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Subhajit Poddar
Washington University School of Medicine in St. Louis

Jennifer L. Hyde
Washington University School of Medicine in St. Louis

Matthew J. Gorman
Washington University School of Medicine in St. Louis

Michael Farzan
The Scripps Research Institute

Michael S. Diamond
Washington University School of Medicine in St. Louis

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The Interferon-Stimulated Gene IFITM3 Restricts Infection and Pathogenesis of Arthritogenic and Encephalitic Alphaviruses

Subhajit Poddar, Jennifer L. Hyde, Matthew J. Gorman, Michael Farzan, Michael S. Diamond

Departments of Pathology and Immunology, Medicine, and Molecular Microbiology and the Center for Human Immunology and Immunotherapy Programs, Washington University School of Medicine, St. Louis, Missouri, USA; Department of Immunobiology and Microbial Sciences, The Scripps Research Institute, Jupiter, Florida, USA

ABSTRACT

Host cells respond to viral infections by producing type I interferon (IFN), which induces the expression of hundreds of interferon-stimulated genes (ISGs). Although ISGs mediate a protective state against many pathogens, the antiviral functions of the majority of these genes have not been identified. IFITM3 is a small transmembrane ISG that restricts a broad range of viruses, including orthomyxoviruses, flaviviruses, filoviruses, and coronaviruses. Here, we show that alphavirus infection is increased in Ifitm3<sup>−/−</sup> and Ifitm locus deletion (Ifitm-del) fibroblasts and, reciprocally, reduced in fibroblasts transcomplemented with Ifitm3. Mechanistic studies showed that Ifitm3 did not affect viral binding or entry but inhibited pH-dependent fusion. In a murine model of chikungunya virus arthritis, Ifitm3<sup>−/−</sup> mice sustained greater joint swelling in the ipsilateral ankle at days 3 and 7 postinfection, and this correlated with higher levels of proinflammatory cytokines and viral burden. Flow cytometric analysis suggested that Ifitm3<sup>−/−</sup> macrophages from the spleen were infected at greater levels than observed in wild-type (WT) mice, results that were supported by experiments with Ifitm3<sup>−/−</sup> bone marrow-derived macrophages. Ifitm3<sup>−/−</sup> mice also were more susceptible than WT mice to lethal alphavirus infection with Venezuelan equine encephalitis virus, and this was associated with greater viral burden in multiple organs. Collectively, our data define an antiviral role for Ifitm3 in restricting infection of multiple alphaviruses.

IMPORTANCE

The interferon-induced transmembrane protein 3 (IFITM3) inhibits infection of multiple families of viruses in cell culture. Compared to other viruses, much less is known about the antiviral effect of IFITM3 on alphaviruses. In this study, we characterized the antiviral activity of mouse Ifitm3 against arthritogenic and encephalitic alphaviruses using cells and animals with a targeted gene deletion of Ifitm3 as well as deficient cells transcomplemented with Ifitm3. Based on extensive virological analysis, we demonstrate greater levels of alphavirus infection and disease pathogenesis when Ifitm3 expression is absent. Our data establish an inhibitory role for Ifitm3 in controlling infection of alphaviruses.
mortality following IAV infection (19–21). However, some studies have questioned the significance of this truncated IFITM3 allele in the susceptibility to IAV and other viral infections (22, 23).

The mechanisms by which IFITM3 restricts viral infection are not fully elucidated. Studies have shown that IFITM3 affects pH-dependent fusion in the late endosome, which potentially traps entering virions in a hemifusion state (24–26). IFITM3 expression also can modulate the efficiency of cathepsin-mediated proteolysis in an as-yet-undefined manner, which is required for the cleavage of the fusion proteins of reoviruses, filoviruses, and coronaviruses and release of the viral genome from the endolysosome into the cytosol (9, 15). Additionally, IFITM3 is incorporated into the plasma membrane of budding HIV particles, which restricts their fusogenic capability (27). Finally, ectopic expression of IFITMs appears to alter the physical characteristics of the endosome, resulting in increased size, reduced membrane fluidity, and increased cholesterol content, which subsequently impact the efficiency of viral fusion (26, 28, 29).

Alphaviruses are enveloped single-stranded positive-sense RNA viruses of the Togaviridae family, many of which are transmitted by mosquitoes. The binding, entry, and pH-dependent fusion of alphaviruses are directed by the structural glycoproteins E1 and E2 (30, 31). E1 and E2 are arranged as heterodimers and assembled into trimeric spikes on the surface of the virion (32). E1 is classified as a type II membrane fusion protein, whereas E2 contains the putative receptor binding site (30).

