/\beta$ R<sup>eff</sup>), Ifnar1<sup>tm1Agt</sup> (herein called IFN- $\alpha/\beta$ R<sup>-/-</sup>), and IRF3<sup>-/-</sup> and IRF7<sup>-/-</sup> mice were generously provided by Ulrich Kalinke (Paul-Ehrlich-Institut, Langen, Germany), Michel Aguet (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland), and Tadatsugu Taniguchi (University of Tokyo, Tokyo, Japan), respectively. Wild-type C57BL/6 (B6) mice (catalog no. 000664) were purchased from the Jackson Laboratory (Bar Harbor, ME). IFN- $\alpha/\beta$ R<sup>-/-</sup>, IRF-3<sup>-/-</sup>, IRF-7<sup>-/-</sup>, IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup>, IFN- $\alpha/\beta$ R<sup>eff</sup>, CD11c-Cre, and LysM-Cre mice (15, 19, 20, 26, 48, 57, 65), as well as IFN- $\alpha/\beta$ R<sup>eff</sup>  $\times$  LysM-Cre and IFN- $\alpha/\beta$ R<sup>eff</sup>  $\times$  CD11c-Cre mice, were bred and housed at Washington University, St. Louis, MO, under specific-pathogen-free conditions (8), in accordance with federal and university guidelines. All of these mice were fully backcrossed to the C57BL/6 background.

Seven- to eight-week-old, sex-matched mice were orally inoculated with 25  $\mu$ l of MNV diluted in phosphate-buffered saline (PBS) or mock inoculated with 25  $\mu$ l of PBS. Mice were sacrificed 3 days after inoculation, and tissue and serum were harvested (10).

**Virus stocks and plaque assays.** A concentrated stock of MNV-1.CW3 (69), herein referred to as MNV, was generated using RAW 264.7 cells (American Type Culture Collection [ATCC], Manassas, VA) as described previously (10) and used in all experiments. MNV titer was determined by plaque assay (10, 76) with the following modifications: suspension-adapted RAW 264.7 cells were plated in DMEM supplemented with 10% fetal bovine serum (FBS), 100  $\mu$ g/ml penicillin and streptomycin (P/S), 2 mM L-glutamine, and 10 mM N-2-hydroxyethylpiperazine-N<sub>2</sub>-2-ethanesulfonic acid (HEPES) (pH 7.3). Cells were inoculated with MNV and overlaid with 2 ml/well of overlay medium [MEM supplemented with 10% FBS, 100  $\mu$ g/ml P/S, 2 mM L-glutamine, and 10 mM HEPES and containing 1% methylcellulose (viscosity, 1,500 cP) (Sigma, St. Louis, MO)]. After 2 to 4 days of incubation at 37°C in 5% CO<sub>2</sub>, the overlay medium was removed and plaques were visualized with a 1% crystal violet–20% ethanol solution.

**Cell culture, interferon treatment, and infection.** RAW 264.7 cells were grown as described previously (76). Suspension-adapted RAW 264.7 cells were grown at 35°C and maintained in MEM supplemented with 10% bovine serum, a 1% final concentration of nonessential amino acids, 100  $\mu$ g/ml P/S, 2 mM L-glutamine, 10 mM HEPES, and 0.225% (wt/vol) sodium bicarbonate. Primary bone marrow-derived macrophages (herein referred to as macrophages) were generated as described previously (79) with the following modification: bone marrow cells were incubated in culture medium for 7 days, after which adherent cells were harvested and replated in fresh culture medium. Primary bone marrow-derived dendritic cells (herein referred to as dendritic cells) were generated as de-

scribed previously (36) with the following modification: bone marrow cells were incubated in culture medium for 7 days, after which nonadherent cells were harvested and replated in fresh culture medium. For *in vitro* infections, macrophages and dendritic cells were plated on day 7 at a density of  $2 \times 10^5$  cells/well in 24-well plates or  $1 \times 10^6$  cells/well in 6-well plates. Macrophages and dendritic cells were either untreated or treated with 100 U/ml of recombinant IFN- $\alpha$ 4 or recombinant IFN- $\beta$  12 to 16 h before inoculation (PBL InterferonSource, Piscataway, NJ). Macrophages were inoculated on day 10 of *in vitro* culture with MNV at a multiplicity of infection (MOI) of 0.05 (76) and either left untreated or treated with 100 U/ml of IFN- $\alpha/\beta$  after inoculation. Dendritic cells were inoculated on day 7 of *in vitro* culture with MNV at an MOI of 0.05 (76) and either left untreated or treated with 100 U/ml of IFN- $\alpha/\beta$  after inoculation. Cells and supernatant were harvested at 48 hours postinfection (hpi) and then stored frozen at  $-80^\circ\text{C}$ .

**Quantification of IFN- $\alpha/\beta$  activity.** Levels of biologically active IFN- $\alpha/\beta$  were determined using an EMCV cytopathic effect bioassay performed in L929 cells as described previously (55) with the following modification: prior to the assay, serum samples were treated with citrate buffer (40 mM citric acid, 10 ml KCl, 135 mM NaCl [pH 3.0]) for 5 min and neutralized with medium containing 45 mM HEPES. The amount of IFN- $\alpha/\beta$  per ml of serum was calculated from a standard curve using IFN- $\beta$  (PBL InterferonSource) and adjusted for the background inhibitory activity of naïve serum (approximately 0.1 IU/ml). The inhibitory activity of naïve serum was IFN- $\alpha/\beta$  independent because it was acid labile but was resistant to treatment with heat (56°C) and the IFN- $\alpha/\beta$ -blocking antibody MAR1-5A3 (61).

