Type I interferon protects neurons from prions in vivo models

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Infectious prions comprising abnormal prion protein, which is produced by structural conversion of normal prion protein, are responsible for transmissible spongiform encephalopathies including Creutzfeldt-Jakob disease in humans. Prions are infectious agents that do not possess a genome and the pathogenic protein was not thought to evoke any immune response. Although we previously reported that interferon regulatory factor 3 (IRF3) was likely to be involved in the pathogenesis of prion diseases, suggesting the protective role of host innate immune responses mediated by IRF3 signalling, this remained to be clarified. Here, we investigated the reciprocal interactions of type I interferon evoked by IRF3 activation and prion infection and found that infecting prions cause the suppression of endogenous interferon expression. Conversely, treatment with recombinant interferons in an ex vivo model was able to inhibit prion infection. In addition, cells and mice deficient in type I interferon receptor (subunit interferon alpha/beta receptor 1), exhibited higher susceptibility to 22L-prion infection. Moreover, in vivo and ex vivo prion-infected models, treatment with RO8191, a selective type I interferon receptor agonist, inhibited prion invasion and prolonged the survival period of infected mice. Taken together, these data indicated that the interferon signalling interferes with prion propagation and some interferon-stimulated genes might play protective roles in the brain. These findings may allow for the development of new strategies to combat fatal diseases.

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Keywords: prion infection; type I interferon (I-IFN); innate immune system.
Abbreviations: I-IFN = type I interferon; IRF = interferon regulatory factor; MEF = mouse embryonic fibroblast; TLR = Toll-like receptor

Introduction

Human prion diseases, including Creutzfeldt-Jakob disease, are transmissible neurodegenerative disorders for which no effective treatment is currently available. Prions are proteinaceous infectious pathogens distinct from bacteria or viruses. Accumulation of the structurally abnormal prion protein (PrPSc) causes pathological changes such as
neuronal death in the brain, resulting in rapidly progressive cognitive disorders. PrPSc, which is highly enriched in β-sheet secondary structures, causes misfolding of normal cellular PrP (PrPC) (Prusiner, 1998; Weissmann et al., 2002). In a widely-accepted model of the molecular mechanism of prion pathogenesis, following the expression or introduction of PrPSc, the pathogenic proteins accumulate in and on neurons; subsequently, various inflammatory responses occur and induce neuronal death (Tamgune et al., 2008; Aguzzi et al., 2013). One pathological feature associated with prion infection is widespread diffuse gliosis. However, the host-pathogen interaction in these diseases remains incompletely understood.

Viral and bacterial pathogens induce various immunological responses in the host to eliminate foreign pathogens from the body. Because the amino acid sequence of PrPSc is identical to that of PrPC, which is encoded by a host gene, the host immune system was initially thought not to recognize the prion pathogen (Aguzzi and Polymenidou, 2004). However, accumulated evidence has shown that prion infection stimulates pattern recognition receptor (PRR)-related molecules and related signalling pathways, including Toll-like receptors (TLRs), interferon regulatory factors (IRFs), and some cytokines (Prinz et al., 2003; Spinner et al., 2008; Bradford and Mabbott, 2012; Ishibashi et al., 2012a; Nuvolone et al., 2013; Kang et al., 2016). We have also reported that IRF3, which upregulates type I interferon (IFN) in various cell types, including neurons, plays a role in host defence against prion infection (Ishibashi et al., 2012a, b), and that persistent prion infection negatively regulates IRF3 via suppression of the transcription factor Octamer-binding protein-1 (Oct-1) (Homma et al., 2014a). Prions also exhibit strain diversity and reciprocal interference between strains, analogous to viral infections (Dickinson et al., 1972, 1975; Manuelidis, 1998). In addition, the levels of interferon-stimulated genes with anti-viral functions, including myxovirus resistance protein, protein kinase R, and 2′-5′ oligoadenylate synthetase, are significantly elevated at the clinical stage of prion disease in animal models (Riemer et al., 2000; Baker et al., 2004; Xiang et al., 2004; Stobart et al., 2007), suggesting that innate immunity protects the host, at least partially, against prion infection. In this study, we focused on the relationship between I-IFN and prion disease and sought to determine which interferon-stimulated gene induced by I-IFN signalling plays a direct protective role against prion invasion.

Materials and methods

Ethics statement

All animal experiments were conducted with approval from Nagasaki University Institutional Animal Care and Use Committee (IACUC, approval No. 150371202) and the Safety Committee for Recombinant DNA Experiments (approval No. 1503311317). The animals were cared for following the Nagasaki University Guidelines for Animal Experimentation.

Animals

C57BL/6 J mice were purchased from SLC Japan. Tga20 mice overexpressing normal PrPC were provided by Prof. M. Horisuchi, Hokkaido University, Japan (Fischer et al., 1996). Interferon alpha/beta receptor 1 (Ifnar1) knockout mice were provided by Prof. T. Taniguchi and Dr K. Honda (Current affiliation: Keio University, Professor), The University of Tokyo, Japan (Muller et al., 1994). All animals were maintained in a pathogen-free environment, at a temperature of 22 ± 2°C and humidity of 40 to 70%; food and autoclaved water were available ad libitum. Mice were periodically inspected for hantavirus, lymphocytic choriomeningitis virus, Sendai virus, parainfluenza virus type 3, pneumonia virus of mice, rat coronavirus, Mycoplasma pulmonis, and Clostridium piliforme, and results were consistently negative.

Antibodies and Reagents

Polyinosinic-polycytidylic acid (Poly I:C, Invivogen), Pentosan polysulphate (PPS, Sigma-Aldrich) and RO4948191, which is an imidazonaphthyridine with the structural formula 8-(1, 3, 4-oxadiazol-2-yl)-2, 4-bis (trifluoromethyl) imidazo [1, 2-a] [1, 8] naphthyridine (BIONET: RO8191), were purchased from the indicated vendors. Recombinant mouse IFN-α and β were purchased from Calbiochem (Merck). The mouse monoclonal antibody 359, recognizing the helix 1 region of PrP, was a kind gift of Prof. Matsuda, Hiroshima University (Miyamoto et al., 2005). The anti-PrP polyclonal mouse antiserum (SS), M20 (Santa Cruz Biotechnology), and the monoclonal (SAF32 and SAF83, SPL-Bio) antibodies have been described previously (Atarashi et al., 2006; Ishibashi et al., 2007, 2011; Homma et al., 2014b). Anti-IFNAR1 (MAR1–5A3, BioLegend), anti-IFN-β (7F-D3, Santa Cruz Biotechnology), anti-ionized calcium binding adaptor molecule 1 (Iba-1; Wako Pure Chemical Industries, Ltd), anti-glial fibrillary acidic protein (GFAP; Dako), anti-murine 2′-5′ oligoadenylate synthetase (OAS1a; C-5, Santa Cruz Biotechnology), anti-HA (Invitrogen), and β-actin (Sigma) mouse, rat, and rabbit monoclonal antibodies, and were purchased from commercial sources. The horseradish peroxidase-conjugated anti-goat (Jackson ImmunoResearch Laboratories), anti-rat (Cell Signaling Technology, Inc.: CST), anti-mouse, and anti-rabbit (GE healthcare Life Sciences) IgG antibodies were used for immunoblotting.

