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Interferon-induced transmembrane protein 1 and Myxovirus resistance protein 1 are induced by polyinosinic-polycytidylic acid in cultured hCMEC/D3 human cerebral microvascular endothelial cells

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ABSTRACT

The molecular mechanisms of antiviral innate immune reactions in brain microvascular endothelial cells remain unclear. Interferon (IFN)-induced transmembrane protein 1 (IFITM1) and Myxovirus resistance protein 1 (MX1), the members of IFN-stimulated genes, are known as antiviral molecules. IFITM1 inhibits virus entry into host cell cytoplasm, whereas MX1 antagonizes virus replication. Here we observed that IFITM1 and MX1, and a proinflammatory cytokine IL-6 expression was induced by polyinosinic-polycytidylic acid (poly IC) in hCMEC/D3 human brain microvascular endothelial cells. Poly IC-induced IFITM1 and MX1 expression were decreased by NF-κB inhibitor SN50, IFN regulatory factor 3 inhibitor MRT67307 and human type I IFN neutralizing antibody mixture. These findings suggest that IFITM1 and MX1 may help protect the brain from viruses.

1. Introduction

The blood-brain barrier (BBB) protects the central nervous system (CNS) from foreign pathogenic substances. Despite the presence of this barrier, the CNS is occasionally infected by neurotropic viruses that cause meningitis and encephalitis. Although these infections can cause significant neurological damage and death, the main treatment strategy for the patients with these diseases remains symptomatic therapy (Manglani and McGavern, 2018). Meningitis, encephalitis and related CNS diseases can be caused by numerous viruses. For example, herpes simplex viruses (Gnann Jr. and Whitley, 2017) can cause encephalitis, and human immunodeficiency virus type-1 induces HIV-associated neurocognitive disorders (Gougeon, 2017). In addition, autoimmune encephalitis such as anti-*N*-methyl-D-aspartate receptor encephalitis and febrile infection-related epilepsy syndrome can be triggered by viral infection in CNS (Venkatesan and Benavides, 2015). To develop more specific and effective treatments for these diseases, it is crucial to understand the CNS defense mechanisms against viral infections.

In the CNS parenchyma, microglia (Chen et al., 2019) and astrocytes (Soung and Klein, 2018) are the major resident cells that respond to viral infections. Brain microvascular endothelial cells are the principal components of the BBB, and comprise the first line of the defense against foreign pathogens in the blood stream. Therefore, brain microvascular endothelial cells are considered to play a crucial role in antiviral reactions in the CNS (Liu et al., 2019). However, the antiviral role of brain microvascular endothelial cells has not been completely elucidated.

The innate immune system is essential for host defense against infectious pathogens including viruses. Pattern recognition receptors recognize the pathogen-associated molecular patterns of the invading microbes and initiate innate immune reactions. Toll-like receptors (TLRs) are a family of pattern recognition receptors, of which TLR3 is the receptor for viral double-stranded RNA (dsRNA) that is generated during most viral infections (Zhang et al., 2013) and is expressed in human cerebral endothelial cells (Nagyoszi et al., 2010). Interactions between TLR3 and dsRNA trigger innate immune reactions against viral infection and induce the expression of downstream genes including type I interferons (IFNs). Type I IFNs are the key cytokines in the innate immune reactions and induce the synthesis of a number of genes, designated as IFN-stimulated genes (ISGs). ISGs include various molecules such as cytokines, adhesion molecules, enzymes, receptors, transcriptional factors, and signaling molecules. Together, these ISGs form a

Abbreviations: BBB, Blood-brain barrier; CNS, Central nervous system; ELISA, Enzyme-linked immunosorbent assay; HIV, Human immunodeficiency virus; IFN, Interferon; PCR, Polymerase chain reaction; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; Poly IC, Polyinosinic-polycytidylic acid

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network of antiviral immune reactions that can inhibit various stages of the viral life cycle (Shives et al., 2017).

IFN-induced transmembrane protein 1 (IFITM1) (Deblandre et al., 1995) and Myxovirus resistance protein 1 (MX1) (Horisberger et al., 1983; Staeheli and Haller, 1985) are ISGs. IFITM1 is a cell surface protein that can function as an antiviral protein by inhibiting virus entry into host cell cytoplasm (Brass et al., 2009). MX1, also named as MXA, encodes a dynamin like guanosine triphosphatase and exerts its antiviral function by antagonizing viral replications (Haller et al., 2015). In cerebral white matter obtained from patients with human immunodeficiency virus infection during autopsy, both of IFITM1 and MX1 mRNA trended toward higher expression compared with that in controls (Solomon et al., 2017). IFITM1 is induced by H9N2 virus in human umbilical vein endothelial cells (Feng et al., 2017). IFNs induce MX1 expression in human uterine microvascular endothelial cells (Kitaya et al., 2007) and human pulmonary arterial endothelial cells (Yuan and Sehgal, 2016). Considering these data, it is suggested that IFITM1 and MX1 may play important roles in antiviral activity of vascular endothelial cells. Because brain microvascular endothelial cells are components of BBB, they are considered as a specific type of endothelial cell. Therefore, examining IFITM1 and MX1 expression in human brain microvascular endothelial cells in detail is important.

Polyinosinic-polycytidylic acid (poly IC) is an authentic analogue of viral dsRNA and is a ligand for TLR3. The activation of TLR3 signaling by poly IC induces the expression of downstream genes as well as initiates antiviral innate immune reactions.