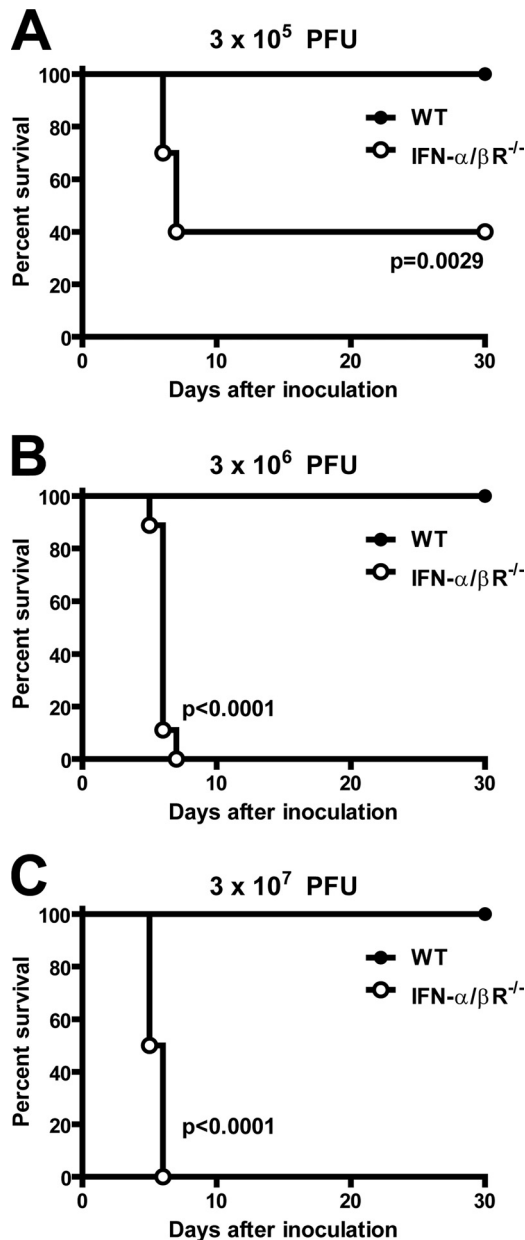
**Quantitative RT-PCR.** Macrophages and dendritic cells were either mock inoculated or inoculated with MNV at an MOI of 10 for 8 or 12 h as described above. Total RNA was isolated and cDNA was synthesized as described previously (23). Quantitative reverse transcription-PCR (qRT-PCR) was performed with SYBR green (Invitrogen, Carlsbad, CA) using primers for IFN- $\alpha$ 4 (5'-TGT GTG ATG CAG GAA CCT CCT-3' and 5'-GGT ACA CAG TGA TCC TGT GG-3'), IFN- $\beta$  (5'-ATA AGC AGC TCC AGC TCC AAG-3' and 5'-GTCTCATTCCACCCAGTGCTG-3') and 18S (5'-CGC CGC TAG AGG TGA AAT TCT-3' and 5'-CGA ACC TCC GAC TTT CGT TCT-3'). Viral RNA transcript levels were normalized to the level of transcription of 18S rRNA within each sample using the  $\Delta\Delta C_T$  method (where  $C_T$  is the threshold cycle) (40).

**Statistical analyses.** All data were analyzed using Prism 5 software (Graph-Pad Software, San Diego, CA). Mean values were used to calculate fold change.

## RESULTS

**IFN- $\alpha/\beta$  responses control MNV infection *in vivo*.** A combination of IFN- $\alpha/\beta$  and IFN- $\gamma$  responses mediate control of MNV infection *in vivo* (28, 31, 49). To further elucidate the role of IFN- $\alpha/\beta$  in control of MNV infection *in vivo*, we examined the specific contribution of IFN- $\alpha/\beta$  responses in preventing lethal MNV infection. Consistent with previous studies (28, 31), significant lethality was not observed in IFN- $\alpha/\beta$ R<sup>-/-</sup> mice inoculated with  $3 \times 10^4$  PFU of MNV (data not shown). However, when mice were inoculated with higher doses of MNV ( $3 \times 10^5$ ,  $3 \times 10^6$ , or  $3 \times 10^7$  PFU), we observed statistically significant lethality in IFN- $\alpha/\beta$ R<sup>-/-</sup> mice compared to C57BL/6 mice (wild-type control) (Fig. 1). No lethality was observed in infected wild-type control mice at any of the tested doses of MNV. These data demonstrate a key role for IFN- $\alpha/\beta$  responses in preventing lethal MNV infection *in vivo*.

Since we observed lethality in IFN- $\alpha/\beta$ R<sup>-/-</sup> mice inoculated with  $3 \times 10^5$  PFU of MNV (Fig. 1A), we assessed the contribution of IFN- $\alpha/\beta$  responses in restricting MNV replication in tissues after inoculation of mice with this dose of virus. Three days after inoculation, we observed a significant increase in MNV replica-



**FIG 1** IFN- $\alpha/\beta$  responses prevent lethal MNV infection. Wild-type (WT) and IFN- $\alpha/\beta$ R<sup>-/-</sup> mice were inoculated with the indicated doses of MNV, and survival was recorded for two independent experiments of five mice each. Statistical significance was determined using the log-rank test.

tion in the livers (300,000-fold), spleen (200,000-fold), mesenteric lymph nodes (70,000-fold), ilea (2,000-fold), and lungs (70,000-fold) of IFN- $\alpha/\beta$ R<sup>-/-</sup> mice compared to wild-type controls (Fig. 2). These data demonstrate that IFN- $\alpha/\beta$  responses are critical for host restriction of MNV replication in multiple tissues.

**IFN- $\alpha/\beta$  responses in lysozyme M- and CD11c-expressing cells restrict MNV replication *in vivo*.** MNV replicates in macrophages and dendritic cells *in vivo* (75, 76), and IFN- $\alpha/\beta$  and IFN- $\gamma$  responses restrict MNV replication in these cells *in vitro* (13, 28, 76). To assess the role of IFN- $\alpha/\beta$  responses in macrophages and dendritic cells in restricting MNV replication *in vivo*, we examined acute MNV replication in mice with floxed alleles of the IFNAR1