Plasmid constructions

The subcloning primers used in this study are summarized in Supplementary Table 1. The pCNA3.1 plasmids coding murine IFN-β (Ifnb) (NCBI accession number: NM_010510.1) were constructed by inserting the open reading frame (ORF) region of each gene amplified by nested polymerase chain reaction (PCR) protocol and digested by arbitrary restriction enzymes. The murine stem cell virus (MSCV) retroviral expression vectors, pMSCV (Clontech Laboratories) plasmids, coding murine IFNAR1 (NCBI accession number: M89641.1) and Large T antigen from Simian virus 40 (NCBI accession number: NC_001669) were constructed by inserting the ORF region of each gene amplified by PCR.
using the primers listed in Supplementary Table 1 and digested by arbitrary restriction enzymes, respectively. Lentiviral self-inactivating (SIN) vectors were kindly provided by Dr Miyoshi of the RIKEN BioResource Center (BRC), Ibaraki, Japan. CSII-CMV-MCS-IRESE2-Venus plasmid coding murine IFN-β were constructed by inserting the ORF region digested by NheI and NotI from pcDNA3.1-IFN-β-venus. Experiments using plasmids inserted with genes coding for the fluorescence protein ‘Venus’, were performed by Dr Miyawaki of the RIKEN Brain Science Institute. To manufacture an insertion region for pUNO-interferon-stimulated response element (ISRE)-luc, the mammalian expression pUNO vector (Invivogen), which contains a strong and ubiquitous composite promoter designated EF1α/HTLV, was used. The PCR products were amplified from pTAL-luc (Promega) with specific primers for a single-round PCR or with two-round PCR. The PCR products were inserted into the pCR-TOPO vector (Invitrogen) and digested by XhoI and NheI after they were confirmed by sequencing. Finally, the pUNO-ISRE-luc plasmid was constructed by inserting the purified product into the ORF region of the pUNO vector.

**Retroviral vectors using lentiviral MSCV virus-based system**

To prepare the MSCV virus, RetroPack PT67 (Clontech) cells, containing the Moloney murine leukemia virus (MoMuLV) gag, pol, and env (10A1 virus-derived) genes, were transfected with either pMSCV-INFNR1 or -Large T plasmids using Lipofectamine® 2000 (Invitrogen) after adding 25 μM chloroquine for 1 h prior to transfection. The medium was changed to a fresh growth medium 8 h after transfection. The culture supernatants were collected 72 h after medium replacement and filtrated with a 0.45 μm cellulose acetate membrane. Nalgene Syringe Filter (Thermo). For measurement of MSCV virus titration, NIH3T3 cells were seeded at densities of 10^5 cells per well in 6-well plates and grown in a humidified incubator at 37°C and 5% CO₂ overnight to 70-80% confluence. The cells were treated with serial diluted viral solution (10^-1 to 10^-12) with 4 μg/ml polybrene and incubated for 24 h. The remaining colonies in each well were measured by Crystal violet staining after selection with antibiotics for 1 week. To prepare the lentivirus, HEK293T cells were co-transfected with these constructs and lentiviral packaging vectors (SIN vector plasmid: CSII-CMV-IRESE2, packaging plasmid: pCAG-HIVgp and VSV-G/Rev plasmid: pCMV-VSV-G-RSV-Rev) using Lipofectamine® LTX (Invitrogen). After 16 h, the transfected cells were added to 10 μM forskolin. After 48 h, the growth medium, including the lentivirus, was collected and filtrated with 0.45 μm cellulose acetate membranes, and concentrated by the Lenti-X™ Concentrator as per the manufacturer’s instructions (Clontech). The resultant lentivirus titration was checked by quantitating the p24 protein using the Lenti-X™ p24 Rapid Titer Kit (Clontech) in the culture medium.

**Cell cultures**

Murine neuroblastoma cells (N2a) and fibroblast cells (NIH3T3) were obtained from the American Type Culture Collection. To create an in vitro model using cells persistently infected by prions, N2a-58 cells overexpressing PrPC<sup>C</sup>, which were established from N2a cells integrating mouse Prnp gene in N2a cells, were subjected to prion infection with a mouse-adapted 22L strain from scrapie as previously described (Nishida et al., 2000; Ishibashi et al., 2012a). As packaging cells, HEK293T cells for lentivector system with SIN virus (provided by Dr Miyoshi in RIKEN BRC) and NIH3T3-derived RetroPack PT67 cells (Clontech) expressing 10A1 viral envelope protein for MSCV retroviral expressing system were obtained from commercial sources. All cells were grown at 37°C in 5% CO₂ in Dulbecco’s-modified Eagle medium (DMEM, Wako) containing 4500 mg/l glucose, 10% heat-inactivated foetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (Nacalai Tesque). Prion-infected and non-infected cells were transfected with Ifnar1 gene plasmids using Fugene® 6 (Roche) as per the manufacturer’s protocol, and, grown in 6-well plates for 2 days. In the anti-prion treatment, 20 μg/ml PPS (Caughhey and Raymond, 1993), 10 μg/ml anti-PrP antibody (359) (Miyamoto et al., 2005) or 0.5 to 500 μM RO8191 was added to the prion-infected cells and incubated for 48 h. In stable line construction, non-tagged IFN-β-overexpressing cells were established and in vitro 22L scrapie infection experiments were performed using their clonal descendants. To establish cell lines stably expressing target proteins, pcDNA3.1 plasmids containing target genes were transfected, using Fugene® 6 (Roche), into N2a-58 cells, the cells were then selected by 350 to 500 μg/ml HygroGold™ (Invivogen) treatment, and drug-resistant colonies were isolated.