Poly IC has been shown to induce interleukin-6 (IL-6) expression via TLR3 in primary culture of human brain vascular endothelial cells (Bhargavan and Kanmogne, 2018). Moreover, poly IC induces CCL5, IFN-β, CXCL10, retinoic acid-inducible gene-I, melanoma differentiation-associated gene 5, ISG60 via TLR3 in hCMEC/D3 cells (Arai et al., 2017; Imaizumi et al., 2018a,b), a cell line derived from human cerebral microvascular endothelial cells (Weksler et al., 2013). However, the role of TLR3 signaling in IFITM1 expression remains unclear. In addition, the effect of poly IC on MX1 expression in human brain microvascular endothelial cells remains unknown.

In the present study, we examined the effect of poly IC on IFITM1 and MX1 expression in cultured hCMEC/D3 cells. Furthermore, we investigated the roles of nuclear factor (NF)-κB, IFN regulatory factor (IRF) 3 and IFN-β in the induction of IFITM1 and MX1 by poly IC. Finally, we compared the expression patterns of IFITM1 and MX1 with that of IL-6.

2. Methods

2.1. Reagents

Poly IC, an IRF3 inhibitor MRT67307 and anti-actin rabbit antibody were purchased from Sigma (St. Louis, MO, USA). M-MLV reverse transcriptase was obtained from Thermo Fisher Scientific (Asheville, NC, USA). Recombinant human (r(h)) IFN-β was purchased from ProSpec (Rehovot, Israel). The NF-κB translocation inhibitor SN50 was purchased from ENZO Life Sciences (Farmingdale, NY, USA). The illustra RNA spin kit was obtained from GE Healthcare (Buckinghamshire, UK). Oligo $(dT)_{18}$ and oligonucleotide primers for polymerase chain reaction (PCR) were custom synthesized by Fasmac (Atsugi, Japan). Sso-advanced Universal SYBR Green Supermix was obtained from Bio-Rad (Hercules, CA, USA). The anti-MX1 and anti-IFITM1 rabbit polyclonal antibodies were purchased from GeneTex (Irvine, CA, USA). The human type I IFN neutralizing antibody mixture (mixture of monoclonal and polyclonal antibodies directed against human type I IFNs and type I IFN receptor subunit 2) and human IFN-β enzyme-linked immunosorbent assay (ELISA) kit were from PBL assay science (Piscataway, NJ, USA). Immobilon Crescendo western HRP substrate was from Millipore (Billerica, MA). The ELISA kit for human IL-6 was purchased from Proteintech (Rosemont, IL, USA).

2.2. Cell culture, RNA interference and inhibitors

The hCMEC/D3 cells were purchased from Merk Millipore (Temecula, CA, USA), and the cells at passage 4–8 were used in the experiments. Human umbilical vein endothelial cells (HUVEC) and endothelial cell growth medium-2 (EGM-2) were from Lonza (Walkersville, MD, USA). hCMEC/D3 cells and HUVEC were cultured in EGM-2. The cells were treated with 0.4–50 μg/mL poly IC for up to 24 h. In the experiments using inhibitors, the cells were pretreated with 10 μM SN50 or 1 μM MR67307 for 1 h before the addition of 30 μg/mL poly IC. In the neutralizing experiments, the cells were pretreated with a human type I IFN neutralizing antibody mixture (1:20 dilution) for 1 h before the addition of poly IC. Moreover, the cells were treated with 1 ng/mL r(h) IFN-β for 16 h.

2.3. RNA extraction and quantitative real-time reverse transcription (RT)-PCR

Total RNA was extracted from the cells after incubation, and purified using the illustra RNA spin kit. cDNA was generated from RNA using oligo $(dT)_{18}$ primer and M-MLV reverse transcriptase. The cDNA for IFITM1, MX1, IL-6, IFN-β, claudin-5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified via 40 cycles using Sso-advanced Universal SYBR Green Supermix; thereafter, the amplifications was analyzed by a CFX96 real-time PCR detection system (Bio-Rad). Data for IFITM1, MX1, IL-6 and claudin-5 mRNA were shown as fold increases compared with unstimulated cells. Because IFN-β mRNA was not detectable in unstimulated cells, the data for IFN-β are shown as arbitrary units. The sequences of primers were as follows.

IFITM1-F: 5′-TCGCCTACTCCGTGAAGTCTA-3′,
IFITM1-R: 5′- TGTCACAGAGCCGAATACCAG-3′,
MX1-F: 5′- GCCAGGACCAGGTATACAG -3′,
MX1-R: 5′- GCCTGCGTCAGCCGTGC -3′,
IL-6-F: 5′- ATGAACTCCTTCTCCACAAGC -3′,
IL-6-R: 5′- AAGAGCCCTCAGGCTGGACTG -3′,
IFN-β-F: 5′- CCTGTGGCAATTGAATGGGAGGC -3′,
IFN-β-R: 5′- CCAGGCACAGTGACTGTACTCCTT −3′,
Claudin-5-F: 5′-ACCGGCGACTACGACAAGAAGA-3′,
Claudin-5-R: 5′-GCCCTGCCGATGGAGTAAAGA-3′,
GAPDH-F: 5′- GCACCGTCAAGGCTGAGAAC -3′,
and GAPDH-R: 5′- ATGGTGGTGAAGACGCCAGT −3′.

2.4. Western blotting

Cells were lysed after incubation using Laemmli's sample buffer and subjected to 10–20% polyacrylamide gel electrophoresis. Thereafter, proteins were transferred to a polyvinylidene difluoride membrane that was probed with anti-IFITM1 antibody (1:2500), anti-MX1 antibody (1:2000), or anti-actin antibody (1:3000) for 16 h at 4 °C. The membrane was subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody, and the bands were detected using Immobilon Crescendo western HRP substrate. Integrated optical density of the band was semi-quantified using ImageJ 1.49i software (National Institute of Health, Bethesda, MD, USA).

2.5. ELISA

The concentration of IL-6 and IFN-β proteins in the culture medium were measured using commercially available ELISA kits, according to the manufacturers' protocols.