Chikungunya virus (CHIKV) has emerged rapidly over the last decade, causing outbreaks in the islands of the Indian Ocean, in southern Europe, and in Southeast Asia. In 2013, CHIKV spread to the Western Hemisphere and by the end of 2015 had infected more than 1.7 million people in North, Central, and South America (33). Other arthropod-borne alphaviruses have a more limited distribution in parts of Oceania, Africa, and South America, whereas outbreaks of encephalitic alphaviruses occur sporadically in North, Central, and South America (34). Infection by arthropod-borne alphaviruses, including CHIKV and Sindbis (SINV), Ross River, and Mayaro viruses, results in a febrile illness associated with rash, myalgia, and moderate to severe joint pain (35). The musculoskeletal disease caused by these viruses is associated with direct infection of myocytes, synovial fibroblasts, and osteoblasts (35–39) and the ensuing infiltration of inflammatory cells. Infection by encephalitic alphaviruses, including Venezuelan (VEEV), Eastern, and Western equine encephalitis viruses, causes a severe febrile illness associated with infection and injury to neurons, encephalitis, long-term debilitating neurological sequelae, and death (34). To date, there are no licensed alphavirus vaccines available for use in humans.

Several ISGs have been characterized as restriction factors against alphavirus infection, including ISG15, PKR, ZAP, and BST-2; these genes target viral protein translation and virion egress, respectively (40–44). Ectopic expression-based screens against alphaviruses also have revealed putative inhibitory genes, including Isg20, Ifit1, Ifit2, Ifit3, and Rasad2 (45). However, in the case of Ifit1, which recognizes RNA lacking a 2′-O methylation on the 5′ cap structure and prevents translation, alphaviruses subvert its antiviral function via RNA secondary structure motifs that inhibit binding (46). Recent studies suggest that ectopic expression of IFITM genes in cell culture can restrict infection of Sindbis (SINV) and Semliki Forest (SFV) viruses in cell culture by inhibiting viral fusion with cellular membranes (47). Other ISGs (e.g., HSPE and P2RY6) have been identified, with little information regarding their mechanism of restriction (48, 49). Finally, ISGs can act in synergy to inhibit alphavirus infection (50).

In this study, we evaluated the antiviral activity of IFITM3 against several alphaviruses by comparing infection of IFN-treated wild-type (WT), Ifitm3−/−, and Ifitm locus deletion (Ifitm-del) mouse fibroblasts with CHIKV, SFV, SINV, O’nyong-nyong virus (ONNV), and VEEV. In the absence of Ifitm3 gene expression, we observed an increase in alphavirus replication in vitro, which was inhibited following transcomplementation with Ifitm3.

**In vivo**, Ifitm3−/− mice inoculated with CHIKV sustained higher viral burdens in the spleen, serum, and joint tissues at early times after infection. This was associated with higher levels of proinflammatory cytokines and increased joint swelling along with greater replication in macrophages in some tissues. Consistent with the latter observation, bone marrow-derived macrophages from Ifitm3−/− mice sustained higher levels of CHIKV infection than WT cells. Analogous to our observed phenotypes with CHIKV, Ifitm3−/− mice infected with VEEV exhibited greater weight loss and mortality and supported greater replication in the liver, spleen, spinal cord, and brain. Collectively, our data suggest that Ifitm3 contributes to an early host defense response against multiple alphaviruses of global concern.

**MATERIALS AND METHODS**

**Ethics statement.** This study was carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (51). The protocols were approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine (assurance number A3381-01). Dissections and injections were performed under anesthesia that was induced with ketamine hydrochloride and xylazine.

**Mice.** WT C57BL/6 mice were obtained commercially from Jackson Laboratories. Ifitm-del and Ifitm3−/− mice have been described previously (52). Ifitm3−/− mice were described in reference 73. All transgenic mice were backcrossed to 99% purity using speed congenic analysis (53). Four-week-old mice were inoculated in the left footpad with 105 focus-forming units (FFU) of CHIKV-LR in 10 μl of phosphate-buffered saline (PBS). Ankles were measured (width by height) for joint swelling on days 3 and 7 postinfection. On selected days after infection, mice were sacrificed for the collection of serum and tissues. After intracardiac perfusion with PBS, organs were harvested, weighed, and homogenized to determine viral titers by a focus-forming assay. For studies with VEEV, a vaccine-derived recombinant strain with a point mutation (TC83-A3G) was used; this mutation confers partial virulence in WT mice as it restores the capacity to antagonize the inhibitory actions of the ISG Ifit1 (46). Four-week-old mice were inoculated in the left footpad with 105 FFU of VEEV-TC83-A3G in 10 μl of PBS. Mice were followed daily for survival and weighed every 2 days. On selected days, infected mice were sacrificed and organs were harvested as described above.