**Mouse embryonic fibroblast isolation, immortalization and establishment of a stable line**

To prepare primary mouse embryonic fibroblasts (MEFs), mouse embryos from the C57BL/6 and Ifnar<sup>1/-</sup> pregnant female mice were isolated and dissected out of the uterine horns at Days 13 to 15. After rinsing them in 70% ethanol, each embryo was separated from its placenta, and the brain and internal organs were removed and placed in a petri dish containing phosphate-buffered saline (PBS). Sequentially, the tissues were chopped using razor blades and were suspended and subdivided by trypsin-EDTA. All of these procedures were performed using aseptic technique in a biological safety cabinet. After centrifugation, these collected cells were resuspended and seeded with fresh DMEM, containing 10% FBS, as described above, in culture dishes. The medium was changed on the following day and the adhered fibroblast cells were cultured and passaged until adequate cell volume was achieved. To establish the immortalizing MEFs, the amplified fibroblasts from Ifnar<sup>1/-</sup> mice were subcloned and passaged by serial replacing with 50 to 100 μg/ml HydroGold™ selections of the cells, following infection of MSCV-Large T vector at a rate to 10<sup>12</sup> colony forming units (cfu) per 100 mm culture dish. The Ifnar<sup>1/-</sup> gene-introduced Ifnar<sup>1/-</sup> MEFs were established by subcloning and passaging with 2.5 μg/ml puromycin. Selection of the cells occurred following infection with the MSCV-IFNR1 vector with final concentration of 4 μg/ml polybrene at a rate to 10<sup>12</sup> cfu per 100 mm culture dish.
Luciferase assay

Ifnar1−/− and Ifnar1 gene-transduced Ifnar1−/− (Ifnar1−/−/Ifnar1−/−) MEF cells were transiently co-transfected with the plasmids pUNO-ISRE-luc and pRL-null as internal standard plasmids, using LipofectamineTM LTX, and cultured for 24 h. The treated cells were continuously transfected with 30 μg/ml Poly I:C using LipofectamineTM LTX. After 20 h, the cells were lysed using Passive Lysis Buffer (Promega) and the luciferase activity was detected by the Dual Reporter Assay System (Promega) and quantified using the Mithras LB940 luminometer instrument (Berthold Technologies). Data of luciferase activity were normalized by the value of co-expressed Renilla activity as previously described (Homma et al., 2014a).

Prion infection in ex vivo and in vivo

In ex vivo prion infection in cell culture, the cells were infected with 22L scrapie strain-infected brain homogenate prepared from mice terminally sick with the 22L strain (final concentration: 2 × 10−3%/ brain homogenate for neuronal cells; 2 × 10−2%/ for NIH3T3 cells; 6 × 10−3%, 3 × 10−2%, 1.5 × 10−1% brain homogenate for MEF cells) in a 6-well culture plate for 48 h, and subsequently grown and scaled up to a 75 cm2 flask. Once confluent, the subcultures were diluted 5- or 10-fold in fibroblast or neuronal cells. In experiments of the inhibitory effect against prion infection at early phase, I-IFNs and RO8191 (0.5–500 μM) were treated in the cells and incubated for 24 h before prion infection simultaneously with recombinant mouse I-IFNs, and cultured until the fifth passage (#1 to #5) after scale-up. Poly I:C (0.2 to 2 μg/well) stimuli were transfected by LipofectamineTM LTX and incubated for 8 h before prion infection. In in vivo prion infection, 4-week-old male mice (wild-type and Ifnar1−/− mice of the same C57BL/6-derived genetic background) were inoculated via the intracerebral (i.c.) and intraperitoneal (i.p.) route with 22L scrapie strain-infected brain homogenate prepared with 22L scrapie strain-infected brain homogenate 103 μl (viral titer: 1.5 × 106 IFU/ml). The volume of the lentivirus via intracerebral injection was 20 μl (viral titer: 1.5 × 106 IFU/ml). The flow rate was 1 μl/min, using auto-microinjector-equipped Hamilton syringe 700 series (Narishige) (10 μl), and mounting a 30 gauge needle on the left hemisphere brain (thalamus coordinates: +2 mm anterior to bregma, +1 mm lateral to the midline and +4 mm ventral form the skull surface). Lentivirus coding the Venus gene vector (LV-venus) was used as a control. To confirm gene expression by the lentiviral vector, the fluorescent Venus protein was visualized in mice brain tissues 3 weeks after viral inoculation using the confocal laser-scanning microscope LSM 700 (Carl Zeiss). Nuclei were stained with VECTASHIELD mounting medium containing DAPI (Vector Laboratories).

Immunoblotting

Immunoblotting was performed as previously described (Homma et al., 2015; Ishibashi et al., 2015). The culture cells and animal tissues treated with various experimental conditions were lysed in 1 × lysis buffer [50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 0.5% TritonX-100, 0.5% sodium deoxycholate, 2 mM EDTA, and protease inhibitors (Nacalai Tesque)], for 30 min at 4°C. The lysates were then treated by sodium dodecyl sulphate (SDS) sample buffer and were separated with 15% SDS-polyacrylamide gel electrophoresis, and blotted to a polyvinylidene difluoride membrane. Bands were detected by appropriate primary antibodies and horseradish peroxidase-labelled secondary antibodies, and visualized using the Chemi-Lumi One L (Nacalai Tesque), ECL prime Western Blotting Detection Kit (GE Healthcare Life Sciences), or ClarityTM Western ECL Substrate (Bio-Rad) to get appropriate results by sufficient enzymatic reaction. Band intensities were quantified using ImageJ software (NIH). For PrPSc detection, lysates were digested with 20 μg/ml of proteinase K (Nacalai Tesque) at 37°C for 30 min.

Immunofluorescence analysis

In a fluorescence-activated cell sorter analysis, immortalized cells established from IFNAR1-deficient mouse embryonic fibroblasts (Ifnar1−/− MEF) and the Ifnar1−/− cells reintroduced with the MSCV retrovirus coded mouse Ifnar1 gene and overexpressing exogenous IFNAR1 (IFNAR1/Ifnar1−/−) were harvested in PBS containing 20 mM EDTA. Cells were suspended, fixed, and permeabilized with BD Cytofix/ CytopermTM Fixation/Permeabilization Solution Kit (BD Biosciences), and reacted with anti-SAF32 and MARI1–SA3 (1:100) as primary antibodies, and Alexa Fluor® 488 goat control, age- and strain-matched mice were intraperitoneally inoculated with the solvents without the drug. Vehicle and RO8191-treated mice were weighed three times a week from the moment before onset to the terminal stage of the disease.