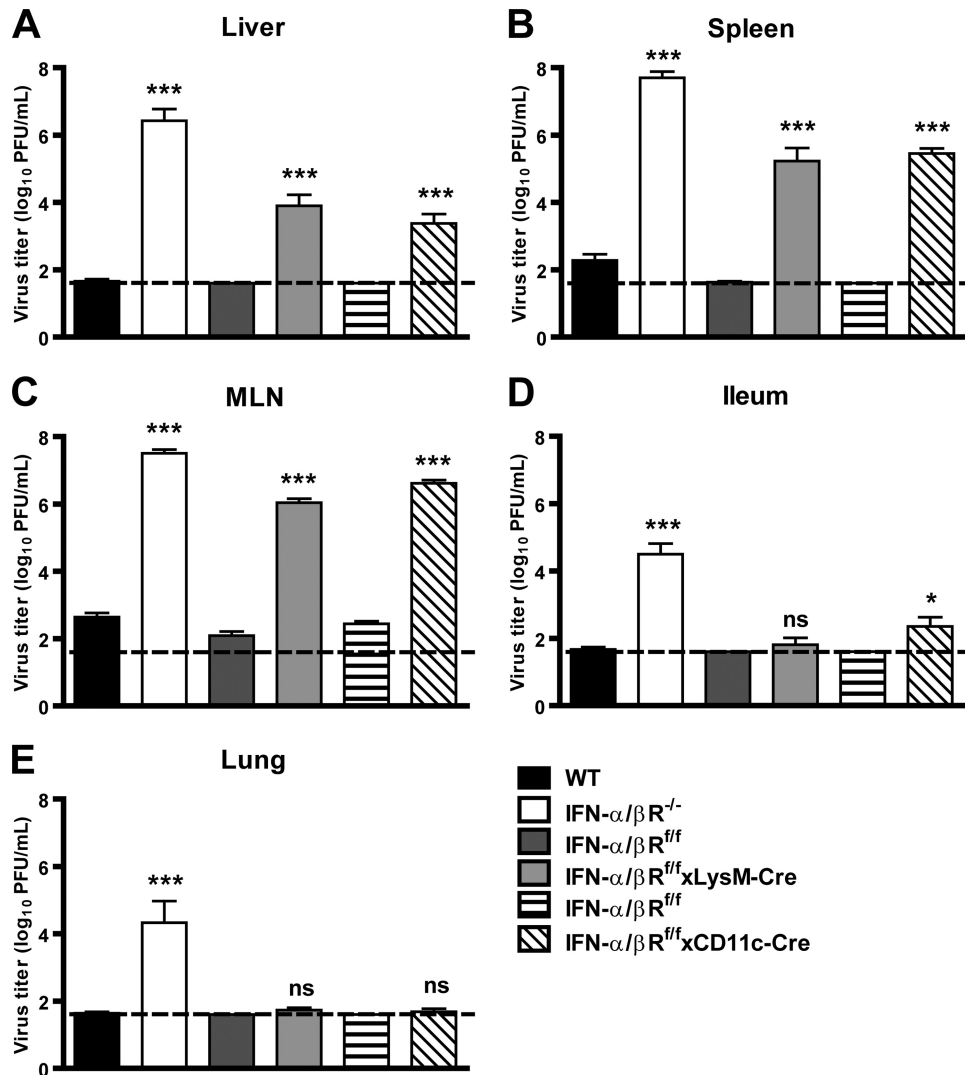
chain of the IFN- $\alpha/\beta$ R in cells expressing either lysozyme M (IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  LysM-Cre cells; macrophages/neutrophils) or CD11c (IFN- $\alpha/\beta$ R<sup>fl/fl</sup>/fxcCD11c-Cre cells; dendritic cells) (20, 30).

Three days after inoculation, we observed a significant increase in MNV replication in the livers (900-fold), spleens (20,000-fold), and mesenteric lymph nodes (8,000-fold) of IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  LysM-Cre mice compared to control IFN- $\alpha/\beta$ R<sup>fl/fl</sup> mice, which carry floxed alleles of the IFNAR1 chain of the IFN- $\alpha/\beta$ R (Fig. 2A to C). We also observed a significant increase in MNV replication in the livers (223-fold), spleens (10,000-fold), and mesenteric lymph nodes (15,000-fold) of IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  CD11c-Cre mice compared to levels in IFN- $\alpha/\beta$ R<sup>fl/fl</sup> controls. In contrast to the elevated titers observed in IFN- $\alpha/\beta$ R<sup>-/-</sup> mice, a significant increase in MNV replication was not observed in the lungs of IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  LysM-Cre or IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  CD11c-Cre mice compared to levels in IFN- $\alpha/\beta$ R<sup>fl/fl</sup> controls (Fig. 2E). These data demonstrate that IFN- $\alpha/\beta$  responses in lysozyme M- and CD11c-expressing cells restrict MNV replication *in vivo* and suggest that further analysis of the mechanisms of IFN- $\alpha/\beta$ -dependent resistance to NoV should include examination of the role of macrophages and dendritic cells, for which MNV has a tropism (75, 76).

Since we observed elevated titers in several tissues of IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  LysM-Cre and IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  CD11c-Cre mice, we assessed the contribution of IFN- $\alpha/\beta$  responses in lysozyme M- and CD11c-expressing cells in preventing lethal MNV infection. In contrast to the lethality observed in IFN- $\alpha/\beta$ R<sup>-/-</sup> mice, no lethality was observed in IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  LysM-Cre ( $n = 10$ ), IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  CD11c-Cre ( $n = 6$ ), or IFN- $\alpha/\beta$ R<sup>fl/fl</sup> ( $n = 16$ ) mice inoculated with  $3 \times 10^7$  PFU (data not shown). Thus, the extent of IFN- $\alpha/\beta$ R deletion in IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  LysM-Cre and IFN- $\alpha/\beta$ R<sup>fl/fl</sup>  $\times$  CD11c-Cre mice was not sufficient to result in lethality, potentially because of residual IFN- $\alpha/\beta$  signaling due to incomplete deletion of the IFN- $\alpha/\beta$ R in specific lysozyme M or CD11c cells or subsets (20, 30) or to a role for IFN- $\alpha/\beta$  signaling in other cell types.