**Flow cytometric analysis of CHIKV-infected splenocytes.** Splenocytes of CHIKV-infected mice were harvested after perfusion with PBS. Splenocytes were obtained by generating a single cell suspension, passing it through a 70-μm filter, and lysing red blood cells with ACK buffer (Invitrogen). Splenocytes were maintained on ice in PBS supplemented with 2% fetal bovine serum (FBS) and 1 mM EDTA. After blockade of Fc receptors with anti-CD16/32 (eBioscience; clone 93), staining for viability (eBioscience; FVD eFluor 506) and cell surface antigens CD45, CD3, CD19, CD4, CD8, Ly6G, Ly6C, CD11b, CD11c, major histocompatibility complex (MHC) class II, and F4/80 was performed. Viral antigen (E1 and E2 proteins on the surface of cells) was detected using biotinylated human anti-CHIKV-A1 and streptavidin-PE (eBioscience). Antibody isotype control (IgG1) was used for background staining. Optical density was measured using a FACSCalibur (Becton Dickinson) and analyzed using FLOWJO software (Treestar, Inc.).

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isolate controls, respectively. Secondary staining was followed with streptavidin-conjugated Alexa 647 (Invitrogen). Cells were fixed subsequently using the ebioScience Foxp3 fixation buffer set and processed for flow cytometry with the BD LSRII flow cytometer. Data were analyzed with FlowJo software.

**Bio-Plex cytokine assay.** To measure cytokine levels, a Bio-Plex Pro assay was performed according to the manufacturer’s protocol (Bio-Rad) on homogenized ankle tissues isolated at day 1 and 2 postinfection. The cytokine screen included interleukin-1α (IL-1α), IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFN-γ), KC, monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, RANTES (CCL5), and tumor necrosis factor alpha (TNF-α).

**Cells and viruses.** Primary WT, Ifitm-Δdel, Ifitm3−/−, and Ifitm3−/− mouse-derived mouse embryonic fibroblasts (MEFs) and bone marrow-derived macrophages were generated according to published methods (57). Transformed MEFs were generated by transfection of the SV2 plasmid, which encodes the large T antigen of SV2 polyomavirus (58), and passage −10 times. All MEFs were cultured in complete Dulbecco’s modified Eagle’s medium (DMEM), which was supplemented with 10% fetal bovine serum and 10 mM each (GluMax) glutamine, sodium pyruvate, nonessential amino acids, and HEPES, pH 7.3. MEFs that ectopically express c-Myc-tagged firefly luciferase or c-Myc-tagged Ifitm3 were generated by lentiviral transduction of the pFCIV vector, which contains an internal ribosome entry site-green fluorescent protein (IRES-GFP) (59, 60). Lentivirus was produced by transfecting 293T cells with pSIN.PX2 addgene catalog no. 12260, pMD2G (Addgene catalog no. 12259), and pFCIV. Supernatants were harvested at 48 to 72 h posttransfection. WT, Ifitm-Δdel, and Ifitm3−/− transformed MEFs were incubated with lentiviral supernatants and 10 μg/ml of Polybrene and spinoculated (300 × g) at room temperature for 30 min. The inoculum was replaced with complete DMEM 24 h later and incubated at 37°C. Transduction efficiency was determined by expression of GFP, and sorting of GFP+ cells was performed on a FACS Aria II cell sorter (Becton, Dickinson). After repeated passages to ensure stable expression, the MEFs were tested for GFP and protein expression by flow cytometry and Western blotting, respectively. Vero and 293T cells were cultured and passage in complete DMEM.

The CHIKV-LR (La Reunion OPY1 p142) strain was a gift from S. Higgs (Kansas State University). SINV (Toto) was a gift from C. Rice and P. MacDonald (Rockefeller University). VEVE-Tc83 was a gift from W. Klimstra (University of Pittsburgh). These strains were produced from infectious cDNA clones (61, 62). CHIKV 181/25, ONNV (MP30), and SFV (Kumba) were provided by the World Reference Center for Arboviruses (R. Tesh, University of Texas Medical Branch). Virus propagation and titration were performed in Vero cells.