In vivo administration of lentiviral vector

As lentiviral vectors were intracerebrally or stereotaxically administered on the ipsilateral side at 3 weeks after the intracerebral injection of 1% 22L prion brain homogenate injection in Tga20 mice. The volume of the lentivirus via intracerebral injection was 20 μl (viral titer: 1.5 × 106 IFU/ml). The volume of the lentivirus via stereotaxic microinjection was 4 μl (viral titer: 1.5 × 105 IFU/ml). The flow rate was 1 μl/min, using auto-microinjector-equipped Hamilton syringe 700 series (Narishige) (10 μl), and mounting a 30 gauge needle on the left hemisphere brain (thalamus coordinates: +2 mm anterior to bregma, +1 mm lateral to the midline and +4 mm ventral form the skull surface). Lentivirus coding the Venus gene vector (LV-venus) was used as a control. To confirm gene expression by the lentiviral vector, the fluorescent Venus protein was visualized in mice brain tissues 3 weeks after viral inoculation using the confocal laser-scanning microscope LSM 700 (Carl Zeiss). Nuclei were stained with VECTASHIELD mounting medium containing DAPI (Vector Laboratories).
anti-rabbit and anti-mouse IgG (Molecular Probes, 1:100). The cells were analysed using a flow cytometer (BD FACSCalibur™, BD Biosciences).

**Histology**

After the mice were sacrificed at the time point, the brain and spleen tissues were fixed in 10% formalin and sectioned into 3µm slices that were then embedded in paraffin. To evaluate spongeform change, the sections were analysed by haemaoylin and eosin staining as previously described (Ishibashi et al., 2012a; Nakagaki et al., 2013). As per the immunohistochemistry protocol, after deparaffination and rehydration as preparation for pre-staining, the sections were boiled with Target Retrieval Solution (Dako, S2369), and treated with 0.3% hydrogen peroxide in methanol to inactivate endogenous peroxidase, and sequentially incubated with 1% bovine serum albumin in Tris-buffered saline-Tween-20 (TBST) at room temperature for 1 h. The sections were reacted with primary antibodies and envision polymer horseradish peroxidase-conjugated anti-rabbit and anti-rabbit immunoglobulin G antibodies (Dako, K4002 and K4000) for 1 h at 37°C, respectively, and visualized using 3,3'-diaminobenzidine. To detect glosis, lba-1 (WAKO, 019–19741) for microglia and GFAP (Dako, Z033429) for astrocytes, were applied to the sections as primary antibodies. For PrPSc staining, autoclaving was performed using the hydrochloric acid protocol described previously (Nakagaki et al., 2013). In this pathological analysis, brain tissues from the regions of the cortex, hippocampus, thalamus, cerebellum, and Pons were evaluated. For semi-quantification in histopathological analysis, the pathological degree of each region in the tissues was scored on a 0 to 5 scale (i.e. non-detectable, a few, mild, moderate, severe, and status spongiosis) as described previously (Ishibashi et al., 2012a).

**Quantitative PCR**

After prion infection, total RNA was harvested from the cells and brain tissues using a GenElute™ Mammalian Total RNA Miniprep kit (Sigma), according to the manufacturer’s protocol. RNA was reverse-transcribed with SuperScript VILO™ (Invitrogen), and the resulting cDNA was amplified using 5'-ACT GAA AAC CGT GGA CCT GC-3' as the sense primer and 5'-AGT CCA TGT CCT CCA CCA AG-3' as the antisense primer; murine Irf3 (NCBI accession number: NM_016849) cDNA, using 5'-GGT ATG CCG CCC AAT CTA AA-3' and 5'-GGT GAG GTC CCA AAT TTC CA-3'; murine Il6b (NCBI accession number: NM_010510.1) cDNA, using 5'-CCC TAT GGA GAT GAC GGA GA-3' and 5'-CTG TCT GGT GGG GTA GTT CA-3'; murine melanoma differentiation-associated gene 5 (Ifih1) cDNA, using 5'-AGA GGT GGT GTA AGC m, 1.0 mmol in a 4-fold volume and were incubated at –80°C for 1 h. The standard samples were prepared from serial dilutions (0.1, 1, 10, 100 and 1000 nM) of compounds mixed with 20% normal brain homogenate. After centrifugation at 20 000g for 15 min, supernatants from the sample tissues and standards were collected, and then subjected to LC-MS/MS. LC-MS/MS was performed by an acquity UPLC-I class system coupled with a Xevo™ TQ-S triple quadrupole-mass spectrometer (Waters). Under UPLC conditions, 5 μl of sample volume was delivered to the ACQUITY UPLC BEH C18 column (1.7 μm, 1.0 × 100 mm, Waters) for reversed-phase chromatography, with the mobile phase consisting of solvent A (0.1% formic acid in water) and solvent B (99% acetonitrile, 0.1% formic acid). The gradient elution, solvent A was used in the order corresponding to ratios of 10%, 10%, 95% and 10% at each time point. The standard samples were prepared from serial dilutions (0.1, 1, 10, 100 and 1000 nM) of compounds mixed with 20% normal brain homogenate. After centrifugation at 20 000g for 15 min, supernatants from the sample tissues and standards were collected, and then subjected to LC-MS/MS. LC-MS/MS was performed by an acquity UPLC-I class system coupled with a Xevo™ TQ-S triple quadrupole-mass spectrometer (Waters). Under UPLC conditions, 5 μl of sample volume was delivered to the ACQUITY UPLC BEH C18 column (1.7 μm, 1.0 × 100 mm, Waters) for reversed-phase chromatography, with the mobile phase consisting of solvent A (0.1% formic acid in water) and solvent B (99% acetonitrile, 0.1% formic acid). The gradient elution, solvent A was used in the order corresponding to ratios of 10%, 10%, 95% and 10% at each time point.

**Measurement of blood–brain barrier permeability**

To investigate the permeability of RO8191, the BBB Kit™ was used as an in vitro blood–brain barrier screening model (RBT-24H, PharmaCo-Cell Co), consisting of co-cultures of endothelial cells, pericytes and astrocytes, according to the manufacturer’s protocol (Pervin et al., 2017). In brief, 10 μM of RO8191 was added to the luminal side as the blood-side in the BBB Kit™ using clear transwell inserts in 12-well plates. After incubation for 1 h, 200 μl of the luminal side medium and 900 μl of the abluminal side as the brain-side medium were harvested from the culture plate. The concentration of RO8191 (MW: 373.21) in each sample, including the rest of the administered original sample, were measured using lipid chromatography with tandem mass spectrometry (LC-MS/MS). To quantify blood–brain barrier permeability, the apparent permeability coefficient (Papp) (× 10−6 cm/s) was calculated as described previously (Tominaga et al., 2015).

