Hantaviruses Induce Antiviral and Pro-Inflammatory Innate Immune Responses in Astrocytic Cells and the Brain

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Abstract

Although hantaviruses are not generally considered neurotropic, neurological complications have been reported occasionally in patients with hemorrhagic fever renal syndrome (HFRS). In this study, we analyzed innate immune responses to hantavirus infection *in vitro* in human astrocytic cells (A172) and *in vivo* in suckling ICR mice. Infection of A172 cells with pathogenic Hantaan virus (HTNV) or a novel shrew-borne hantavirus, known as Imjin virus (MJNV), induced activation of antiviral genes and pro-inflammatory cytokines/chemokines. MicroRNA expression profiles of HTNV- and MJNV-infected A172 cells showed distinct changes in a set of miRNAs. Following intraperitoneal inoculation with HTNV or MJNV, suckling ICR mice developed rapidly progressive, fatal central nervous system-associated disease. Immunohistochemical staining of virus-infected mouse brains confirmed the detection of viral antigens within astrocytes. Taken together, these findings suggest that the neurological findings in HFRS patients may be associated with hantavirus-directed modulation of innate immune responses in the brain.

Introduction

THE SEMINAL DISCOVERY OF Hantaan virus (HTNV), as the prototype virus of hemorrhagic fever with renal syndrome (HFRS), in the striped-field mouse (*Apodemus agrarius*) in Korea in 1976 (37), led to the detection of several other HFRS-causing hantavirus species. These include Seoul virus (SEOV) (12,23,32,36,45), Puumala virus (PUUV) (11,42), Dobrava/Belgrade virus (DOBV) (7,8,33,34,44), and Amur/Soochong virus (AMRV/SOOV) (9,26). The discovery of HTNV also made possible the identification of hantaviruses such as Sin Nombre virus (SNV), Black Creek Canal virus (BCCV), and Andes virus (ANDV), harbored by sigmodontine and neotomine rodent species, which cause hantavirus cardiopulmonary syndrome (HCPS) in the Americas (39,43,51). Recently, the reservoir host range has been expanded with the discovery of highly divergent lineages of hantaviruses in shrews (2,3,5,28,30,52–54), moles (4,27–29) and bats (57,58), but their pathogenic potential in humans is not known.

HFRS-associated hantaviruses cause vascular leakage and renal dysfunction, whereas HCPS-associated hantaviruses cause acute pulmonary edema (15,47). The mechanisms of hantavirus-induced pathogenesis are not fully understood, but both HFRS and HCPS share several clinical and pathologic features, including high fever, thrombocytopenia,

increased capillary permeability and upregulation of tumor necrosis factor- α (TNF- α). Recent reports indicate less well-studied complications of HFRS and HCPS, such as central nervous system (CNS) dysfunction (10,19–22,24,55). However, hantaviral antigens have not been detected in the brains of patients, so the mechanisms by which hantavirus infection might lead to neurological and neuropsychological complications are almost entirely unknown.

Both antiviral and pro-inflammatory responses to hantavirus infection play an important role in the modulation of host defense and disease manifestation (1,6,18,25,38,46,48). Pathogenic rodent-borne hantaviruses, such as HTNV, evade early innate immune responses by downregulating antiviral responsive gene expression immediately after infection. By contrast, upregulation of antiviral gene expression is observed in nonpathogenic Prospect Hill virus (PHV) infection (35). The pathogenesis of HFRS and HCPS is hypothesized to be mediated by increased pro-inflammatory responses (46). TNF- α , interleukin-1 β (IL-1 β), interleukin-6 (IL-6), chemokine (C-C motif) ligand-5 (CCL-5), C-X-C motif ligand-8 (CXCL-8), and C-X-C motif ligand-10 (CXCL-10) are elevated in the kidneys and lungs of HFRS and HCPS patients, respectively, and in hantavirus-infected endothelial cells, macrophages, or epithelial cells *in vitro* (17,40,41,50). Although the major target organs of HFRS and HCPS are the

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kidney and lungs, respectively, the possibility that other organs, such as the brain, serve as target sites remains to be determined.