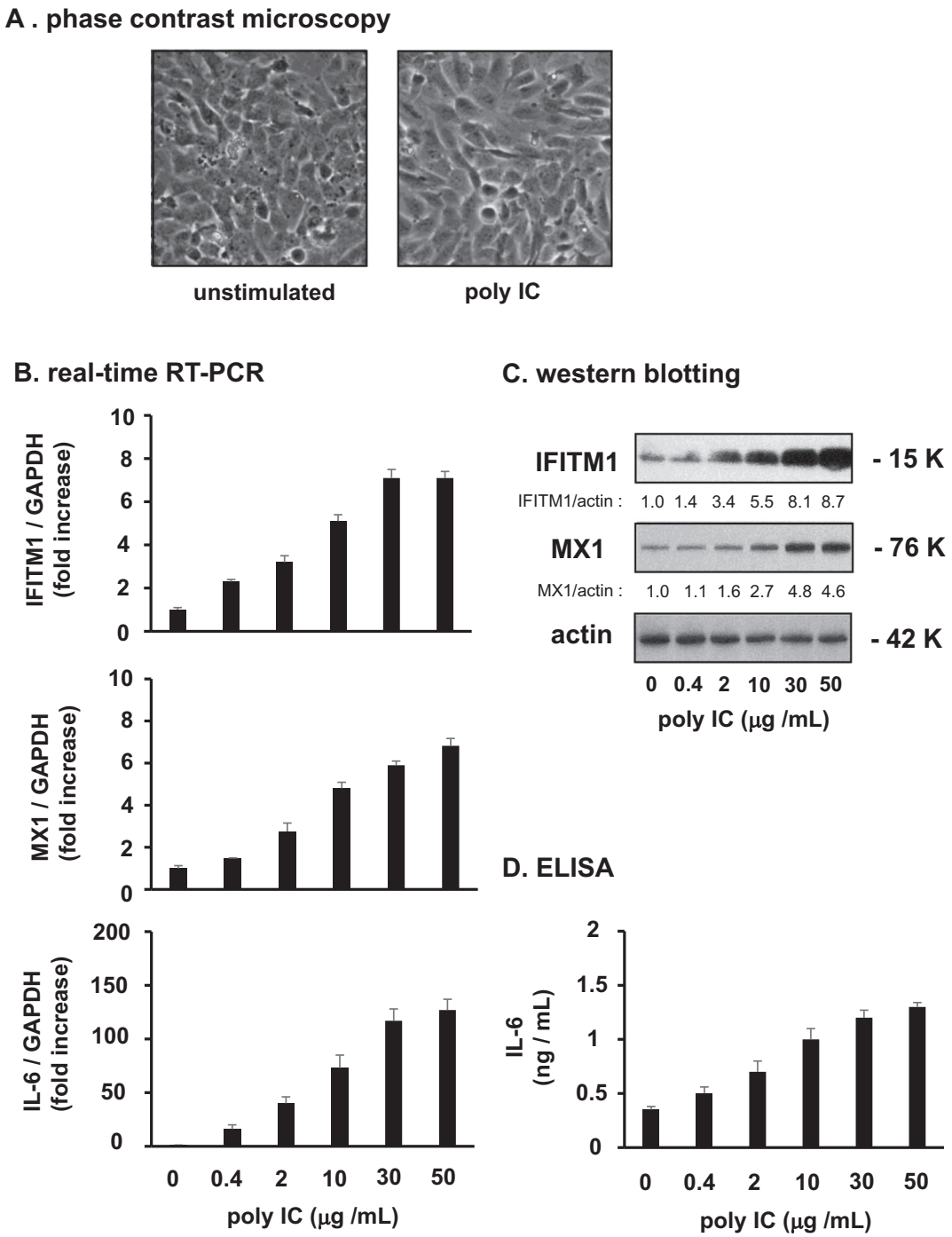

Fig. 1. Treatment of hCMEC/D3 cells with poly IC induced IFITM1, MX1 and IL-6 expression in a concentration-dependent manner. (A) The hCMEC/D3 cells were cultured and stimulated with 30 μg/mL poly IC for 16 h, and were observed using phase contrast microscopy. (B) The cells were cultured and treated with 0.4–50 μg/mL poly IC for 2 h (for the analysis of IL-6 mRNA) or 16 h (for the analysis of IFITM1 and MX1 mRNA). RNA was extracted from the cells after the incubation, and reverse-transcribed to cDNA using oligo(dT)$_{18}$ and M-MLV reverse transcriptase. The cDNA was used as a template for quantitative real-time RT-PCR analysis for IFITM1,MX1, IL-6 and GAPDH expression. (C) Cells were treated with poly IC as in (B), incubated for 16 h, and then lysed using Laemmli's reducing sample buffer. Lysates were subjected to western blot analysis for IFITM1, MX1 and actin proteins. The band of the proteins was semi-quantitated using ImageJ 1.49i. (D) Cells were treated with poly IC as in (B) and incubated for 24 h. The culture medium was collected and subjected to a specific ELISA for IL-6. Data in (B) and (D) are shown as mean +/− SD (n = 3).