**IRF-3 and IRF-7 responses restrict MNV replication *in vivo* but are dispensable for preventing lethal MNV infection.** IRF-3 and IRF-7 are key transcription factors that regulate IFN- $\alpha/\beta$  responses following virus infection (26, 57). To assess their role in control of MNV infection *in vivo*, we examined acute MNV replication in mice lacking both IRF-3 and IRF-7 (IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup>) compared to wild-type and IFN- $\alpha/\beta$ R<sup>-/-</sup> mice. Three days after inoculation, we observed a significant increase in MNV replication in the livers (370-fold), spleens (14,000-fold), mesenteric lymph nodes (3,400-fold), ilea (56-fold), and lungs (200-fold) of IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup> mice compared to wild-type controls (Fig. 3A to E). Of note, we also observed a further increase in MNV replication in the livers (487-fold), spleen (14-fold), mesenteric lymph node (13-fold), ilea (20-fold), and lungs (14-fold) of IFN- $\alpha/\beta$ R<sup>-/-</sup> mice compared to levels in IRF-3<sup>-/-</sup> IRF-7<sup>-/-</sup> mice. These data demonstrate that IRF-3 and IRF-7 function to restrict MNV replication *in vivo* but also suggest that additional factors are involved in IFN- $\alpha/\beta$  responses during MNV infection *in vivo*.

IRF-3 is constitutively expressed in most cell types, whereas IRF-7 expression is induced by IFN- $\alpha/\beta$  in most cell types (56, 60). Since we observed elevated MNV titers in IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup> mice, we examined acute MNV replication in IRF-3<sup>-/-</sup> mice and in IRF-7<sup>-/-</sup> mice to establish the relative hierarchy of these transcription factors in the innate immune responses to



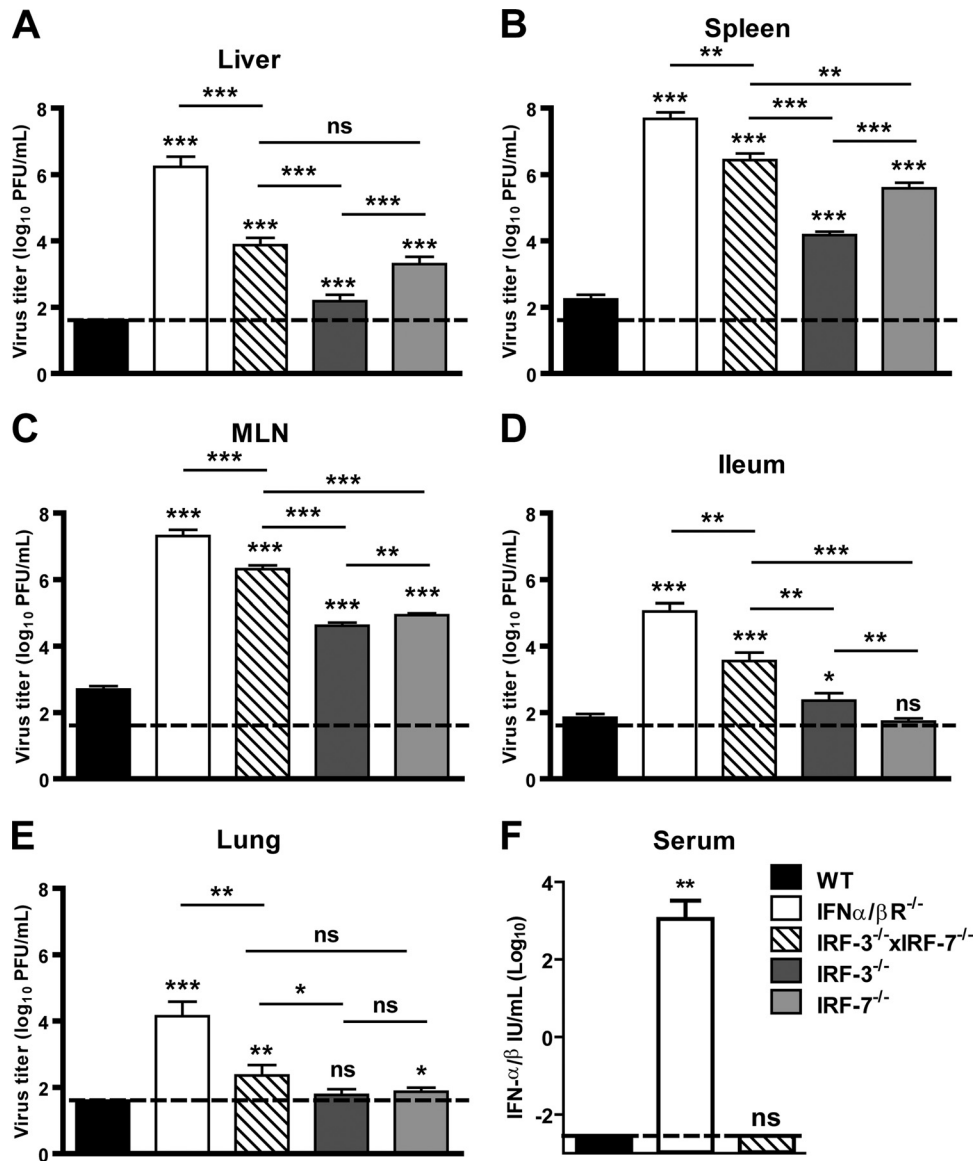
**FIG 2** IFN- $\alpha/\beta$  responses restrict MNV replication *in vivo*. WT, IFN- $\alpha/\beta$ R<sup>-/-</sup>, IFN- $\alpha/\beta$ R<sup>eff</sup>, IFN- $\alpha/\beta$ R<sup>eff</sup> × LysM-Cre, and IFN- $\alpha/\beta$ R<sup>eff</sup> × CD11c-Cre mice were inoculated with  $3 \times 10^5$  PFU of MNV and tissues were harvested 3 days postinfection (dpi). MLN, mesenteric lymph node. Data are the means and standard errors of the means from at least two independent experiments of at least four mice each. The dashed line represents the limit of detection. Titers from IFN- $\alpha/\beta$ R<sup>-/-</sup> mice were compared with those from wild-type controls; titers from IFN- $\alpha/\beta$ R<sup>eff</sup> × LysM-Cre and IFN- $\alpha/\beta$ R<sup>eff</sup> × CD11c-Cre mice were compared with those from IFN- $\alpha/\beta$ R<sup>eff</sup> controls. Statistical significance was determined using the nonparametric Mann-Whitney test. ns, not significant; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ .

MNV. Three days after inoculation, we observed an increase in MNV replication in the livers (20-fold), spleens (42-fold), and mesenteric lymph nodes (60-fold) of IRF-3<sup>-/-</sup> mice and in the livers (163-fold), spleens (1,500-fold), and mesenteric lymph nodes (105-fold) of IRF-7<sup>-/-</sup> mice compared to wild-type controls (Fig. 3A to C). We also observed a significant increase in MNV replication in the ilea (4-fold) of IRF-3<sup>-/-</sup> mice and in the lungs (3-fold) of IRF-7<sup>-/-</sup> mice compared to wild-type controls (Fig. 3D and E). These data suggest that while IRF-3 and IRF-7 independently restrict MNV replication *in vivo*, IRF-7 is a greater participant in innate immune responses during MNV infection.

Since we observed roles for IRF-3 and IRF-7 in restricting MNV replication in the ilea and lungs, we examined the relative requirements for IRF-3 and IRF-7 in restricting MNV replication *in vivo* by comparing titers in IRF-3<sup>-/-</sup>, IRF-7<sup>-/-</sup>, and IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup> mice. We observed increased MNV replication in the

livers (8-fold), spleens (36-fold), and mesenteric lymph nodes (2-fold) of IRF-7<sup>-/-</sup> compared to IRF-3<sup>-/-</sup> mice (Fig. 3A to C). Furthermore, enhanced MNV replication was observed in the livers (19-fold), spleens (330-fold), mesenteric lymph nodes (56-fold), ilea (13-fold), and lungs (19-fold) of IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup> mice compared to IRF-3<sup>-/-</sup> mice and in the spleens (9-fold), mesenteric lymph nodes (326-fold), and ilea (152-fold) of IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup> mice compared to IRF-7<sup>-/-</sup> mice (Fig. 3A to E). These data are consistent with the idea that IRF-3 and IRF-7 work together to induce both unique and overlapping tissue-specific antiviral responses, as observed for other viruses (19, 58), to restrict MNV replication *in vivo*.

Because elevated MNV titers were observed in IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup> mice, we evaluated whether IRF-3 and IRF-7 are required to prevent lethal MNV infection. In contrast to results obtained with IFN- $\alpha/\beta$ R<sup>-/-</sup> mice, no lethality was observed in



**FIG 3** IRF-3 and IRF-7 restrict MNV replication *in vivo*. (A to E) WT, IFN- $\alpha/\beta$ R<sup>-/-</sup>, IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup>, IRF-3<sup>-/-</sup> and IRF-7<sup>-/-</sup> mice were inoculated with  $3 \times 10^5$  PFU of MNV, and tissues were harvested 3 dpi. Virus titers shown are from three independent experiments of at least three mice each. Titers were compared with those from wild-type controls. ns, not significant; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ . (F) WT, IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup>, and IFN- $\alpha/\beta$ R<sup>-/-</sup> mice were mock inoculated or inoculated with  $3 \times 10^5$  PFU of MNV, and serum was harvested 3 dpi. Data are from two independent experiments of three mice each. Serum concentrations were compared with those from wild-type controls. Statistical significance was determined using the Mann-Whitney test. ns, not significant; \*\*,  $P = 0.0043$ .

IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup> mice inoculated with  $3 \times 10^7$  PFU of MNV ( $n = 10$ ). Thus, IRF-3 and IRF-7 are dispensable for preventing lethal MNV infection. When combined with the observation that IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup> mice had reduced MNV titers compared to IFN- $\alpha/\beta$ R<sup>-/-</sup> mice, these data suggest that IRF-3, IRF-7, and additional factors control MNV infection *in vivo* through the induction of IFN- $\alpha/\beta$  responses.

Since we observed decreased MNV titers and no lethality in IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup> mice compared to IFN- $\alpha/\beta$ R<sup>-/-</sup> mice, we investigated the production of IFN- $\alpha/\beta$  during MNV infection *in vivo*. High levels of IFN- $\alpha/\beta$  were observed in IFN- $\alpha/\beta$ R<sup>-/-</sup> mice 3 days after MNV infection, likely due to elevated virus titers and the lack of the IFN- $\alpha/\beta$ R to bind and internalize IFN- $\alpha/\beta$  (1, 34)

(Fig. 3F). In contrast, circulating IFN- $\alpha/\beta$  was not detected in either wild-type or IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup> mice despite the elevated MNV titers observed in IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup> mice. Of note, circulating IFN- $\alpha/\beta$  was not detected in mock-inoculated wild-type, IRF-3<sup>-/-</sup> × IRF-7<sup>-/-</sup> and IFN- $\alpha/\beta$ R<sup>-/-</sup> mice (data not shown). Our inability to detect serum IFN- $\alpha/\beta$  in wild-type mice is unexpected, as IFN- $\alpha/\beta$ R<sup>-/-</sup> mice are profoundly susceptible to MNV infection, and suggests that limited IFN- $\alpha/\beta$  production at the site of virus infection may be sufficient to control MNV infection.

**IRF-3 and IRF-7 restrict MNV replication in macrophages and dendritic cells.** To directly assess the role of IRF-3 and IRF-7 in restricting MNV replication, we generated primary macro-

phages and dendritic cells from wild-type, IFN- $\alpha$ / $\beta$ R $^{-/-}$ , and IRF-3 $^{-/-}$   $\times$  IRF-7 $^{-/-}$  mice. Consistent with a prior study (76), we observed enhanced MNV replication in IFN- $\alpha$ / $\beta$ R $^{-/-}$  macrophages (12-fold) and dendritic cells (4-fold) compared to wild-type controls (Fig. 4A and B). We also observed similarly increased MNV replication in IRF-3 $^{-/-}$   $\times$  IRF-7 $^{-/-}$  macrophages (19-fold) and dendritic cells (3-fold) compared to wild-type controls (Fig. 4A and B). These data demonstrate that IRF-3 and IRF-7 restrict MNV replication in primary macrophages and dendritic cells.