We recently determined that a novel shrew-borne hantavirus, Imjin virus (MJNV) (23), causes rapid and intense innate immune responses in human endothelial cells and macrophages, similar to that of the pathogenic rodent-borne HTNV (50). In this report, we demonstrate that both HTNV and MJNV show similar replication kinetics and efficient viral protein synthesis in human astrocytic cells. Distinct patterns of microRNA (miRNA) expression were also associated with HTNV and MJNV infection, and the top-predicted gene targets were found to play a role in inflammatory cytokine and chemokine pathways and integrin signaling. Moreover, HTNV and MJNV produced profound increases in cytokine and chemokine expression in brains of experimentally infected mice. Therefore, our data suggest that profound innate immune stimulation may be a potential underlying mechanism for CNS dysfunction in HFRS patients.

Materials and Methods

Cells lines

A human astrocytoma cell line (A172), derived from the brain tissue of a 53-year-old man with glioblastoma, was obtained from the Korean Cell Line Bank (Seoul, Korea) and propagated in RPMI-1640 (Lonza) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Lonza), 10 mM HEPES, 2 mM L-glutamine, and antibiotics (penicillin-streptomycin) at 37°C at 5% CO₂. Vero E6 cells (African green monkey kidney epithelial cell line, CRL1586; ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Lonza) with 5% FBS, 2 mM L-glutamine, and antibiotics (penicillin-streptomycin) at 37°C at 5% CO₂.

Viruses

HTNV strain 76-118 and MJNV strain 04-55 were used. Virus stocks and cells were determined to be free of *Mycoplasma* contamination by polymerase chain reaction (PCR) analysis. Infectivity titers of virus stocks were measured by plaque assay on Vero E6 cell monolayers, as previously described (48).

Animals and virus inoculation

Pregnant outbred ICR mice (Dbl Laboratory, Korea) were maintained in the vivarium at Korea University. Newborn ICR mice (<24 h after birth; 13-15 per group) were inoculated intraperitoneally with 1,000 plaque forming units (PFU) of HTNV or MJNV by the route. Mice were monitored daily for clinical signs, and were euthanized if moribund or at prescribed time points post-inoculation. The Institutional Animal Care and Use Committee approved all experimental animal protocols.

Virus replication kinetics

A172 cells were inoculated with HTNV and MJNV at a multiplicity of infection (MOI) of 0.5. After adsorption for 90 min at 37°C, cell monolayers were washed with phosphate buffered saline (PBS) and maintained in complete medium. Virus infectivity titers of supernatants were determined on each post-infection day by plaque assay (48).

Immunofluorescence microscopy

A172 cells, seeded on coverslips in 24-well plates, were inoculated with HTNV or MJNV at a MOI of 0.5 for 90 min at 37°C. Thereafter, fresh cell culture medium was added and incubated for 72 h. Slides were prepared as described previously (50) and examined by fluorescence microscopy, using a Zeiss Axioplan 2 microscope. Images were captured with a digital CCD camera (Hamamatsu).

Western blot analysis

Protein lysates were prepared as described previously (50). Briefly, HTNV- and MJNV-infected A172 cell lysates were separated on 10% SDS-PAGE and blotted onto PVDF membranes. HTNV and MJNV N proteins were detected with mouse monoclonal N protein-specific antibodies followed by anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody.

Quantitative real time reverse transcription PCR

RNA isolation and cDNA synthesis were described previously (50). Briefly, total RNA (1 µg), isolated with Trizol reagent (Invitrogen), was reverse transcribed using the RT system (Promega) for 1 h at 42°C. The resulting cDNA was used as template for real-time PCR quantification. Using the power SYBR Green supermix (Invitrogen), mRNA expression of human and mouse genes was measured. Human primers were previously published (14,50,60), and mouse primers are provided (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/vim). Cycling parameters were 95°C for 15 min, followed by 40 cycles, with one cycle consisting of 30 sec at 95°C and 1 min at 60°C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an endogenous reference to normalize the quantities of target mRNAs and presented as the change in induction relative to that of untreated control cells. Calculations of expression were normalized by using the ΔC_t method, where the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta C_t}$, where C_t is the cycle number of the detection threshold.