**Genotyping of MEFs.** Genomic DNA was extracted from MEFs with the Qiagen DNeasy blood and tissue kit and was characterized by PCR. The Ifitm2 WT allele or knockout (KO) construct was genotyped using the following primers: Ifitm2 WT F, 5′-ATGTGGTCGCTGTCCTCCGTGGTCCT-3′, and Ifitm2 WT R, 5′-AGTGGCTGTCGGTCCTCATTTC-3′ (WT band, 520 bp); Ifitm2 KO F, 5′-CTCATTCAGTATGTTGCTGCC-3′, and Ifitm2 KO R, 5′-TGGAGACCGAAGGCTGAC-3′ (KO band, 373 bp). PCR conditions for both Ifitm2 WT and KO alleles were as follows: 94°C for 3 min, 94°C for 45 s, 55°C for 30 s, and 70°C for 1 min 30 s, for 35 cycles, and 70°C for 10 min. The Ifitm3−/− mouse can be identified by the in-frame insertion of GFP within the Ifitm3 allele (52). The WT allele or the knockout construct was genotyped using the following primers: WT Ifitm3 F, 5′-ATCTCCGGTCCGGTATGATG-3′, and Ifitm3 WT R, 5′-AC GATGCATCCTTCACATTTCCCTCC-3′ (WT band, 355 bp; KO band, 1,321 bp). PCR conditions for both Ifitm3 WT and KO were as follows: 94°C for 1 min 30 s, 94°C for 25 s, and 60°C for 30 s, reducing temperature by 0.1°C per cycle; 72°C for 1 min 30 s, 35 cycles, and 72°C for 5 min. The IFITM-del allele was determined using the following primers (52): IFITM-del WT F, 5′-AACATCGGCTTGTACCTTGACGAGGGCTTC-3′, and IFITM-del WT R, 5′-CCCTAAACACTCTGACGTCCTCCCTACAA-3′ (WT band, 500 bp); IFITM-del KO F, 5′-ACCTTACGAGAAGCCTTC-3′, and IFITM-del KO R, 5′-TCTAGTACAGTCGTTAGACAAAATATGTCCTTCAT-3′ (KO band, 600 bp). PCR conditions for Ifitm-del alleles were as follows: 95°C for 30 s, 54°C for 30 s, and 68°C for 1 min 30 s, for 29 cycles, and 68°C for 5 min.

**qRT-PCR measurement of Ifitm genes.** WT, Ifitm2−/−, Ifitm3−/−, and Ifitm-del MEFs (105 cells per condition) were seeded in a 96-well plate. After 6 h of incubation with IFN-β at various doses, MEFs were lysed and total RNA was extracted with the Qiagen RNeasy kit. Ifitm2 and Ifitm3 were detected using quantitative reverse transcription-qPCR (qRT-PCR) and normalized to Gapdh expression, using the following primetime assays (IDT) according to the manufacturer’s instructions: Ifitm2, Mm.PT.58.33172327.g; Ifitm3, Mm.PT.51.6979575.g; and Gapdh, Mm.PT.39.a.

**Western blotting.** MEFs were lysed in radioimmunoprecipitation assay (RIPA) buffer and electrophoresed under reducing conditions on a 12% Bis-Tris NuPAGE gel with morpholineethanesulfonic acid (MES) buffer according to the manufacturer’s instructions (Thermo Fisher). After transfer onto polyvinylidene dioxide (PVDF) membranes (Thermo Fisher) using an iBlot apparatus (Thermo Fisher), proteins of interest were detected with mouse anti-β-actin (CST; 8H10D10), mouse anti-c-Myc (Sigma; #9E10), goat anti-ifitm3 (R & D; AF337), horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma Chemical), and HRP-conjugated anti-goat IgG (Santa Cruz; sc2304). For quantification of protein, secondary donkey anti-mouse IRDye 680 (Li-Cor; 925-68072) and anti-rabbit-IRDye 800CW (Li-Cor; 926-32214) were used instead of HRP conjugates and visualized on the Odyssey Imager (Li-Cor). Polyclonal rabbit anti-ifitm3 (Proteintech; 11714-1-AP) was used for Ifitm3 detection in these experiments. Quantification was performed with LiCor Odyssey software.