3. Results

3.1. Treatment of hCMEC/D3 cells with poly IC induces IFITM1, MX1 and IL-6 expression

The cultured hCMEC/D3 cells showed a cobblestone appearance at 100% confluence, and significant morphological changes were not observed in cells treated with poly IC (Fig. 1A). The IFITM1, MX1 and IL-6 mRNA expression levels were low in unstimulated hCMEC/D3 cells. The poly IC treatment upregulated the expression of these molecules in a concentration-dependent manner (Fig. 1B). Moreover, IFITM1 and MX1 protein levels in the cell lysate (Fig. 1C) and IL-6 protein level in

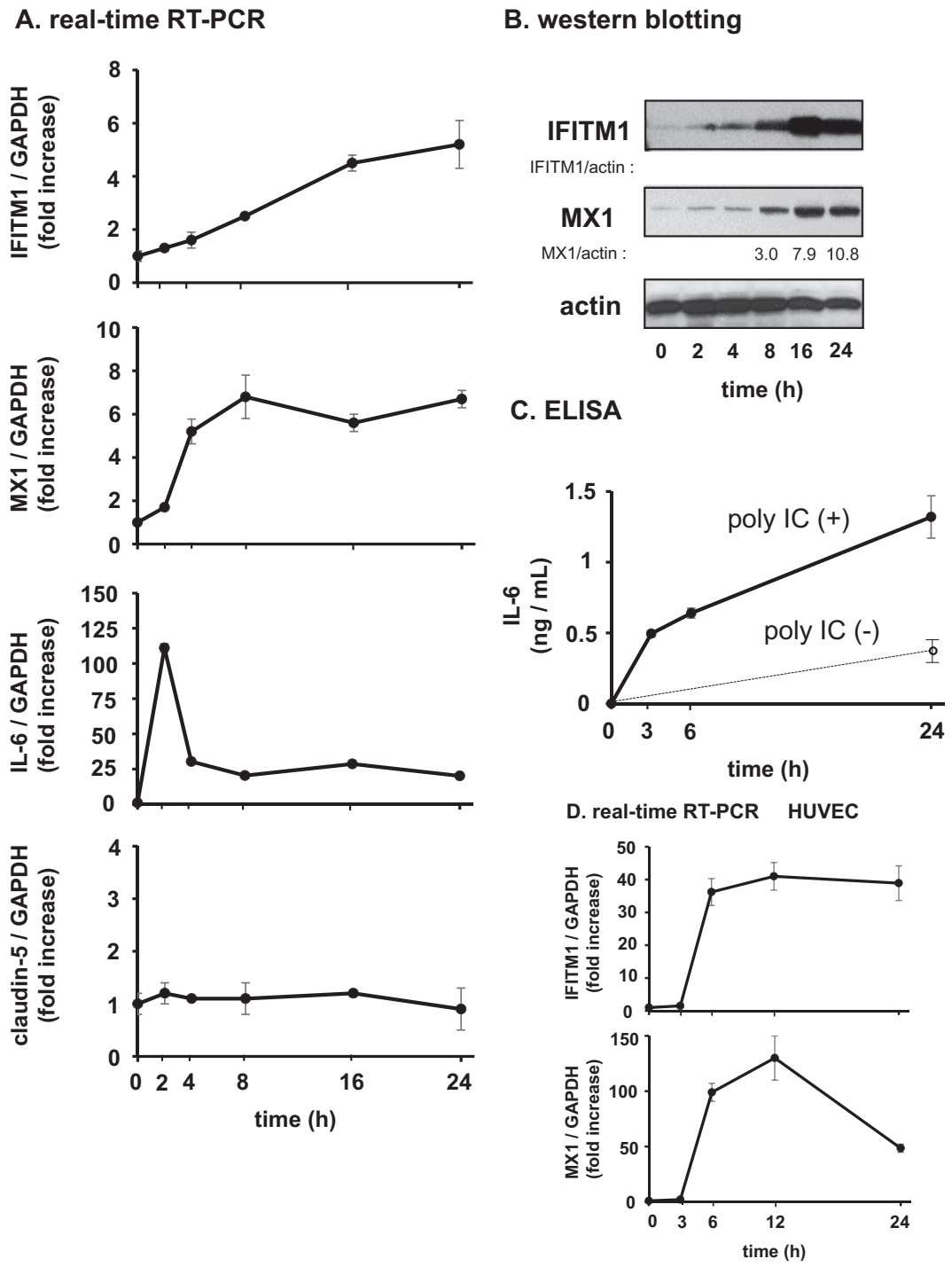

Fig. 2. Poly IC induced IFITM1, MX1 and IL-6 expression in hCMEC/D3 cells in a time-dependent manner. hCMEC/D3 cells were treated with 30 μg/mL poly IC for up to 24 h. (A) RNA was extracted from the cells and subjected to quantitative real-time RT-PCR for IFITM1, MX1, IL-6 and claudin-5. (B) Cells were lysed and western blotting for MX1, IFITM1, and actin was performed. (C) The culture medium was collected and the concentration of IL-6 was measured using ELISA. (D) Cultured human umbilical vein endothelial cells (HUVEC) were treated with 30 μg/mL poly IC for up to 24 h. Quantitative real-time RT-PCR for IFITM1 and MX1 were performed. Data in (A), (C) and (D) are shown as mean +/− SD ($n = 3$).

the culture medium (Fig. 1D) increased with poly IC treatment in a concentration-dependent manner. Poly IC induced the mRNA expression for IFITM1, MX1, and IL-6 in a time-dependent manner (Fig. 2A). However, time course of expression differed among these molecules. The IFITM1 mRNA expression gradually increased until 24 h, whereas MX1 mRNA significantly increased 4 h after poly IC treatment, reached the maximum expression after 8 h, and then nearly plateaued until 24 h. In contrast, IL-6 mRNA expression was rapidly induced, reached maximum level after 2 h, and decreased thereafter. Substantial amount of claudin-5 mRNA was detected in cells without poly IC treatment, and the expression level of claudin-5 mRNA was not changed by poly IC treatment. IFITM1 protein expression was almost similar to the IFITM1 mRNA expression, while MX1 protein expression lagged behind the MX1 mRNA expression and reached maximal level from 16 to 24 h (Fig. 2B). In unstimulated cells, a small amount of IL-6 protein accumulated in the culture medium for up to 24 h, whereas in poly IC-