**IRF-3 and IRF-7 are required for IFN- $\alpha$ / $\beta$  induction but are dispensable for the downstream antiviral activity mediated by IFN- $\alpha$ / $\beta$ .** To further elucidate the role of IRF-3 and IRF-7 during MNV infection, we examined MNV replication in macrophages and dendritic cells from wild-type, IFN- $\alpha$ / $\beta$ R $^{-/-}$ , and IRF-3 $^{-/-}$   $\times$  IRF-7 $^{-/-}$  mice after treatment with IFN- $\alpha$ 4 or IFN- $\beta$ . We elected to examine the ability of IFN- $\alpha$ 4 to inhibit replication of MNV *in vitro*, as virus infection in the mouse can induce both IFN- $\alpha$ 4 and IFN- $\beta$  with immediate-early kinetics (38, 42). Treatment with IFN- $\alpha$ 4 and IFN- $\beta$  significantly decreased MNV replication in wild-type and IRF-3 $^{-/-}$   $\times$  IRF-7 $^{-/-}$  macrophages and dendritic cells compared to untreated controls but had no significant effect, as expected, in IFN- $\alpha$ 4- and IFN- $\beta$ -treated IFN- $\alpha$ / $\beta$ R $^{-/-}$  macrophages and dendritic cells compared to untreated controls (Fig. 4A and B). Thus, IRF-3 and IRF-7 are dispensable for the antiviral activity mediated by IFN- $\alpha$ 4 or IFN- $\beta$  in macrophages and dendritic cells.

To assess whether IRF-3 or IRF-7 is important for induction of IFN- $\alpha$ / $\beta$  in cells infected with MNV, we measured IFN- $\alpha$ / $\beta$  mRNA during MNV infection of wild-type and IRF-3 $^{-/-}$   $\times$  IRF-7 $^{-/-}$  macrophages and dendritic cells. Notably, we observed a significant increase in IFN- $\alpha$ 4 (850-fold) and IFN- $\beta$  (3,600-fold) mRNA induction in IRF-3 $^{-/-}$   $\times$  IRF-7 $^{-/-}$  macrophages compared to wild-type controls during MNV infection, with virtually no induction of either cytokine seen in cells lacking both IRF-3 and IRF-7 (Fig. 4C). In striking contrast, we observed a limited decrease in IFN- $\alpha$ 4 (7-fold) and IFN- $\beta$  (11-fold) mRNA induction in IRF-3 $^{-/-}$   $\times$  IRF-7 $^{-/-}$  dendritic cells compared to wild-type controls at 8 hpi with MNV, with near-wild-type levels of induction by MNV of both cytokines in cells lacking both IRF-3 and IRF-7 at 12 hpi (Fig. 4D). These data demonstrate that IRF-3 and IRF-7 are essential for induction of IFN- $\alpha$ / $\beta$  during MNV infection of primary macrophages and participate in IFN- $\alpha$ / $\beta$  induction during MNV infection of primary dendritic cells. These results also indicate that there is a cell type-specific role for these transcription factors in IFN- $\alpha$ / $\beta$  induction during MNV infection.

## DISCUSSION

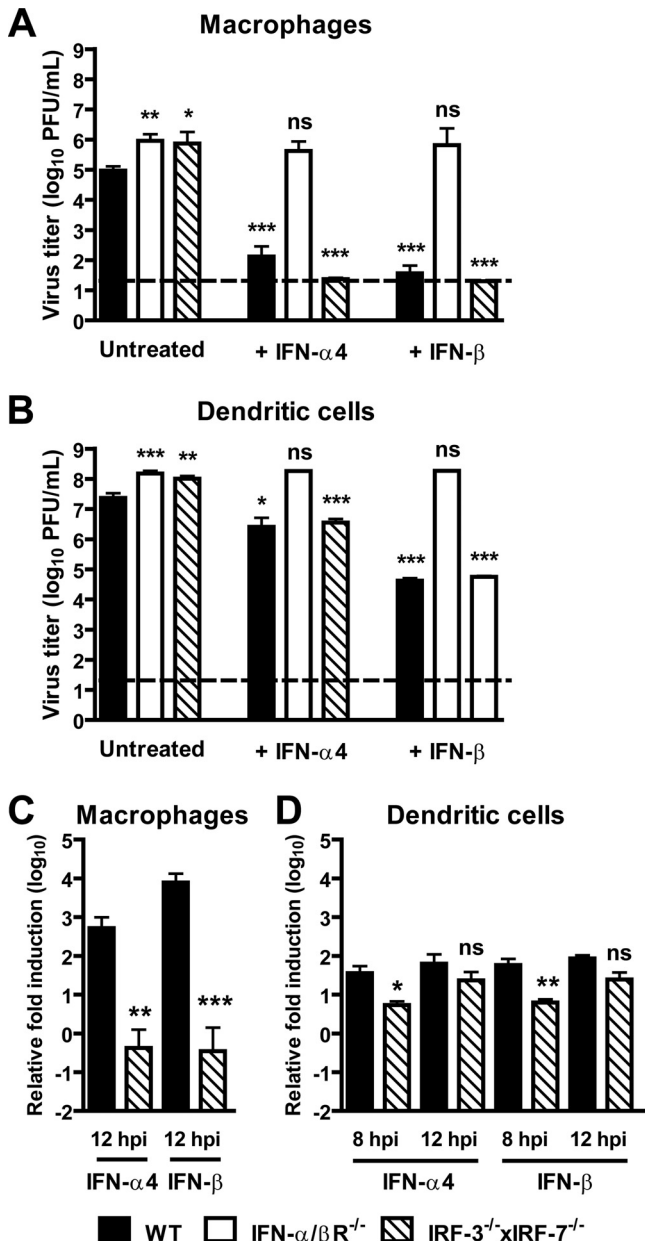
IFN- $\alpha$ / $\beta$  likely plays an important role in the induction of antiviral immune mechanisms during HuNoV infection and has been shown to be important, when combined with IFN- $\gamma$ , for control of MNV replication. However, the contribution of IFN- $\alpha$ / $\beta$  responses to host defense during NoV infection, as well as the upstream initiators of these responses, is not well defined. Previous studies using the MNV model system identified a requirement for MDA-5 in IFN- $\alpha$ / $\beta$  production during MNV infection *in vitro*, as well as a role for MDA-5 in restricting MNV infection both *in vitro* and *in vivo* (46). In this report, we demonstrate an important role for IFN- $\alpha$ / $\beta$  responses in control of MNV replication and pathogenesis *in vivo*. Using mice with floxed alleles of the IFNAR1 chain