**Virus infection of cells.** MEFs were plated (105 cells per well) in a 96-well plate and in some experiments pretreated for 6 h with recombinant mouse IFN-β (PBL Assay Science) at concentrations from 5 to 0.1 IU/ml, as indicated in the figure legends. The cells were inoculated with a given alphavirus (multiplicity of infection [MOI] of 5) and incubated at 37°C. At selected time points, cells were trypsinized, fixed with 1% paraformaldehyde (PFA), and permeabilized with Hanks’ balanced salt solution (HBSS) containing 0.1% saponin and 10 mM HEPES. Infection was determined after sequential staining with cells or human monoclonal antibodies (MAbs) (CHIKV, CHK-11; SFV, 2B4; TC83, IAA4-1; ONNV, 421) (54, 63) against the E2 glycoprotein. SINV infection was detected using murine anti-SINV ascites (ATCC; VR-1248AF). Alexa 647-conjugated goat anti-mouse or human IgG antibody (Life Technologies) was used for secondary antibody staining. Samples were processed by flow cytometry using a BD FACSAria cytometer. Data were analyzed with FlowJo software.

For viral yield assays, cells were plated (105 cells per well in a 12-well plate) and in some experiments pretreated with specific doses of IFN-β for 12 h. Cells then were infected with CHIKV at 37°C. One hour later, the plates were rinsed twice with warm PBS and replaced with fresh DMEM supplemented with 10% FBS. Supernatants were collected at specific time points, and viral titers were determined by focus-forming assay on Vero cells, as described previously (46, 54). After fixation, infected cell foci were detected with CHK-11 and HRP-conjugated anti-mouse IgG (Sigma Chemical) and quantified with an ImmunoSpot analyzer (Cellular Technologies, Ltd.).

**Binding and internalization assays.** MEFs were plated (105 cells/well in a 24-well plate) on the night before use. Cells were chilled on ice for 10 min, exposed to CHIKV-LR at an MOI of 5, and incubated on ice for 1 h. Unbound virus was removed with repeated washes of chilled medium or PBS. To determine binding efficiency of virus, MEFs were lysed with RNeasy lysis buffer (Qiagen), and RNA was extracted using the RNeasy minikit (Qiagen) and analyzed for CHIKV RNA by qRT-PCR. To determine the efficiency of the virus internalization, warm complete DMEM was
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RESULTS

Restriction of alphaviruses by Ifitm proteins in cell culture. Although expression of IFITM genes inhibits infection of several different genera of viruses (7–16), their antiviral activities against alphaviruses have yet to be established. To test whether Ifitm genes restrict alphavirus infection, we developed MEF lines lacking Ifitm2 (Ifitm2−/−), Ifitm3 (Ifitm3−/−), and Ifitm1, -2, -3, -5, and -6 (Ifitm-del) (Fig. 1A). To assess their effects on CHIKV replication, MEFs were first pretreated with 1 IU/ml of recombinant mouse IFN-β to induce Ifitm gene expression (Fig. 1B). Ifitm3 protein induction was confirmed by Western blotting in WT and Ifitm-deficient MEFs after IFN-β treatment, whereas, as expected, Ifitm3−/− and Ifitm-del MEFs lacked Ifitm3 protein (Fig. 1C). IFN-pretreated MEFs were then infected with a high viral dose (MOI of 5) of pathogenic (CHIKV-La Reunion 2006 [LR]) or attenuated (CHIKV 181/25) strains of CHIKV. Fourteen hours later, cells were harvested, and viral antigen was analyzed by flow cytometry. Whereas Ifitm3−/− and Ifitm-del MEFs supported enhanced infection by SFV, ONNV, VEEV (strain TC-83), and SINV compared to WT cells (P < 0.05, Fig. 2).

To corroborate our findings, we transcomplemented WT, Ifitm3−/−, and Ifitm-del MEFs with c-Myc-tagged to the N terminus of Ifitm3 or firefly luciferase protein as a control. After confirmation of ectopic expression by flow cytometry and Western blotting (Fig. 3A and B), MEFs were infected with CHIKV 181/25 (MOI of 5) in the absence of IFN-β treatment and analyzed at 6 h postinfection. MEFs transcomplemented with Ifitm3 showed less CHIKV replication than firefly luciferase-expressing controls (Fig. 3C and D). These data suggest that Ifitm3 inhibits multiple alphaviruses in vitro and does not require expression of Ifitm1, Ifitm2, Ifitm5, and Ifitm6 proteins to exert its antiviral activity.