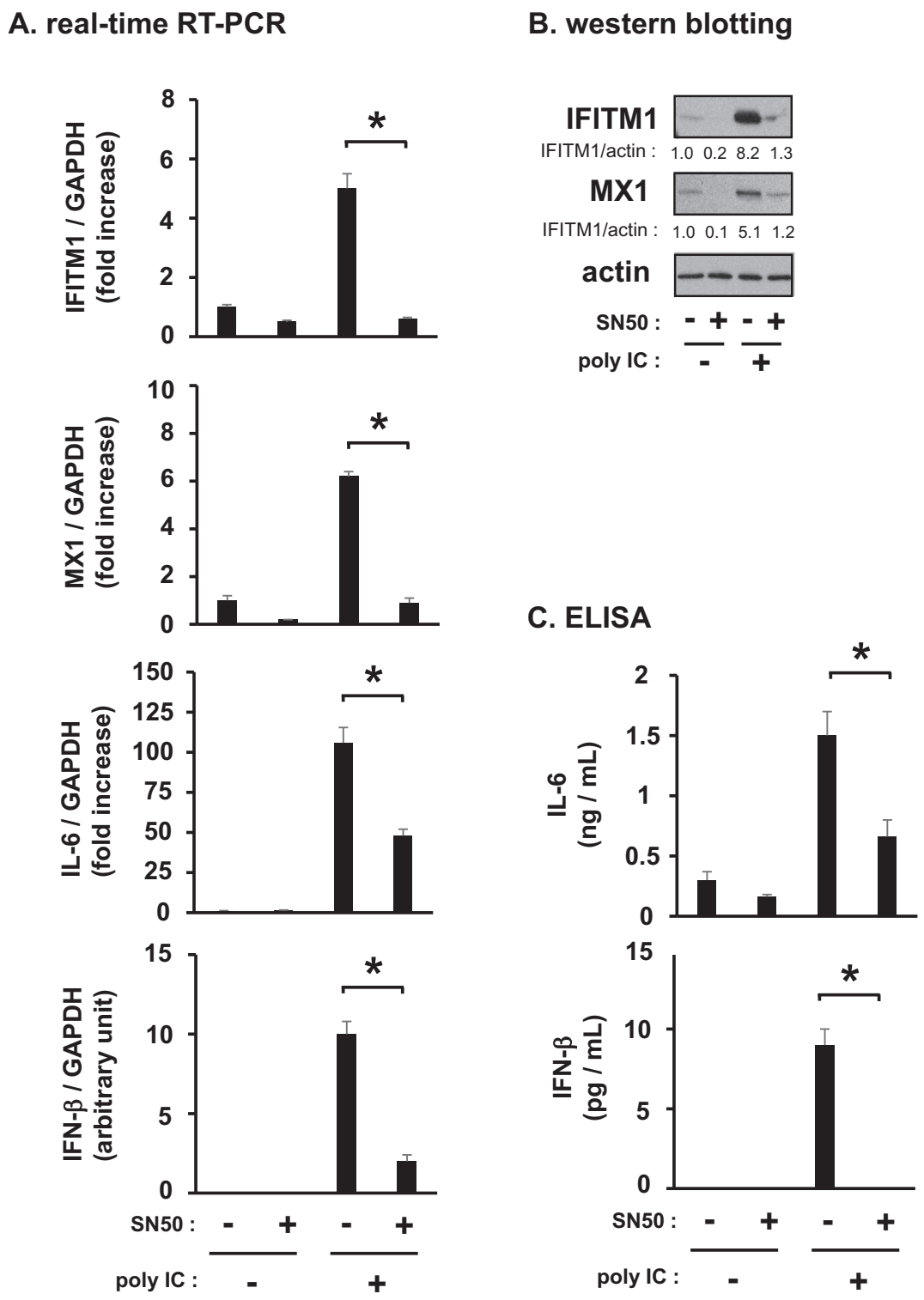

Fig. 3. NF-κB is involved in poly IC-induced IFITM1, MX1, IL-6 and IFN-β expression. (A) hCMEC/D3 cells were pretreated with 10 μM SN50, a specific inhibitor of NF-κB translocation into the nucleus, for 1 h. Subsequently, the cells were treated with 30 μg/mL poly IC for 2 h (for the analysis of IL-6 and IFN-β mRNAs) or 16 h (for the analysis of MX1 and IFITM1 mRNAs). RNA was extracted from the cells and quantitative real-time RT-PCR was performed. (B) The cells were pretreated with SN50 as described in (A) and subsequently treated with poly IC for 16 h. The cell lysate was subjected to western blotting for IFITM1, MX1 and actin. (C) Following the pretreatment with SN50, the cells were treated with poly IC as described in (B). After 24 h incubation, the medium was collected and IL-6 and IFN-β protein concentrations were measured using specific ELISAs. Data in (A) and (C) are shown as mean +/− SD ($n = 3$; $^*p < .01$, by t-test).