Cytokine secretion measurement

Supernatants of HTNV- and MJNV-infected A172 cells were harvested at indicated times post-infection and stored at -80°C until testing. Cytokine levels were measured on a *Magpix* using a human cytokine Milliplex Kit (Millipore). Data analysis was performed with Bio-Plex Manager software (Bio-Rad Laboratories). The *Magpix* experiments were performed in duplicate.

miRNA microarray analysis

Total RNA was isolated followed by quality checks of both total RNA and small RNA using a 2100 Bioanalyzer and software capable of determining 28S and 18S ribosomal RNA ratio, total RNA Integrity Number, and small RNA and miRNA concentrations. Only samples with adequate total RNA and miRNA were used in the study. miRNA expression profiling was performed using the Agilent Human miRNA 8 X 60K (V16). The microarray was designed based on Sanger miRBase (release 16.0) and contained probes for 1,205 human

and 144 human viral miRNAs. One hundred ng of total RNA was labeled using the Agilent miRNA Complete Labeling and Hybridization Kit, according to the manufacturer's instructions. For target gene functional annotation, we chose to use GO categories, canonical Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways maps, and Panther protein classification tools. Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Inc.) was used to analyze miRNA expression data in the context of known biological response, regulatory networks, and specific pathways.

Immunohistochemistry

Brain tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded, 5 μ m thick sections were deparaffinized with xylol, rehydrated using decreasing concentrations of ethanol (100%, 95%, 80%, and 70% for 5 min each). Following antigen retrieval in 10 mM sodium citrate buffer, pH 6.0 at 95–100°C for 20 min, peroxide-blocking solution was applied to slides for 20 min at room temperature. Antigen detection was performed using anti-HTNV and anti-MJNV monoclonal mouse IgG antibodies followed by antibody enhancer and polymer AP (GBI Polink-2 Plus kits; GBI Lab); subsequent color development was performed with AP+red (GBI Labs). For double staining of glial fibrillary acidic protein (GFAP), preblocking solution (10% normal goat serum) was applied to the slides and in-

cubated for 10 min at room temperature. Anti-GFAP primary antibody, at the recommended concentration (1:500; Millipore), was incubated for 2 h at room temperature. Slides were washed in 1 $\times$ TBST four times for 5 min and antibody enhancer and polymer HRP detection kit was applied (GBI Polink-2 Plus kits). After washing the slides four times, color development was performed with Emerald Green (GBI Labs). The slides were mounted with aqueous mounting media.

Statistical analysis

The paired differences of the experimental groups were compared using the nonparametric Mann–Whitney *U*-test. A *p*-value of <0.05 was considered statistically significant (Graph-Pad; Prism software).

Results

Replication kinetics of HTNV and MJNV in A172 human astrocytic cells

To study the immunological responses *in vitro*, we first determined if the A172 human astrocytic cell line could be infected with hantaviruses. Virus-specific fluorescence was detected in A172 cells at 72 h following HTNV or MJNV infection at a MOI of 0.5 (Fig. 1A). Infectivity titers of HTNV and MJNV in A172 cells, at a MOI of 0.5, attained 10^3 – 10^4 PFU/mL at day 2 and persisted until day 5 post-infection

FIG. 1. Hantavirus infection in human astrocytic cells. (A) A172 cells, infected with HTNV or MJNV at a multiplicity of infection (MOI) of 0.5 and incubated for 72 h, were stained by the IFA technique, using virus-specific mouse monoclonal antibodies followed by FITC-conjugated anti-mouse IgG antibodies, to detect virus antigen (green). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue). Original magnification, 400 $\times$. (B) Infectivity titers at 1–5 days post-infection were determined by plaque assay in supernatants of A172 cells infected with HTNV or MJNV at a MOI of 0.5. Days post-infection are indicated on the horizontal axis, and titers are represented on a log scale (PFU/mL). Titrations were repeated twice. (C) Viral proteins at 1–5 days post-infection were determined by Western blot analysis, using mouse monoclonal antibodies against HTNV or MJNV N protein, followed by HRP-conjugated anti-mouse IgG antibodies, in lysates of A172 cells infected with HTNV or MJNV at a MOI of 0.5. Comparable loading of proteins was verified by re-probing the blots with an antibody specific for the housekeeping gene product, β -actin. The Western blots are representative of three independent experiments. HTNV, Hantaan virus; MJNV, Imjin virus.