**Measurement of RO8191 from animal tissues using LC-MS/MS**

RO8191-treated and -untreated mice brains and spleens at 100 dpi and terminal phase were homogenated with PBS. Then 20% homogenate samples were mixed with 99% methanol in a 4-fold volume and were incubated at −80°C for 1 h. The standard samples were prepared from serial dilutions (0.1, 1, 10, 100 and 1000 nM) of compounds mixed with 20% normal brain homogenate. After centrifugation at 20 000g for 15 min, supernatants from the sample tissues and standards were collected, and then subjected to LC-MS/MS. LC-MS/MS was performed by an acquity UPLC-I class system coupled with a Xevo™ TQ-S triple quadrupole-mass spectrometer (Waters). Under UPLC conditions, 5 μl of sample volume was delivered to the ACQUITY UPLC BEH C18 column (1.7 μm, 1.0 × 100 mm, Waters) for reversed-phase chromatography, with the mobile phase consisting of solvent A (5 mM ammonium formate in methanol) and solvent B (ultra-pure water) at a flow rate of 0.35 ml/min at 40°C. In the gradient elution, solvent A was used in the order corresponding to ratios of 10%, 10%, 95% and 10% at each time point.
point (0, 1.5, 2.0 and 4.5 min, respectively). To detect the separated compound, the tandem mass spectrometer was set in ESI positive ionization mode and in multiple reaction-monitoring (MRM) mode. Collision energy and cone voltage were 3000 V and 50 V, respectively. Cone and desolvation gas flow rates, and the desolvation temperature were set to 150 and 6500 l/h, and 350°C, respectively. The system was tuned to monitoring the scan type of MRM with three channels set to 373.98 to 265.00, 373.98 to 331.98 and 373.98 to 347.06 m/z transition (monitor range, precursor ion to product ion). From the MRM data, the concentration of RO8191 was acquired by analysis through MassLynx software (Waters).

**Statistical analysis**

Statistical analysis of all data was performed using Statcel 2 of the Excel and GraphPad Prism software. Student's t-test and Mann-Whitney U-test were used in the comparisons of two groups, and the one-way ANOVA followed by the Tukey-Kramer and Dunnett's tests in multiple comparisons. The log-rank test was used to analyse the mortality of prion-infected mice. Results in the table and graph represent the mean ± standard error of the mean (SEM) or standard deviation (SD) of at least three independent experiments.

**Data availability**

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary material.

**Results**

**Type I IFN is protective against prion infection in pathological models**

In 22L-scrapie prion-infected cell cultures, an ex vivo model of prion disease, the TLRs and I-IFN related genes were significantly downregulated in association with the establishment of infection. Data are represented for PrPSc expression with primary [younger than postnatal Day (P)5] or constitutive (P20) infections in Neuro2-a cells overexpressing mouse PrPSc (N2a-58) and immortalized mouse fibroblast cells (NH3T3) (Fig. 1A–C and Supplementary Fig. 1A–C). The reduction in expression of these genes significantly recovered when PrPSc levels were decreased by treatment with PPS (Caughey and Raymond, 1993) or anti-PrP monoclonal antibody (3S9) (Miyamoto et al., 2005) (see ‘Materials and methods’ section) (Fig. 1D and Supplementary Fig. 1D), suggesting that prion infection might suppress several innate immune-related genes in cultured cells.

I-IFN, which defends the host against infectious agents, is produced not only by immune cells but also by other cell types including fibroblasts and neurons (Delhaye et al., 2006). Hence, we investigated the potential anti-prion effect of recombinant I-IFNs (IFN-α and -β) in cell culture models. In the primary prion-challenge test IFN-β treatment significantly suppressed PrPSc levels in persistently 22L-prion-infected cells, and pretreated cells became resistant to 22 L-prion primary infection in a dose-dependent manner; the effect of IFN-α at similar doses was weaker (Fig. 2A and B). Similar resistance to prion was also observed in IFN-β-overexpressing stable cell lines (58-IFN-β) (Fig. 2C and Supplementary Fig. 2A). Transient IFN-β expression did not reduce endogenous PrPSc levels in persistently 22 L-prion-infected cells (Supplementary Fig. 2C). Pre-transfection of cells with Poly I:C, which activates the innate immune system via I-IFN induction, prevented the establishment of prion infection in a dose-dependent manner (Fig. 2D). To evaluate efficacy of IFN-β against prion propagation in prion infectious animal model, we performed bioassay using 22 L-prion-infected Tga20 mice with lentivirus coding Ifnb gene (LV-IFN). Lentiviral vectors were administered at 3 weeks after prion infection. LV-IFN localized in thalamus at 20 days after stereotaxic injection into Tga20 mouse brain (Fig. 2E). When lentivirus carrying the mouse Ifnb gene (LV-IFN) was injected intracerebrally, PrPSc levels were significantly reduced at the terminal stage in the brain of Tga20 mice after prion inoculation (Fig. 2F). In pathological analysis, vacuolation and PrPSc deposits decreased significantly in the brains of LV-IFN-treated mice (Supplementary Fig. 2B). The endogenous PrPSc level did not change in response to IFN-β overexpression in N2a-58 cells (Supplementary Fig. 2D), indicating that IFN-β might prevent new PrPSc production at the early phase of primary prion infection without affecting PrPSc expression.

**Prion infection is accelerated in IFNAR1-deficient cells and mice**

To confirm that the suppression of prion infection by IFNs was associated with IFN signalling, we generated immortalized MEF cells from wild-type and IFNAR1-deficient (Ifnar1−/−) mice and performed prion infection ex vivo. Susceptibility to 22 L prion infection was remarkably higher in immortalized MEF cells from Ifnar1−/− mice than in MEF cells from wild-type mice. Prions were dose-dependently propagated in both MEF cells. IFNAR1−/− MEFs showed significantly higher sensitivity to prion infection with 1.5 × 10^−1% 22 L-brain homogenate than MEFs from wild-type mice (Fig. 3A). Next, we generated Ifnar1-transduced Ifnar1−/− MEF cells, which were rescued by delivering the Ifnar1 gene to Ifnar1−/− MEFs with an MSCV retrovector, and investigated the efficiency of prion infection in an ex vivo model. The PrPSc level was similar between Ifnar1−/− MEFs and Ifnar1−/− MEFs rescued by delivering the Ifnar1 gene with an MSCV retrovector, but the latter cells were significantly and continuously less susceptible to 22 L prion infection (Fig. 3B, Supplementary Fig. 3A and B). To confirm whether the transduction restored IFN signalling, we performed ISRE-promoter activity analysis using the dual luciferase assay. The results indicated that the promoter activity in Ifnar1-transduced Ifnar1−/− MEFs after poly I:C stimulation was significantly increased (3-fold) compared with non-stimulated cells,
indicating that the transduced cells recovered cell signalling function via IFNAR1 whereas Ifnar1−/− MEFs did not possess this signalling pathway (Supplementary Fig. 3C). To elucidate the relationship between I-IFN dependent signalling pathways and prion infection in vivo, we inoculated 22 L prion into wild-type and Ifnar1−/− mice and monitored prion pathogenesis. When mice were challenged intracerebrally with a 10−1 dilution of 22 L prion brain homogenate, the survival periods of Ifnar1−/− mice were significantly shorter (145 ± 5 days, n = 15; P = 0.0006, Log-rank test) than those of the wild-type mice (153 ± 8 days, n = 17) (Fig. 4A and Table 1). Furthermore, all groups of IFNAR1−/− mice inoculated with 10−2 dilution i.c., or 10−3 and 10−2 dilution i.p., also exhibited significantly shortened survival (Table 1). To confirm pathogenesis in IFNAR1−/− mice after prion inoculation, we performed immunoblotting to examine the PrPSc levels in the brain and spleen at 100 dpi and at the terminal stage. The PrPSc levels in the brain and spleen of Ifnar1−/− mice were similar into those of wild-type mice (Fig. 4B and Supplementary Fig. 3D). However, vacuolation and gliosis with microglia (marker: IBA1) and astrocytes (marker: GFAP) were significantly more severe than in wild-type mice. In addition, accumulation of PrPSc in some regions at 100 dpi was considerably more severe in Ifnar1−/− mice (Fig. 4C, D and Supplementary Fig. 3E). Taken together, these findings indicate that the I-IFN signalling pathway via I-IFN receptor, including IFNAR1, protects to some degree against prion infection.