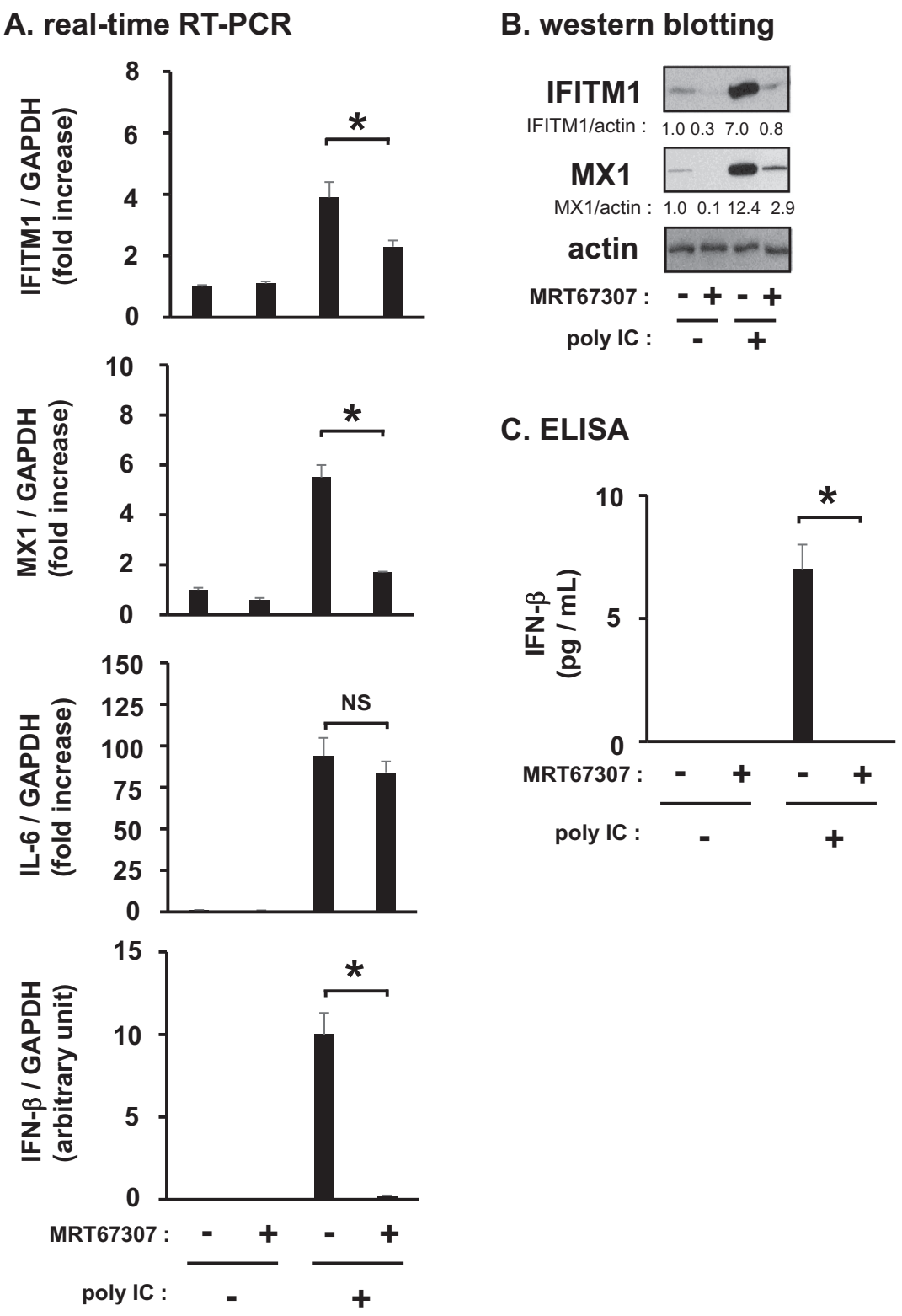

Fig. 4. MRT67307 inhibited poly IC-induced IFITM1, MX1 and IFN-β expression but not IL-6 expression. (A) Cells were pretreated with 1 μM MRT67307 for 1 h followed by treatment with 30 μg/mL poly IC. After further incubation for 2 h (for analysis of IL-6 and IFN-β mRNAs) or 16 h (for analysis of IFITM1 and MX1 mRNAs), RNA was extracted. The extracted RNA was subjected to quantitative real-time RT-PCR analysis for MX1, IFITM1, IL-6 and IFN-β mRNA expression. (B) Cells were pretreated with MRT67307 as described in (A), and then treated with poly IC. After further incubation for 16 h, cells were lysed. Western blotting for IFITM1, MX1, and actin was performed. (C) Cells were pretreated with MRT67307 followed by treatment with poly IC as described above. After further incubation for 24 h, the medium was collected and subjected to an ELISA for IFN-β. Data in (A) and (C) are shown as mean +/− SD (n = 3; *$p < .01$, NS: not significant, by *t*-test).