(Fig. 1B). Nucleocapsid (N) protein synthesis in HTNV- and MJNV-infected A172 cells, as measured by Western blot analysis, was elevated early and persisted until day 5 post-infection (Fig. 1C).

Antiviral and pro-inflammatory responses in HTNV- and MJNV-infected A172 cells

To determine if astrocytes can induce antiviral responses to hantavirus infection, transcripts for myxovirus resistance

gene A (MxA), IFN- β , IL-28 (IFN- λ 2), and IL-29 (IFN- λ 1) were quantitated by real-time reverse transcription PCR (qRT-PCR) at 1, 3, and 5 days post-infection. MxA and IFN- β gene expression peaked at day 3 post-infection, and decreased at day 5 post-infection in cells infected with HTNV or MJNV (Fig. 2A and B). In contrast to the low expression levels of MxA and IFN- β at day 1 post-infection, the induction of type III IFN (both IL-28 and IL-29) preceded induction of MxA and IFN- β by 2 days (Fig. 2C and D). Thus, these data indicate that type III IFN (IFN- λ)

FIG. 2. Kinetics of antiviral and pro-inflammatory gene expression during hantavirus infection. A172 cells were infected with HTNV (black) or MJNV (light grey) (MOI of 0.5) or mock infected. RNA was extracted at days 1, 3, and 5 post-infection. The mRNA expression of (A) MxA, (B) IFN- β , (C) IL-28, (D) IL-29, (E) IP-10, (F) RANTES, (G) VEGF, and (H) IL-8 in A172 cells treated with media was arbitrarily set to 1 and relative expression is shown in the graph. Expression of target genes was normalized to that of β -actin. The quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) experiments were performed in duplicate, and the results are representative of at least three independent experiments.

expression is induced prior to type I IFN (MxA and IFN- β) during HTNV and MJNV infection.

To determine the effect of HTNV and MJNV infection on the pro-inflammatory responses, mRNA expression of interferon gamma-induced protein-10 (IP-10), Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES), vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8) were measured at 1, 3, and 5 days in A172 cells infected with either HTNV or MJNV. Both HTNV and MJNV led to elevated levels of IP-10, RANTES, VEGF, and IL-8 at 3 days post-infection (Fig. 2E–H). In general, HTNV-infected cells show decreased detection of IP-10, RANTES, and IL-8 at day 5 post-infection. However, MJNV-infected cells generally show slight increases in detection of these markers at day 5 post-infection. Next, the levels of secreted cytokine and chemokine proteins were measured in supernatants of HTNV- or MJNV-infected A172 cells. Consistent with the qRT-PCR results, the protein secretion levels of IP-10 and VEGF increased upon infection with HTNV and MJNV (Fig. 3).

Distinct cellular miRNA expression patterns in HTNV- and MJNV-infected A172 cells

To identify differentially expressed miRNAs associated with HTNV and MJNV infection, we compared global cellular miRNA expression profiles in HTNV- and MJNV-infected A172 cells at matching time points. miRNAs that were altered at least twofold were considered significant. Heat maps showed the top 10 up- and downregulated miRNAs induced by both HTNV and MJNV-infected samples (Fig. 4A and Supplementary Table S2). Analysis of 1,205 human miRNAs revealed that HTNV upregulated the expression of 90 miRNAs and downregulated expression of six miRNAs by twofold, whereas MJNV upregulated expression of 64 miRNAs and downregulated expression of two miRNAs by twofold (Fig. 4B). A total of 3,364 target genes were upregulated, whereas 2,803 target genes were downregulated by selected miRNAs. To analyze more fully the top biological functions associated with identified targets, we performed a Gene Ontology (GO) term and KEGG pathway annotation, using the DAVID gene annotation tool. Our pathway-based analysis suggested that the top three putative targets of miRNAs regulated by HTNV and MJNV infection were involved in Wnt, inflammatory cytokine and chemokine production, and integrin signaling (Table 1).