Figure 1 Innate immune-related genes levels are suppressed by prion infection in the cells. (A) Ifnb gene expression in persistently prion-infected N2a-22L and non-infected N2a-S8 cells. Immunoblot shows PrPSc levels in those cells. (B and C) Irf3 and Ifnb levels in N2a-S8 (B) and NIH3T3 (C) cells after ex vivo prion infection with 2 × 10−3 or 2 × 10−2 % 22 L-brain homogenate (BH). Pre-infection of N2a-S8 cells and normal brain homogenate treatment in 3T3 cells, white; 22 L-prion infection, black. Immunoblot shows PrPSc levels in those cells after prion infection. (D) Irf3 and Ifnb levels in N2a-22L cells for 48 h after PPS and 359 treatments. N2a-S8 (white); -22 L (black). Immunoblot shows PrPSc levels in the cells 24 and 48 h after PPS and 359 treatment. Statistical significance was determined using unpaired Student’s t-test (A–C) and one-way ANOVA, followed by Tukey-Kramer test in multiple comparisons (D). *P < 0.05, **P < 0.01, ***P < 0.001. Data are presented as mean ± SEM. Quantitative RT-PCR and western blot results represent at least three independent experiments.
I-IFN agonist suppresses prion formation in pathological models

An imidazonaphthyridine with the structural formula 8-(1,3,4-oxadiazol-2-yl)-2,4-bis (trifluoromethyl) imidazo [1,2-a] [1,8] naphthyridine (RO4948191, referred to as RO8191) is able to block hepatitis C virus infection to several cells including cancer cell lines and human primary hepatocytes. The reported functional mechanism of RO8191 is selective binding to the I-IFN receptor and the sequential induction of many interferon-stimulated genes, followed by the activation of I-IFN signalling.

Figure 2 Type I IFNs inhibit prion propagation in infectious cells and animal models. (A) PrPSc levels in N2a-22 L cells 48 h after treatments with recombinant I-IFNs (rIFNs) (0.1–10 kU/ml). (B–D) Resistance to ex vivo 22 L-prion infection in N2a-58 cells pretreated with rIFNs (0.1–10 kU/ml) (B), constitutively overexpressing IFN-β (C), or transfected with poly I:C (0.2–2 μg) (D). All graphs show quantifications of PrPSc band intensities. (E and F) Bioassay using 22 L-prion-infected mice (Tga20) inducing Ifnb with lentivirus system. Panels show localization of lentivirus in thalamus 20 days after stereotaxic injection to Tga20 mice brain (Venus: green; nuclei: blue) (E). Scale bar = 50 μm. PrPSc levels in brain of 22 L-prion-infected mice in the terminal stage brain pre-injected stereotaxically (s.t.) and intracerebrally (i.c.) with LV-venus and -IFN (F). Graphs show quantifications of PrPSc band intensities in brain (n = 3 mice per each lentiviral injection group). Statistical significance was determined using unpaired Student’s t-test (C and F) and one-way ANOVA, followed by Dunnett’s test in multiple comparisons (A, B and D). *P < 0.05, **P < 0.01 and ***P < 0.001. Data are presented as mean ± SEM. Western blot results represent at least three independent experiments.
against prion propagation in a prion infectious animal model, we performed a bioassay using prion-infected mice. The ddY mice intracerebrally inoculated with 10% 22 L brain homogenate were intraperitoneally administered 2 mg/kg/day RO8191 three times a week from 2 days post-inoculation until sacrifice. The survival periods of RO8191-treated mice were significantly longer (165 ± 7 days, n = 8; P = 0.0123, Log-rank test) than those of the vehicle mice (156 ± 4 days, n = 7) (Fig. 6A). Changes in the body weight of each group were consistent with prolonged survival times. The changes in body weight of the RO8191 group gently decreased compared with those of vehicle group, and this was delayed by about 2 weeks. RO8191-treated mice were observed for toxicity due to chronic administration and the symptoms of prion disease were similar to those of the vehicle mice (Supplementary Fig. 4A). We analysed PrPSc and histological changes in the brains of mice at 100 dpi, which was the time point before disease onset. PrPSc levels in the RO8191-treated brains and spleens were significantly less (50% reduction) than in the vehicle-treated brains at 100 dpi. PrPSc suppression in the spleens of RO8191-treated mice was observed until the terminal stage (Fig. 6B). RO8191 treatment significantly inhibited the levels of vacuolation and PrPSc deposition in some regions, including the cortex and spleen, compared with vehicle treatment at 100 dpi. Likewise, we also evaluated gliosis levels in the brains of RO8191-treated mice at 100 dpi. At 100 dpi, RO8191 treatment significantly reduced the expression of Iba-1 and GFAP, which are markers of activated microglia and astrocytes, in multiple regions of the cortex, thalamus and pons. However, RO8191 treatment resulted in no changes in expression in any of the regions at terminal phase (Fig. 6C, D and Supplementary Fig. 4B). These results indicate that RO8191 might have the potential to suppress prion formation in several tissues of the body following exogenous prion infection.