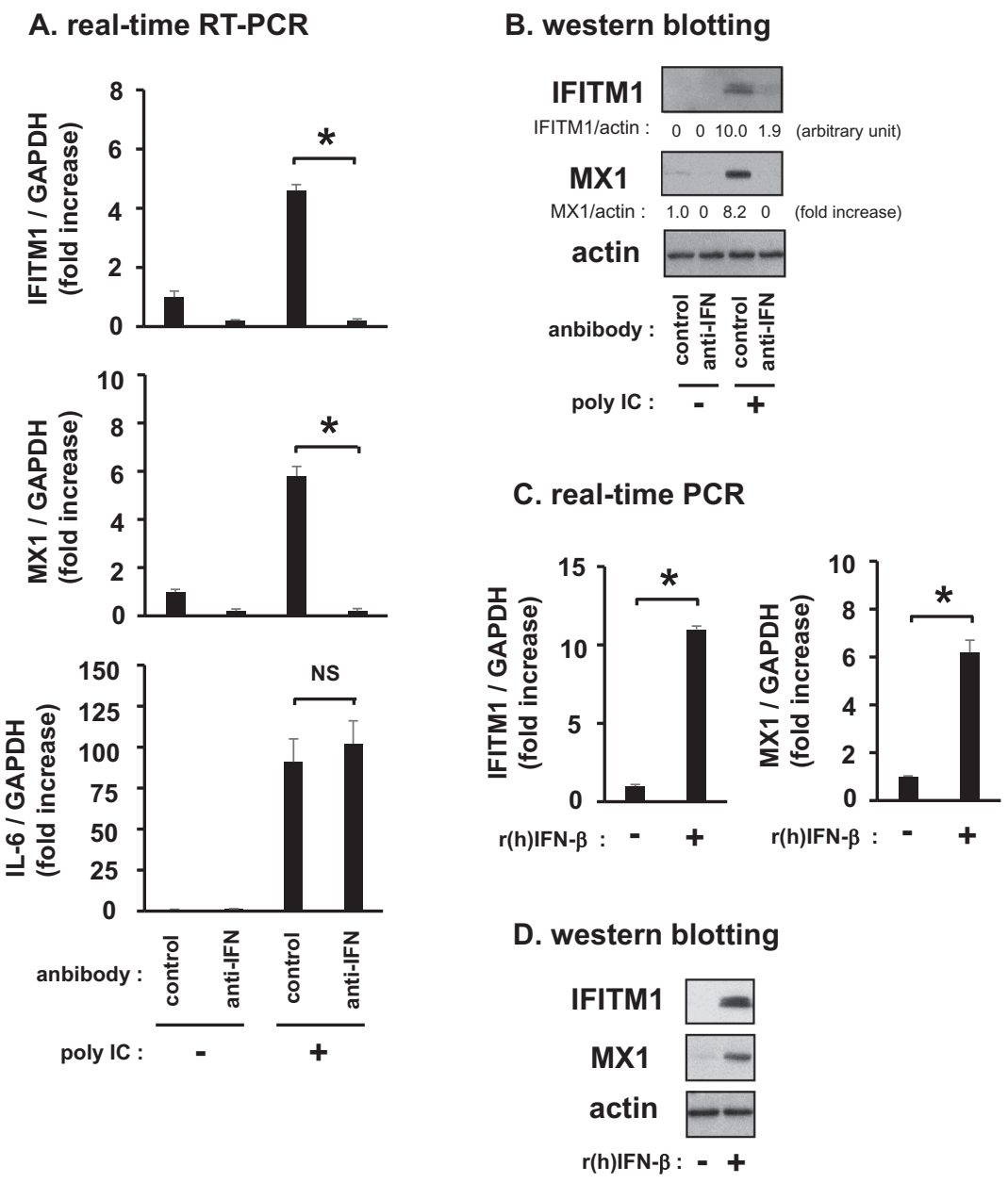

Fig. 5. Type I IFN is involved in poly IC-induced IFITM1 and MX1 expression but not IL-6 expression. (A) Cells were preincubated for 1 h with human type I IFN neutralizing antibody mixture (1:20 dilution), followed by treatment with 30 μg/mL poly IC for 2 h (for analysis of IL-6 mRNA) or 16 h (for analysis of IFITM1 and MX1 mRNAs). RNA was extracted from the cells, and quantitative real-time RT-PCR was performed. (B) Cells were preincubated for 1 h with human type I IFN neutralizing antibody mixture and subsequently treated with 30 μg/mL poly IC for 16 h. Cells were lysed and subjected to western blotting for IFITM1, MX1 and actin. (C) Cells were treated with 1 μg/mL r(h)IFN-β for 16 h. IFITM1 and MX1 mRNA expression was then examined using real-time RT-PCR. (D) Cells were treated with 1 ng/mL r(h)IFN-β for 16 h, and western blotting was performed as in (B). Data in (A) and (C) are shown as mean +/− SD (n = 3; *$p < .01$, NS: not significant, by *t*-test).

treated cells, the secretion of IL-6 protein into the medium markedly increased in a time-dependent manner (Fig. 2C). Poly IC also induced the expression of IFITM1 and MX1 mRNA in HUVEC (Fig. 2D).

3.2. NF-κB is involved in poly IC-induced expression of IFITM1, MX1, IFN-β and IL-6

When cells were pretreated with SN50, an inhibitor of NF-κB translocation into the nucleus, the induction of IFITM1, MX1, IL-6 and IFN-β mRNA was significantly inhibited (Fig. 3A). Moreover, the poly IC-induced IFITM1, MX1 (Fig. 3B), IL-6 and IFN-β (Fig. 3C) protein expression decreased with SN50 treatment.

3.3. MRT67307 inhibits poly IC-induced IFITM1, MX1 and IFN-β expression, but not of IL-6 expression

Pretreatment of cells with MRT67307, an IRF3 inhibitor, inhibited poly IC-induced IFITM1, MX1 and IFN-β mRNA expression but did not affect poly IC-induced IL-6 mRNA expression (Fig. 4A). Moreover, poly IC-induced IFITM1, MX1 (Fig. 4B) and IFN-β (Fig. 4C) protein expression decreased with MRT67307 pretreatment of cells.

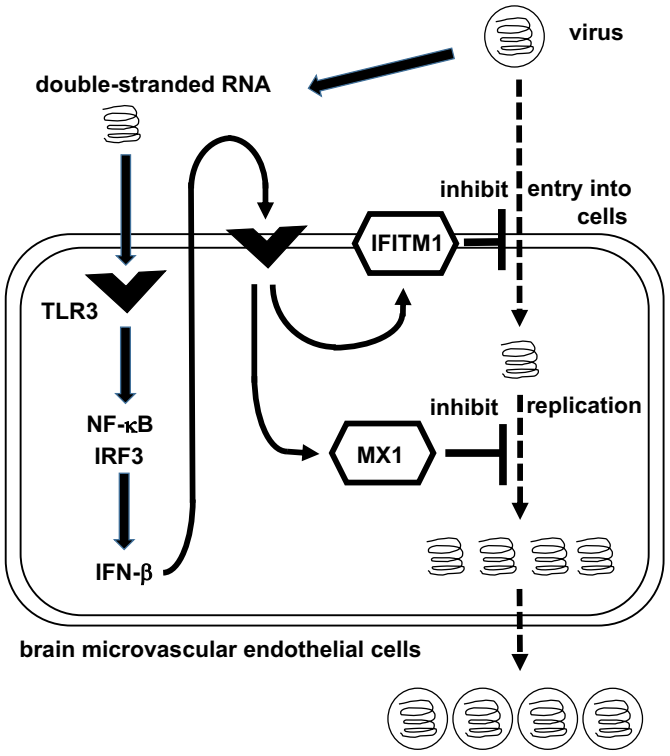

Fig. 6. Supposed role of IFITM1 and MX1 in antiviral responses in brain microvascular endothelial cells. TLR3 signaling induces IFITM1 and MX1 expression via NF-κB, IRF3 and IFN-β. Increased IFITM1 and MX1 protein may contribute to preventing the entry and replication of invading viruses in brain microvascular endothelial cells.