Ifitm3 inhibits pH-dependent fusion of alphaviruses. Studies with IAV have shown that IFITM3 prevents fusion of virions from the late endosome, which is required for release of viral genomic material into the cytosol (24, 25). Correspondingly, IFITM3 is expressed preferentially on membranes of intracellular vesicles, including endosomes (9). However, following gene upregulation, such as after IFN induction or ectopic expression, IFITM3 can accumulate on the plasma membrane (18, 65, 66), which independently could restrict attachment of viruses to the cell surface. To define the stage in the alphavirus life cycle that Ifitm3 inhibits, we assessed its effect on binding, internalization, and fusion.

To determine if expression of Ifitm3 alters binding of alphaviruses to the cell surface, transcomplemented MEFs were incubated with CHIKV at 4°C for 1 h, washed extensively to remove unbound virus, and assayed by qRT-PCR. As no differences in levels of bound CHIKV genomic RNA were detected between Ifitm3-expressing MEFs and their corresponding controls (Fig. 4A), we concluded that binding efficiency was not appreciably affected. To assess whether Ifitm3 affected internalization, CHIKV was prebound to transcomplemented MEFs for 1 h on ice, followed by incubation at 37°C for 1 h. MEFs then were treated with proteinase K to remove residual surface-bound virus before recovery of cellular RNA. Similarly to cell surface binding assays, we observed no difference in the levels of internalized viral RNA (Fig. 4B). As expected, control binding experiments performed at 4°C, proteinase K treatment significantly decreased (11-fold, P < 0.0001) the level of cell-bound viral RNA (Fig. 4C).

As we did not observe effects of Ifitm3 on attachment or internalization, we next evaluated pH-dependent fusion. Alphaviruses can be induced to fuse at the plasma membrane in the presence of an acidic solution (acid bypass or fusion from without [FFWO]) (67), albeit at low efficiency; this required us to infect at a high multiplicity of infection. To test whether FFWO is affected by ectopic expression of Ifitm3, MEFs were preincubated with CHIKV at 4°C, washed to remove unbound virus, and then incubated with prewarmed medium at pH 7.4 or pH 5.5. Subsequently, medium was replaced with normal-pH culture medium supplemented with 20 mM NH4Cl, which prevents alphavirus maturation and fusion (67) and was added to inhibit productive infection of progeny virions. Fourteen hours later, MEFs were analyzed for viral antigen by flow cytometry. Ifitm3-transcomplemented MEFs had lower levels of CHIKV antigen than luciferase-expressing controls in WT, Ifitm3−/−, and Ifitm-
CHIKV infection is enhanced in cells lacking Ifitm3 expression. WT, Ifitm2−/−, Ifitm3−/−, and Ifitm-del MEFs were generated from WT and gene-targeted mice. (A) Genotyping of MEFs was performed by PCR and agarose electrophoresis. Bands corresponding to WT and KO alleles are indicated to the right of each gel. (B) MEFs were pretreated with various doses of IFN-β and tested for Ifitm2 and Ifitm3 gene induction by qRT-PCR. Ifitm2 expression was not detected in Ifitm2−/− and Ifitm-del MEFs, and Ifitm3 expression was not detected in Ifitm3−/− and Ifitm-del MEFs. Bars show the means and standard errors of the means from three independent experiments performed in duplicate. Means were compared between control and IFN-β-treated cells using a nonparametric one-way ANOVA with Dunn’s multiple comparisons (*, P < 0.05; ***, P < 0.001; ****, P < 0.0001). (C) MEFs were pretreated with the indicated doses of IFN-β and tested for Ifitm3 expression by quantitative Western blotting. (Left) Means from three independent experiments were compared between control and IFN-β-treated cells using a nonparametric one-way ANOVA with Dunn’s multiple comparisons (*, P < 0.05). (Right) A representative Western blot with loading controls (β-actin) is shown. (D to H) The indicated MEFs were pretreated with 1 U/ml of IFN-β and subsequently infected with CHIKV 181/25 or CHIKV-LR at an MOI of 5. (D) At 14 h postinfection, MEFs were stained for viral E2 protein and analyzed by flow cytometry. (E and F) Cumulative flow cytometry data for CHIKV 181/25 and CHIKV-LR. Bars show the means and standard errors of the means (SEM) from five independent experiments performed in quadruplicate or duplicate. Means were compared between WT and deficient cell lines using one-way ANOVA with Dunnett’s multiple comparisons (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001). (G and H) Kinetics of CHIKV 181/25 and CHIKV-LR replication in IFN-β-pretreated WT, Ifitm3−/−, and Ifitm-del MEFs infected at an MOI of 5. Supernatant was harvested at indicated time points, and virus titers were determined. Curves show the means and standard errors of the means from the pooled data of two or three independent experiments performed in triplicate. Means at each time point were compared between WT and knockout cell lines using two-way ANOVA with Dunnett’s multiple comparisons (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).
del MEFs (Fig. 4D and E). Consistent with results with IAV (24), expression of Ifitm3 also inhibits pH-dependent fusion of alphaviruses.