To understand better the roles of miRNAs expressed in HTNV- and MJNV-infected cells, Ingenuity Pathway Analysis (IPA) was used to generate gene lists known to have direct or indirect functional relationships with these miRNAs. Using this approach, we were able to show that HTNV- and MJNV-modulated miRNAs and their targets regulated the innate immune response, antiviral response, and pro-inflammatory signaling pathways (Fig. 4C). Furthermore, our network analysis demonstrated a potential correlation between HTNV- and MJNV-modulated miRNAs and mRNA expression data of antiviral or inflammatory molecules (such as MxA, IL-8, IP-10, and VEGF; see Fig. 2E–H) induced by these viruses in the same cell type and same time after infection, suggesting their role in inducing inflammation after HTNV and MJNV infection (Fig. 4D). Moreover, the similar expression profiles of specific miRNAs in HTNV- and

FIG. 3. Cytokine secretion following HTNV or MJNV infection in A172 cells. Supernatants of HTNV- and MJNV-infected A172 cells were harvested at indicated times post-infection, and inflammatory molecule levels of IP-10 and VEGF were determined by *Magpix* analysis in supernatants of A172 cells infected with HTNV or MJNV at a MOI of 0.5, or mock infected and harvested from days 1 to 5 post-infection. The *Magpix* experiments were performed in duplicate, and the results are representative of two independent experiments.

MJNV-infected A172 cells suggested that these two viruses might cause similar cellular pathology.

Immunological responses in the brains of HTNV- and MJNV-infected suckling ICR mice

Suckling ICR mice (<24 h of age), inoculated intraperitoneally with 1,000 PFU of HTNV or MJNV, developed rapidly progressive, severe disease, characterized by weight loss, ruffled fur, and reduced activity, followed by neurological signs, including paralyses and seizures. The onset of disease was more rapid with MJNV than HTNV. That is, clinical signs appeared at 8–9 days post-inoculation in MJNV-infected mice, compared to 12–13 days in HTNV-infected mice, and death ensued within 2 days of disease onset (Table 2).

The transcriptional levels of cytokines and chemokines that might play a role in attracting inflammatory cells to

FIG. 4. Cellular miRNA expression patterns in HTNV- and MJNV-infected human astrocytic cells. **(A)** Heat map expression differences between HTNV and MJNV infection at 3 days post-infection, compared with media control alone, as shown by hierarchical clustering and the top 10 miRNAs (most upregulated and downregulated) were selected, and miRNA names are listed to the right. Average Linkage Clustering was performed using Euclidean Distance, Average Linkage, and Gene Tree. Clustering was visualized using the program MeV 4.7.1. Gene expression ratios are depicted by $\log_2$. **(B)** Venn diagram showing the number of miRNAs differentially expressed in HTNV- and MJNV-infected cells, and in both. For each category, the miRNAs elevated in the *top* row ($>$ twofold) and downregulated in the *bottom* row ($<$ twofold) are indicated. **(C)** Top canonical pathways of target genes of significantly expressed miRNAs. Line shows ratio of genes in network to total number of genes in canonical pathways. **(D)** Network analysis of HTNV- and MJNV-modulated miRNAs with mRNA expression data of inflammatory genes induced by these viruses. Solid lines indicate a direct interaction, whereas a broken line indicates an indirect interaction. The bars in the brackets next to miRNA and mRNA represent fold-change. The first bar is HTNV and the second is MJNV.

affected tissues was assessed by measuring the expression of antiviral responsive genes, including myxovirus (influenza virus) resistance-1 (Mx1), IFN- β , IFN- λ , 2'-5'-oligoadenylate synthetase (OAS), and pro-inflammatory response genes, including IP-10, RANTES, macrophage/monocyte chemoattractant protein-1 (MCP-1), and TNF- α in the brain, kidney, lungs, liver, and spleen of HTNV- and MJNV-infected suckling mice, using qRT-PCR. All four antiviral genes were significantly elevated in the brains of HTNV-infected ($p < 0.05$), and IFN- β and IFN- λ gene expressions were also significantly increased in the brains of MJNV-infected ($p < 0.05$) compared with mock-infected control mice. IFN- β and IFN- λ expression was elevated by ~ 1 log in the virus-

infected groups, whereas Mx1 and OAS expression was 2–3 logs higher in the virus-infected groups (Fig. 5A–D). Along with upregulation of antiviral responsive genes, the levels of IP-10, RANTES, MCP-1, and TNF- α were significantly increased in brains of HTNV-infected mice (Fig. 5E–H). In examining the innate immune responses in other tissues of HTNV- and MJNV-infected mice, we found that the expression of antiviral and pro-inflammatory genes was increased, but the level of induction was not as high as that in the brain (Fig. S1). For example, a 4–6 log-fold higher elevation of IP-10 and RANTES was found in the brain compared to <1 –2 log increase in the kidney, lungs, liver, and spleen.