3.4. IFN-β is involved in poly IC-induced IFITM1 and MX1 expression but not in IL-6 expression

Pretreatment of cells with human type I IFN neutralizing antibody mixture decreased both IFITM1 and MX1 mRNA (Fig. 5A) and protein (Fig. 5B) expression in poly IC-treated cells but showed no effect on IL-6 mRNA expression (Fig. 5A). Treatment of cells with r(h)IFN-β induced IFITM1 and MX1 mRNA (Fig. 5C) and protein (Fig. 5D) expressions.

4. Discussion

In BBB, brain microvascular endothelial cells form paracellular and transcellular barrier (Knowland et al., 2014). Tight junction is the essential structure for paracellular barrier, and several proteins including claudin-5 contribute to the structure of tight junction. In the present study, expression of claudin-5 mRNA was not changed by poly IC treatment in hCMEC/D3 cells. It was reported that paracellular adhesion of hCMEC/D3 cells was reduced for a short period by poly IC treatment, and then it was markedly increased for up to 100 h (Johnson et al., 2018). On the other hand, molecular mechanisms of transcellular barrier against viruses in brain microvascular endothelial cells are not known well.

During the early stages of infection, the invading viruses enters host cell cytoplasm. The viruses then replicates within the host cells and is ultimately released from the cells. However, host cells have various antiviral factors which restrict these steps of the viral lifecycle. IFITM1 and MX1 are some of these antiviral factors: IFITM1 inhibits the virus entry into the cytoplasm (Li et al., 2015), whereas MX1 restricts viral replication (Haller et al., 2015). However, the expression and roles of these molecules in brain microvascular endothelial cells remain unclear.

In the present study, we examined IFITM1 and MX1 expression in hCMEC/D3 cells. First we observed that poly IC treatment induced IFITM1 and MX1, as well as IL-6, in a concentration-dependent manner. This result suggests that increased IFITM1 and MX1 proteins may function to prevent virus entry into the cytoplasm and viral replication in human brain microvascular endothelial cells. Brain microvascular endothelial cells, being a structural component of BBB, may at as a physical barrier as well as an immunologically functional barrier against invading viruses, thereby playing a pivotal role in protecting the brain from viral infections.

We observed that IFITM1, MX1, and IL-6 mRNA expression was induced by poly IC in different time courses. This suggests that induction of these molecules by poly IC is differentially regulated. Because NF-κB and IRF3 are major transcriptional factors in the downstream of TLR3 signaling (Takeda, 2005), we next examined whether NF-κB and IRF3 are involved in the induction of IFITM1 and MX1 expression by poly IC. Pretreatment of cells with either the NF-κB inhibitor SN50 or the IRF3 inhibitor MRT67307 inhibited the poly IC-induced expression of IFITM1 and MX1 expression. Moreover, these inhibitors inhibited the induction of IFN-β, a key type I IFN in antiviral innate immune reactions in this cell type (Imaizumi et al., 2018a). These results suggest that NF-κB and IRF3 are involved in poly IC-induced IFITM1, MX1, and IFN-β expression. On the other hand, poly IC-induced IL-6 expression was inhibited by SN50 but not by MRT67307.

Type I IFN is produced by virus-infected cells and promotes the production of ISGs which modulate antiviral immune reactions (Le Page et al., 2000). In a previous study, we observed that IFN-β is rapidly induced and involved in the following expression of ISGs including CXCL10, melanoma differentiation-associated gene 5, retinoic acid-inducible gene-I (Imaizumi et al., 2018a) and ISG60 (Imaizumi et al., 2018b) in poly IC-treated hCMEC/D3 cells. Because IFITM1 and MX1 are known ISGs, we examined if poly IC induces IFITM1 and MX1 expression in an IFN-β-dependent manner. We observed that the pretreatment of cells with human type I IFN neutralizing antibody mixture inhibited poly IC-induced IFITM1 and MX1 expression; however, poly IC-induced IL-6 expression remained unaffected. In addition, the IFITM1 and MX1 expression was induced by r(h)IFN-β. Taken together, these findings indicate that NF-κB, IRF3 and newly synthesized IFN-β are involved in poly IC-induced IFITM1 and MX1 in hCMEC/D3 cells. In the present study, we were unable to clarify the mechanisms via which the time courses of IFITM1 and MX1 mRNA expression were differentially regulated. On the other hand, we successfully demonstrated that IL-6 was induced by poly IC via NF-κB and not by IRF3 and IFN-β. These results agree with the rapid poly IC-induced IL-6 mRNA expression.

In summary, poly IC induces IFITM1 and MX1 expression in hCMEC/D3 cells. NF-κB, IRF3 and IFN-β are all involved in this reaction. Increased IFITM1 and MX1 protein levels in brain microvascular endothelial cells may contribute, at least in part, to preventing the entry and replication of invading viruses (Fig. 6). These molecules may play important roles in preventing the passage of viruses to pass via the BBB, thereby protecting the brain from viral infection.

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