**Ifitm3 inhibits alphavirus infection in vivo.** To determine whether Ifitm3 has a protective role against alphaviruses in vivo, we used an established mouse model of CHIKV infection and arthritis (68). We inoculated 4-week-old WT and Ifitm3−/− mice with CHIKV-LR in the left footpad and measured joint swelling on days 3 and 7 after infection, which correspond to the peaks of tissue edema and cellular infiltrates, respectively (54, 68). Whereas
no difference was seen in viral titers at these time points (Fig. 5A), greater swelling was observed in ipsilateral ankle joints of Ifitm3−/− mice than in WT mice on both days (Fig. 5B and C, \( P < 0.001 \) and \( P < 0.01 \), respectively). Because of the disparity between clinical signs and virological data, we analyzed viral burden in different tissues (serum, spleen, ankles, wrists, and quadriceps muscles) at earlier time points (days 1 and 2 after inoculation) (Fig. 5D to K). At day 1 after inoculation, the serum, spleen, and ipsilateral ankle (Fig. 5D to F) of Ifitm3−/− mice had higher viral titers than did WT mice (20-fold in serum, \( P < 0.0001 \); 160-fold in spleen, \( P < 0.0001 \); and 2.5-fold in ipsilateral ankle, \( P < 0.01 \)). In comparison, at day 2, the titers in the spleen, serum, and ipsilateral ankle were similar but levels in the contralateral ankle and contralateral quadriceps muscle (Fig. 5G and I) were somewhat higher (4.5-fold, \( P < 0.001 \), and 5-fold \( P < 0.01 \), respectively). However, by day 3, no differences in viral titer were observed in any tissues between WT and Ifitm3−/− mice.

The early higher viral burden in Ifitm3−/− mice corresponded to higher levels of inflammatory chemokines and cytokines in the ipsilateral ankle (Table 1). The mean concentrations of several chemokines and cytokines (e.g., IL-2, MCP-1, TNF-α, IL-1α, IL-12p40, G-CSF, and GM-CSF, \( P < 0.05 \)) were higher in ankles from CHIKV-infected Ifitm3−/− mice than in those from WT mice at days 1 and/or 2 after infection. These data suggest that in the context of CHIKV infection in vivo, Ifitm3 contributes to restriction of early viral infection and spread, which impacts cytokine induction and the development of clinical disease.

Given the increase in viral titers in the spleen of Ifitm3−/− mice on day 1, we hypothesized that Ifitm3 might affect the cellular tropism of CHIKV. To identify the cell subsets that were more susceptible to CHIKV infection, we performed flow cytometric analysis on spleens of infected WT and Ifitm3−/− mice (Fig. 6A to C). Splenocytes were stained for CHIKV envelope (E1 and E2)
proteins using specific MAbs (54) and compared to isotype control MAbs. Inflammatory monocytes (CD11b+ Ly6G+), macrophages (CD11bhi F4/80lo), and red pulp macrophages (CD11blo F4/80hi) expressed high levels of viral antigen (50%, 50%, and 25%, respectively), with no difference in the fraction of infected cells from WT and Ifitm3−/− cells (Fig. 6B and data not shown). Nonetheless, greater numbers of CHIKV antigen-positive CD11bhi F4/80lo and CD11blo F4/80hi macrophages were detected in the spleens of Ifitm3−/− mice than in WT mice (1.3-fold, $P < 0.05$; 1.7-fold, $P < 0.05$; and 2.2-fold, $P < 0.05$, respectively) (Fig. 6C). An increased number of Ifitm3−/− neutrophils expressed CHIKV antigen (1.6-fold, $P < 0.05$), but the overall number of neutrophils was substantially lower than other myeloid cell populations. No differences in viral antigen-positive inflammatory monocytes were observed between the Ifitm3−/− and WT controls, and neither Ifitm3−/− nor WT CD4+, CD8+, CD19+, or NK1.1− cells exhibited detectable viral protein staining (data not shown). To determine if Ifitm3−/− macrophages can support...
Ifitm3 restricts CHIKV pathogenesis in vivo. Four-week-old WT and Ifitm3−/− mice were inoculated with 10^3 FFU of CHIKV-LR in the left footpad. (A) Viral titers in the ipsilateral ankle at days 3 and 7 postinfection. Data were pooled from two independent experiments, and each point represents one mouse (n = 8 to 10). The dotted line represents the limit of detection. No statistical difference was seen by the Mann-Whitney test. (B and C) Swelling of the ipsilateral ankle of infected WT and Ifitm3−/− mice at days 3 and 7 postinfection. Data were pooled from three independent experiments, and each point represents one mouse (n = 13 to 16). Asterisks indicate statistical differences by the Mann-Whitney test (**, P < 0.01; ***,** P < 0.001).