**Blood–brain barrier permeability of RO8191 and concentration in brain and spleen after chronic administration**

To investigate whether RO8191 functions in the brain, we performed a blood–brain barrier permeability assay using the BBB KitTM in vitro. The blood–brain barrier permeability coefficient (Papp), which indicates the ability of the compound to translocate from blood vessels to the brain, was measured using the BBB KitTM (Pervin et al., 2017). After 60 min, RO8191 exhibited a significantly higher blood–brain barrier permeability coefficient (43.94 ± 7.29) than sodium fluorescein (F-Na), the negative control (2.66 ± 0.78) (Fig. 7A). Moreover, to confirm the levels of RO8191 in the tissues of the animal model, we measured the concentration of RO8191 in the brains and spleens of prion-infected mice with RO8191 treatment at

(Konishi et al., 2012). To determine whether IFN-dependent signalling mediated IFN receptor-regulated prion infection, we investigated PrPSc levels in persistently prion-infected cells after RO8191 treatment for 48 h. PrPSc levels in N2a-22 L cells treated with RO8191 were significantly reduced in a dose-dependent manner (0.5, 5, 50, 250 and 500 μM RO8191) (Fig. 5A). However, PrPSc levels in N2a-58 cells treated with RO8191 showed no change (Fig. 5B). In both cells, the level of 2’-5’ oligoadenylate synthetase 1a (OAS1a), encoded by an interferon-stimulated gene, was dose-dependently increased by RO8191 treatment. Moreover, we investigated the potential anti-prion effect of RO8191 in cell culture models. RO8191 pretreated cells became resistant to 22 L-prion primary infection with the increase in expression of OAS1a in a dose-dependent manner (Fig. 5C), suggesting that RO8191, which induces interferon-stimulated genes via IFN signalling during the innate immune response, might prevent the establishment of prion infection without affecting PrPSc expression.

Next, to evaluate the efficacy of RO8191 in both cells, the level of PrPSc in 22 L-brain homogenate (BH). Graphs show quantification of PrPSc levels in wild-type (black) and Ifnar1−/− (white) cells. *p < 0.05 versus wild-type. (B) Efficiency of prion infection in cells established by transducing the Ifnar1 gene into immortalized Ifnar1−/− MEFs. Immunobots show PrPSc after prion infection with 2 × 10−2% 22 L-brain homogenate. Graphs show quantification of PrPSc levels in Ifnar1−/− (white) and Ifnar1-transduced (black) cells. **p < 0.01 versus Ifnar1−/−. Data are presented as mean ± SEM. Statistical significance was determined using one-way ANOVA, followed by Tukey-Kramer test in multiple comparisons (A) and unpaired Student’s t-test (B).
100 dpi and the terminal stage. The concentrations of RO8191 in the brains and spleens of cured mice at 100 dpi were confirmed to be high 8.44\(\frac{\mu g}{g}\) of tissue and 86.46\(\frac{\mu g}{g}\) of tissue, respectively, and these concentrations were maintained at the terminal stage (Fig. 7B). These results suggest that RO8191 might be able to act specifically on the brain and spleen, the organs mainly responsible for establishing prion formation.

**Discussion**

Prion infection suppressed Ifnb gene expression in 22 L-prion infected cells, and the level of Ifnb expression recovered following reduction of PrPSc by treatment with anti-prion compound and antibody (Fig. 1). This suppression of Ifnb gene transcription was followed by the low expression of IRF3 via a reduction of Oct-1 after prion infection.
infection (Homma et al., 2014a). Notably, Thr3, Ddx58/RIGI, Ifih1/MDA5 and Irf7, which are innate immune genes of the upstream signalling pathway relating to I-IFN induction, were suppressed by prion infection (Supplementary Fig. 1). IRF3 can contribute to the regulation of gene expression not only for Ifnb but also for Ifih1/MDA5 (Yount et al., 2007). TLR3 (Heinz et al., 2003), IRF1 (Choi et al., 2009) and Irf7 (Farlik et al., 2012) are evoked by STAT1 and NFκB is activated by intracellular signalling in response to IFN-β stimuli. Moreover, IRF1 is a transcription factor that positively regulates RIGI expression in human cells (Su et al., 2007). These studies indicate that prion infection might result in a dysfunctional immune system following the suppression of the expression of several genes by IRF3 reduction because type I IFN signalling mediated by IRF3 is closely connected with the regulation of expression of several innate immunity-related genes. However, the mechanism of action has not yet been determined and needs to be investigated in the future.

Although we found that IFN expression remained unchanged in the brains of prion-infected mice during the terminal period, it has been reported that the expression of many interferon-stimulated genes was increased in the brains of prion-infected animals (Riemen et al., 2000; Xiang et al., 2004; Stobart et al., 2007). Similarly, the expression of several interferon-stimulated genes was remarkably increased in the glial cells of the brains of patients with Creutzfeldt-Jakob disease (Baker et al., 2004). However, it remains unclear whether IFN gene expression is increased in ex vivo and in vivo prion-infected models at an early phase. Furthermore, it has not been determined whether IFN in the host plays a protective role against prion pathogenesis. In our experiments, prion infection suppressed IFN expression in prion-infected cells (Fig. 1) and low IFN expression levels were recovered by a reduction of PrPSc (Fig. 1). Furthermore, pretreatment with IFN and IFN system stimuli inhibited the new establishment of prion infection (Fig. 2), indicating that the host might combat prion propagation using any defence system available, including IFN. Thus, it will be necessary to investigate IFN induction at an early phase in neurons after prion infection. I-IFN stimulates various cell types, including immune cells such as lymphocytes, glia, and neurons (Delhaye et al., 2006; Paul et al., 2007). Intriguingly, I-IFN is constitutively expressed at low levels even in the absence of pathogens and contributes to the regulation of tumour propagation and cell growth (Taniguchi and Takaoka, 2001). Dysfunction of TLR4 and IRF3 facilitate prion pathogenesis (Spinner et al., 2008; Ishibashi et al., 2012a), suggesting that this process might be negatively regulated by signalling pathways, including I-IFN, that act downstream of PRRs. Pretreatment with complete Freund’s adjuvant and unmethylated CpG DNA to activate TLR signalling delays clinical onset in mice after prion inoculation (Sethi et al., 2002; Tal et al., 2003). Furthermore, glial cells pretreated with poly I:C are strongly resistant to prion infection (Kang et al., 2016), but post-treatment does not protect against prion pathogenesis in mice (Worthington, 1972; Cunningham et al., 2005; Field et al., 2010). Here, we showed that I-IFN signalling mediated by IFNAR1 suppressed prion pathogenesis in cell culture and mouse models after prion inoculation. Although persistent infection by 22L prion decreased expression of innate immunity genes such as Irf3 and Ifnb in an ex vivo system (Homma et al., 2014a), I-IFN protected against primary prion infection in cells (Fig. 2). These findings suggest that I-IFN, induced by TLR signalling, exerts a crucial anti-prion effect in the early phase after prion infection. Tumor necrosis factor-α and interleukin-6, which are induced following stimulation of PRR such as TLR4, may contribute to the acceleration of prion infection as determined by a prion bioassay using gene-deficient mice (Thackray et al., 2004; Tammuney et al., 2008). However, the relationship between suppression and I-IFN signalling has yet to be determined.