TABLE 1. TOP 20 FINDINGS FROM TARGET GENE ANALYSIS, USING GENE ONTOLOGY, PATHWAY, AND PROTEIN CLASSIFICATION TOOLS

	<i>GO</i>	<i>Pathway</i>	<i>Protein class</i>
1	Metabolic process	Unclassified	Unclassified
2	Primary metabolic process	Wnt signaling pathway	Nucleic acid binding
3	Unclassified	Inflammation mediated by chemokine and cytokine signaling pathway	Transcription factor
4	Cellular process	Integrin signaling pathway	Hydrolase
5	Cell communication	Angiogenesis	Transferase
6	Signal transduction	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	Receptor
7	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Huntington disease	Enzyme modulator
8	Protein metabolic process	Interleukin signaling pathway	Transporter
9	Transport	TGF-beta signaling pathway	Signaling molecule
10	Developmental process	PDGF signaling pathway	RNA binding protein
11	Transcription	Cadherin signaling pathway	Zinc finger transcription factor
12	Transcription from RNA polymerase II promoter	Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	Cytoskeletal protein
13	Immune system process	EGF receptor signaling pathway	DNA binding protein
14	Cell surface receptor linked signal transduction	FGF signaling pathway	Kinase
15	Regulation of transcription from RNA polymerase II promoter	Alzheimer disease-presenilin pathway	Oxidoreductase
16	System process	Apoptosis signaling pathway	Protease
17	Protein transport	PI3 kinase pathway	KRAB box transcription factor
18	Intracellular protein transport	p53 pathway	Protein kinase
19	System development	Cytoskeletal regulation by Rho GTPase	Defense/immunity protein
20	Protein modification process	Parkinson disease	Cell adhesion molecule

GO, gene ontology.

To ascertain which cell types were targeted in the brain of hantavirus-infected mice, antibodies against GFAP were used to identify astrocytes. Also, antibodies against HTNV and MJNV N protein were used to localize virus in the brain of HTNV- and MJNV-infected mice. Viral N protein was detected extensively throughout the brain and appeared to be specifically associated with GFAP-staining astrocytes (Fig. 6).

Discussion

While renal insufficiency is the principal target-organ manifestations of HFRS, viral antigens can be found in the brains of animal models (59) and CNS-associated clinical signs and symptoms have been reported in some HFRS patients (10,19–22,24,55). Our data demonstrate that HTNV and MJNV efficiently replicate and induce N protein syn-

thesis in human astrocytic cells and similarly cause the induction of innate immune responsive genes. Also, in suckling ICR mice, HTNV or MJNV elicited production of high levels of pro-inflammatory cytokines and chemokines in the brain, which were associated with an acute, fatal neurological disease. Collectively, these data suggest that the neurological complications associated with HFRS may be triggered by innate immune stimulation in the brain, following infection with either known pathogenic rodent-borne hantaviruses or newfound still-orphan shrew-borne hantaviruses.

We recently reported that HTNV and MJNV replicated to high titer in both human umbilical vein endothelial cells (HUVEC) and macrophages (THP-1) and was associated with increased expression of antiviral responsive genes in a time-dependent manner (50). As in HUVEC and THP-1,

TABLE 2. MORTALITY OF SUCKLING ICR MICE EXPERIMENTALLY INFECTED WITH HTNV OR MJNV

<i>Inoculum</i>	<i>Virus dose</i>	<i>Clinical signs</i>	<i>Day of termination</i>	<i>Number of animals</i>	<i>Mortality</i>
HTNV	1,000 PFU	Reduced activity, paralysis	13	13	100%
MJNV	1,000 PFU	Reduced activity, paralysis	9	15	100%
PBS	0	None	13	14	0%

Two independent experiments were performed for each group.

HTNV, Hantaan virus; MJNV, Imjin virus; PFU, plaque forming units; PBS, phosphate buffered saline.