of the IFN- $\alpha$ / $\beta$ R, we showed that IFN- $\alpha$ / $\beta$  responses in lysozyme M- and CD11c-expressing cells, likely macrophages/neutrophils and dendritic cells, restrict MNV replication *in vivo*. Moreover, by analyzing cultured macrophages and dendritic cells, we demonstrated that IRF-3 and IRF-7 are key mediators of the antiviral immune response during MNV infection. The role for IRF-3 and IRF-7 is prominently in the induction of IFN- $\alpha$ / $\beta$  rather than in the induction of an antiviral state by IFN- $\alpha$ / $\beta$ .

**Macrophages and dendritic cells participate in IFN- $\alpha$ / $\beta$  immune responses against MNV.** The tropism of HuNoV is not completely understood. The binding of NV-like particles to enterocytes, as well as histopathology in the intestines, of human volunteers inoculated with NV or recombinant virus-like particles (43, 59) suggests that HuNoV replicates in intestinal epithelial cells. However, the detection of NV capsid antigen in mononuclear cells in the intestinal lamina propria of an infected person, as well as in experimentally infected chimpanzees (5, 35), suggests that HuNoV may also infect macrophages and/or dendritic cells in gut lymphoid tissues.

MNV infects mononuclear cells in the intestinal lamina and has a tropism for macrophages and dendritic cells *in vitro* (49, 75, 76). Furthermore, IFN- $\alpha$ / $\beta$  responses are important for restricting MNV replication in macrophages and dendritic cells *in vitro* (13, 76). Our data suggest that IFN- $\alpha$ / $\beta$  responses in CD8 $\alpha^+$  dendritic cells, which lack the IFN- $\alpha$ / $\beta$ R (20), likely play a role in restricting MNV replication *in vivo*. Data obtained from IFN- $\alpha$ / $\beta$ R $^{fl/fl}$   $\times$  LysM-Cre mice is more complicated in that the IFNAR1 allele is floxed in macrophage-lineage cells, in particular resident macrophages, and in neutrophils (20). To date, the role of neutrophils in NoV infection has not been explored. The lack of MNV titers observed in the lung of IFN- $\alpha$ / $\beta$ R $^{fl/fl}$   $\times$  LysM-Cre and IFN- $\alpha$ / $\beta$ R $^{fl/fl}$   $\times$  CD11c-Cre mice is consistent with the idea that IFN- $\alpha$ / $\beta$  responses in additional as-yet-uncharacterized cell types may also prevent MNV replication. Alternatively, incomplete deletion of the IFN- $\alpha$ / $\beta$ R in macrophages or neutrophils and dendritic cells may occur in certain tissues of IFN- $\alpha$ / $\beta$ R $^{fl/fl}$   $\times$  LysM-Cre and IFN- $\alpha$ / $\beta$ R $^{fl/fl}$   $\times$  CD11c-Cre mice (20, 30). Given that IFN- $\alpha$ / $\beta$  levels were not significantly decreased in plasmacytoid dendritic cells in IFN- $\alpha$ / $\beta$ R $^{fl/fl}$   $\times$  CD11c-Cre mice in a prior study (20), it is interesting to speculate that plasmacytoid DC that constitutively express IRF-7, which was critical for restriction of MNV replication in livers, spleens, and mesenteric lymph nodes, may regulate IFN- $\alpha$ / $\beta$  responses at distal sites during MNV infection. Additional studies will be needed to elucidate the role of neutrophils, and subsets of macrophages and dendritic cells, during NoV infection.

**IRF-3 and IRF-7 are key components of the transcriptional antiviral response upon MNV infection.** Our studies with primary macrophages revealed a requirement for IRF-3 and IRF-7 in the induction of IFN- $\alpha$ 4 and IFN- $\beta$  during MNV infection. In addition, we demonstrated that both IFN- $\alpha$ 4 and IFN- $\beta$  can inhibit MNV replication in macrophages and dendritic cells in the absence of IRF-3 and IRF-7. Consistent with these observations, we observed elevated MNV titers in all tissues of IRF-3 $^{-/-}$   $\times$  IRF-7 $^{-/-}$  mice compared to wild-type controls. We previously showed that MDA-5 and TLR-3 are involved in IFN- $\alpha$ / $\beta$  release during MNV infection (46). These new data, in combination with prior findings, are consistent with a recent paradigm suggesting that upon virus infection, cells utilize a subset of both pattern



**FIG 4** IRF-3 and IRF-7 are required for IFN- $\alpha/\beta$  induction during MNV infection but are dispensable for the antiviral activity of IFN- $\alpha/\beta$ . (A) Primary bone marrow-derived macrophages (macrophages) from WT, IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup>, and IFN- $\alpha/\beta$ R<sup>-/-</sup> mice were untreated or treated with recombinant IFN- $\alpha$ 4 or recombinant IFN- $\beta$  and inoculated with MNV (MOI of 0.05). Titers at 48 hpi are the mean and the standard error of the mean from four independent experiments. Titers from untreated IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup> and IFN- $\alpha/\beta$ R<sup>-/-</sup> macrophages were compared with those from wild-type controls; titers from IFN- $\alpha$ 4 or IFN- $\beta$  treated wild-type, IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup>, and IFN- $\alpha/\beta$ R<sup>-/-</sup> macrophages were compared with those from untreated controls. Statistical significance was determined using the unpaired *t* test. ns, not significant; \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.005. (B) Primary bone marrow-derived dendritic cells from WT, IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup>, and IFN- $\alpha/\beta$ R<sup>-/-</sup> mice were untreated or treated with recombinant IFN- $\alpha$ 4 or recombinant IFN- $\beta$  and inoculated with MNV (MOI of 0.05). Virus titers at 24 hpi are from at least three independent experiments. Titers from untreated IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup> and IFN- $\alpha/\beta$ R<sup>-/-</sup> dendritic cells were compared with those from wild-type controls; titers from IFN- $\alpha$ 4 or IFN- $\beta$ -treated wild-type, IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup>, and IFN- $\alpha/\beta$ R<sup>-/-</sup> dendritic cells were compared with those from untreated controls. (C) Macrophages from WT and

recognition receptors and IRF transcription factors to induce cell-specific IFN- $\alpha/\beta$  responses (reviewed in reference 45).