Using quantitative high-throughput screening, RO8191 was identified as a novel small molecule that acts like I-IFNs by directly interacting with the I-IFN receptor to drive interferon-stimulated gene expression. RO8191 can positively bind to the IFNAR2 receptor and strongly evoke an IFN signal inducing interferon-stimulated gene expression (Konishi et al., 2012). Thus, RO8191 can markedly block hepatitis C virus replication and cell death after encephalomyocarditis virus infection in cell culture as demonstrated with recombinant IFN treatment (Konishi et al., 2012; Wang et al., 2015). Moreover, mice orally inoculated with RO8191 showed significantly higher expression of
several interferon-stimulated genes compared with the vehicle group (Konishi et al., 2012). RO8191 inhibited PrPSc in prion-infected cells but did not change the PrPc level (Fig. 5). Prion-infected mice that were intraperitoneally treated with RO8191 showed significantly prolonged survival periods and were resistant to pathological changes in the brain after prion infection (Fig. 6). Several low molecular weight compounds (NPRs), which provide stabilization...
of protein structure by binding to PrP\textsuperscript{C} and inhibit conversion to PrP\textsuperscript{Sc}, suppressed the prion pathology in prion-infected mice following intraperitoneal chronic treatment. Several NPRs were linked with significantly reduced PrP\textsuperscript{Sc} levels only in persistently prion-infected cells, with no effect on PrP\textsuperscript{C} levels (Ishibashi et al., 2016). This implies that NPRs may show medical efficacy in peripheral areas such as the spleen, resulting in delayed PrP\textsuperscript{Sc} synthesis and accumulation in the brain. However, it remains to be clarified whether NPR acts in the brain. In our experiments, RO8191 was detected in high concentration in the brain and spleen of prion-infected mice at 100 dpi and at terminal

Figure 6 RO8191-treated mice have resistance against prion infection. (A–D) Evaluation of prion pathogenesis in RO8191-treated mice. (A) Survival curves for the vehicle and RO8191-treated mice after inoculation intracerebrally (i.c.) with a 10\textsuperscript{-1} dilution of 22L-brain homogenate (P < 0.05, log-rank test). PrP\textsuperscript{Sc} levels (B) and histological changes (C) in the brain (cortex, Cx) and spleen are shown at 100 dpi and in the terminal phase of prion infection. (D) Lesion profiles in the same five brain regions are similar to those shown in Fig. 4. Circle and triangle symbols indicate vehicle and RO8191-treated mice, respectively, at 100 dpi (black) and in the terminal stage (red). *P < 0.05 and **P < 0.01 versus vehicle mice at 100 dpi. Error bars represent SEM. Statistical significance was determined using an unpaired Student’s t-test (B and D). These results represent at least three independent experiments.
phase. RO8191 showed high blood–brain barrier permeability in an in vitro system compared with the control (Fig. 7), indicating that RO8191 might block prion propagation by acting in not only peripheral tissues but also in the brain. Furthermore, RO8191-treated mice did not have a shortened life span or show changes in phenotype, such as rapid weight loss from the side effects, after continuous treatment (Fig. 6 and Supplementary Fig. 4A), suggesting that the compound may be safe for clinical application.

In conclusion, the I-IFN pathway contributes to host defence mechanism against prion infection. Although we do not yet know exactly how host cells can recognize the prion pathogen, which lacks its own genetic material, the IFR3–IFN pathway serves as an important line of defence. Interference with this pathway permits persistent prion infection. The reciprocal interaction between innate host immunity and invading prions may explain the long latency of prion diseases. I-IFN and TLR signalling have diverse functions, including activation of autophagic systems that promote degradation of α-synuclein-derived aggregations (Ejlerskov et al., 2015; Kim et al., 2015) and contribute to neuronal homeostasis (Taniguchi and Takaoka, 2001). Therefore, effects of I-IFN should be considered in further investigations. Elucidating the role of host immunity in prion diseases could facilitate development of novel therapeutics for this deadly disease.

Acknowledgements

We thank Prof Horiuchi from Hokkaido University for the kind gift of Tga20 mice, Prof Taniguchi and Dr Honda from The University of Tokyo for the kind gift of IFNAR1 knockout mice, Dr Miyawaki from the RIKEN Brain Science Institute for the CSII lentivirus vector, and Daisuke Watanabe and Shinsuke Nakagawa from PharmaCo-Cell Co. Ltd. and Nagasaki University for helpful discussions and experiments on BBB permeability. We thank Yuzuru Taguchi, Hanae Takatsuki, Katori Ono-Ubagai, Yukiko Miyazaki-Hirota and Hiroya Tange from Nagasaki University, for helpful discussions and critical assessment of the manuscript, and Atsuko Matsuo, Hanako Nakayama, Marie Yamaguchi and Megumi Tanaka for technical assistance. We thank Edanz Group (www.edanzediting.com) and ZENIS Co., Ltd (www.zenis.co.jp/eng/index.html) for editing a draft of this manuscript.

Funding

This work was supported in part by the a grant from a Grant-in-Aid for Young Scientists (B) (DI: number 22790955), Scientific Research (C) (DI: number 24591482 and 16K07042) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; a Grant-in-Aid of the Research Committee of Prion Disease and Slow Virus Infection from the Ministry of Health, Labour and Welfare of Japan (N.N.); a Grant-in-Aid of the Research Committee of Molecular Pathogenesis and Therapies for Prion Disease and Slow Virus Infection, the Practical Research Project for Rare and Intractable Disease from the Japan Agency for Medical Research and Development, AMED (D.I.); a grant from the Takeda Science Foundation (N.N. and D.I.); a grant from the Japan Intractable Disease Research Foundation (D.I.); a Grant-in-Aid from the Tokyo Biochemical Research Foundation (D.I.); a grant provided by the YOKOYAMA Foundation for Clinical
Pharmacology (Grant No. YRY1502) (D.I.); the grant provided by the Ichiro Kanehara Foundation (D.I.); a grant provided by the Mochida Memorial Foundation for Medical and Pharmaceutical Research (D.I.); and a grant provided by the Waksman Foundation of Japan Inc. (D.I.).

Competing interests
The authors report no competing interests.

Supplementary material
Supplementary material is available at Brain online.

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