FIG. 5. Hantavirus-induced antiviral and pro-inflammatory gene expression patterns *in vivo*. Suckling ICR mice were injected intraperitoneally with HTNV or MJNV and sacrificed 9 (MJNV) or 13 (HTNV) days after infection. Total RNA was extracted from the brain, and the expression of (A) Mx1, (B) IFN- β , (C) IFN- λ , (D) OAS, (E) IP-10, (F) RANTES, (G) MCP-1, and (H) TNF- α was examined by qRT-PCR. *p*-Values at the top of individual graphs are comparisons between mock-infected and virus-infected mice. Expression of target genes was normalized to that of the housekeeping gene, GAPDH. The qRT-PCR experiments were performed in duplicate and each symbol represents the value for an individual mouse, and the bar indicates the mean for the group. An asterisk indicates that the values for the HTNV-infected or MJNV-infected samples were significantly different from those in the buffer control samples ($p < 0.05$). Kruskal–Wallis test (one way analysis of variance) and Dunn’s Multiple Comparison Test (post-test) were performed. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

HTNV and MJNV replicated in A172 cells with similar kinetics and efficiency. Hantavirus infection in A172 cells was accompanied by type I and type III IFN responses and increased production of pro-inflammatory mediators, such as IP-10, RANTES, and VEGF. It has been previously demonstrated that hantaviruses induces the expression of type I IFN (α/β), type III IFN- λ , and interleukin-28 and -29

(IL-28 and IL-29) (56). Type I IFN has been demonstrated to play a central role in the regulation of innate immune responses and in the control of hantavirus infection *in vitro* (1,35). Interestingly, type III IFN was upregulated more rapidly at 1 day post-infection than type I IFN in A172 cells, although this was not sufficient to control viral replication at early time points.

FIG. 6. Immunohistochemical detection of GFAP and viral N protein in the brain of HTNV- and MJNV-infected mice. Robust cytoplasmic staining of GFAP and hantavirus N protein was found in brains of mice experimentally infected with HTNV and MJNV. (A) GFAP staining only (emerald green); (B) double staining of GFAP and HTNV N protein; (C) double staining of GFAP and MJNV N protein. Magnification: 400 $\times$. GFAP, glial fibrillary acidic protein.

Mounting evidence suggests that specific miRNA expression profiles may be associated with clinical outcomes or treatment responses in human diseases (13). We also previously reported distinct changes in miRNA expression profiles in a hantavirus species-specific manner from human endothelial, epithelial, and macrophage cells (49). In this study, we demonstrated that similar miRNAs were up- or downregulated in HTNV- and MJNV-infected A172 cells, and the top three putative targets of miRNAs appeared to be involved in Wnt, inflammatory cytokine/chemokine production pathways, and integrin signaling, which were previously shown to be dysregulated in pathogenic hantavirus infection (1,16). Moreover, expression-pairing analysis using Ingenuity demonstrated that HTNV- and MJNV-modulated miRNAs can target inflammatory genes induced by these viruses in A172 cells. Thus, our miRNA expression analysis confirms previous reports and these dysregulated innate immune responses may be attributed in part to the miRNA-mediated expression changes of target genes.

In their natural rodent reservoir hosts, hantaviruses cause asymptomatic and persistent infections with no apparent pathology. However, suckling ICR mice, inoculated with HTNV develop lethal CNS infection (31). Thus, we utilized suckling ICR mice to study innate immune responses. Both HTNV and MJNV infection caused similar pathology in suckling mice, but there were minor differences in disease kinetics. That is, MJNV caused disease and death more rapidly than HTNV. Furthermore, higher induction of pro-inflammatory responsive genes was found in the brains of HTNV-infected mice compared to MJNV-infected mice. It is possible that the more pronounced cytokine and chemokine response in HTNV-infected mice may have been protective in delaying pathology.

In conclusion, this study indicated that MJNV triggered distinct activation of innate immune responses, resembling that of HTNV, in human astrocytic cells and mice brain. The differentially expressed miRNAs detected by microarray analysis provide potential candidates of viral infection-related host cellular miRNAs. Although the disease kinetics was accelerated in MJNV-infected suckling mice, the severity of CNS-mediated paralysis was similar to HTNV. Taken together, these findings suggest that distinct innate immune responses occur in the brain following hantavirus infection and may account for the neurological signs and symptoms in some HFRS patients.

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Author Disclosure Statement

No competing financial interests exist.

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