Although IFN- $\alpha$ 4 and IFN- $\beta$  mRNA induction was abolished in IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup> macrophages, we observed IFN- $\alpha$ 4 and IFN- $\beta$  mRNA induction in IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup> dendritic cells, especially at later time points. Consistent with these observations, despite elevated MNV titers in all tissues examined, IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup> mice failed to succumb to lethal MNV infection, in contrast to IFN- $\alpha/\beta$ R<sup>-/-</sup> mice, which were highly vulnerable. These observations show that expression of the IFN- $\alpha/\beta$ R is more important for control of MNV infection than the combination of IRF-3 and IRF-7. These observations are reminiscent of recent studies that uncovered an IRF-3- and IRF-7-independent antiviral mechanism during WNV and chikungunya infection *in vivo* (6, 16, 19), and they suggest that one or more transcription factors in addition to IRF-3 and IRF-7 are involved in IFN- $\alpha/\beta$  induction or IFN- $\alpha/\beta$  antiviral effects during MNV infection *in vivo*. To date, these additional transcription factor(s) remain unknown; a significant decrease in IFN- $\alpha$ 4 and IFN- $\beta$  mRNA induction during MNV infection was not observed in macrophages lacking IRF-1 compared to wild-type controls (L. Thackray, unpublished observations), but this may reflect compensatory effects of IRF-3 or IRF-7. Alternatively, IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup> mice may be protected against lethal MNV infection by compensatory immune mechanisms, such as enhanced IFN- $\gamma$  production, in a manner similar to that of murine cytomegalovirus infection, or by enhanced IFN- $\lambda$  production (33, 62, 64). Future studies will be needed to identify other transcription factors involved in initiating antiviral host responses during NoV infection.

**IRF-3 and IRF-7 prevent disseminated MNV infection.** HuNoV infection is typically associated with gastrointestinal symptoms, including nausea, vomiting, and watery diarrhea (3, 24), all consistent with the current paradigm that the gastrointestinal tract is the primary site of HuNoV replication. However, the more recent association of HuNoV infection with extraintestinal symptoms such as benign infantile seizures and encephalopathy, as well as the detection of HuNoV RNA in serum samples from patients (14, 47, 50, 68), suggests that HuNoV may accumulate in other tissues of infected patients. Of note, while HuNoV RNA was not detected in serum of experimentally infected chimpanzees, it was detected in the liver (5). The mechanisms that restrict HuNoV replication to the intestine or limit the replication of virus that is released into the lymphatic or somatic organ systems remain unknown.

MNV replicates in the livers, spleens, mesenteric lymph nodes, and intestines of immunocompetent mice (27, 49, 69) and thus can spread to extraintestinal sites after peroral inoculation. Mice lacking B or T cells, STAT-1, or the IFN- $\alpha/\beta$ R and IFN- $\gamma$  receptor (IFN- $\gamma$ R) sustain high levels of disseminated MNV infection, in-

IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup> mice were mock inoculated or inoculated with MNV (MOI of 10). Induction of IFN- $\alpha$ 4 and IFN- $\beta$  mRNA levels at 12 hpi was normalized to 18S RNA levels and calculated using the  $\Delta\Delta C_T$  method. Relative induction was determined from four independent experiments and compared to induction in wild-type controls. Statistical significance was determined using the unpaired *t* test. (D) Dendritic cells from WT and IRF-3<sup>-/-</sup>  $\times$  IRF-7<sup>-/-</sup> mice were mock inoculated or inoculated with MNV (MOI of 10). Induction of IFN- $\alpha$ 4 and IFN- $\beta$  mRNA levels at 8 and 12 hpi was normalized to 18S RNA levels and calculated using the  $\Delta\Delta C_T$  method. Relative induction was determined from three independent experiments and compared to induction in wild-type controls.

cluding in the brain and lung (31, 49, 66). Our data show that IRF-3 and IRF-7, as well as signaling through the IFN- $\alpha/\beta$ R, induce an antiviral response that limits dissemination of MNV and/or the replication of the virus in extraintestinal organs. These effects could be due to increased MNV replication at primary sites of replication such as the intestine, changes in the release of the virus into the circulation, or uncontrolled MNV replication in other organs. In this study, we observed that MNV titers in mice lacking IRF-3 and IRF-7 or the IFN- $\alpha/\beta$ R increased more dramatically in extraintestinal sites than in the intestine. Combined with previous observations of high MNV titers in the intestine of mice lacking components of both the IFN- $\alpha/\beta$ R and IFN- $\gamma$ R pathways (28, 49, 66), the data presented in this study suggest a key role for IFN- $\alpha/\beta$  responses in controlling norovirus replication at extraintestinal sites and a role for additional antiviral responses, such as those mediated by IFN- $\gamma$ , in controlling norovirus replication in the intestine. Although the idea is speculative, impaired IFN- $\alpha/\beta$  production during HuNoV infection in a subset of individuals, such as children or immunocompromised adults, could result in disseminated HuNoV infection and more severe disease.

IFN- $\beta$  limits expression of HuNoV RNA and protein expression in cells expressing an NV replicon (12), indicating that the cytokine is important for control of both human and murine norovirus replication. It is interesting to speculate that the findings here regarding mechanisms of IFN- $\alpha/\beta$  control of MNV reflect similar antiviral mechanisms that control other NoV. Future studies that elucidate the molecular pathways that induce IFN- $\alpha/\beta$  and initiate IFN- $\alpha/\beta$  responses in specific cells and tissues during MNV infection may identify novel targets for vaccination or therapeutic strategies to prevent or treat NoV infection.

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