FIG 5 Ifitm3 restricts CHIKV pathogenesis in vivo. Four-week-old WT and Ifitm3−/− mice were inoculated with 10^3 FFU of CHIKV-LR in the left footpad. (A) Viral titers in the ipsilateral ankle at days 3 and 7 postinfection. Data were pooled from two independent experiments, and each point represents one mouse (n = 8 to 10). The dotted line represents the limit of detection. No statistical difference was seen by the Mann-Whitney test. (B and C) Swelling of the ipsilateral ankle of infected WT and Ifitm3−/− mice at days 3 and 7 postinfection. Area was determined by measuring the width and height of the ankle using digital calipers. Data are pooled from three independent experiments and are normalized to the measured area of the ankles just prior to infection. Each dot represents one mouse (n = 8 to 10). Asterisks indicate statistical differences by the Mann-Whitney test (**, P < 0.01; ***,** P < 0.001). (D to K) Four-week-old WT and Ifitm3−/− mice were inoculated with 10^3 FFU of CHIKV-LR in the left footpad. Viral burdens in the serum (D), spleen (E), ankles (F and G), muscles (H and I), and wrists (J and K) at days 1 and 2 postinfection were determined by focus-forming assay. Dotted lines represent the limit of detection. Data are pooled from two independent experiments and are normalized to the measured area of the ankles just prior to infection. Each dot represents one mouse (n = 8 to 10). Asterisks indicate statistical differences by the Mann-Whitney test (**, P < 0.01; ***,** P < 0.001).

**DISCUSSION**

To evaluate the potential antiviral role of Ifitm3 in restricting alphaviruses in vivo, we infected WT, Ifitm3−/−, and Ifitm-del MEFs with CHIKV, SFV, ONNV, VEEV, and SINV. All alphaviruses tested exhibited some degree of enhanced infection in Ifitm3 cells. In contrast, studies with CHIKV and Ifitm2−/− MEFs showed infection comparable to that of WT MEFs, suggesting that Ifitm2 is not the predominant Ifitm gene responsible for inhibiting alphaviruses in the context of an intact type I IFN response. The antiviral function of Ifitm3 against alphaviruses was validated using transcomplemented MEFs that ectopically express Ifitm3. Analogous to how IFITM3 inhibits IAV infection (24, 26), our mechanism-of-action studies suggest that Ifitm3 does not affect the binding or internalization of CHIKV but instead prevents pH-dependent fusion events.

We also observed greater CHIKV infection and disease pathogenesis in vivo in animals lacking Ifitm3 expression. Ifitm3−/− mice developed greater ankle swelling than did WT animals, and this difference correlated with an increased viral burden and inflammatory chemokine and cytokine levels at early times postinoculation. Notably, at later time points, titers became equivalent to that of WT MEFs, suggesting that Ifitm2 is not the predominant Ifitm gene responsible for inhibiting alphaviruses in the context of an intact type I IFN response.

We observed greater CHIKV infection in animals lacking Ifitm3 expression. Ifitm3−/− mice developed greater ankle swelling than did WT animals, and this difference correlated with an increased viral burden and inflammatory chemokine and cytokine levels at early times postinoculation. Notably, at later time points, titers became equivalent to that of WT MEFs, suggesting that Ifitm2 is not the predominant Ifitm gene responsible for inhibiting alphaviruses in the context of an intact type I IFN response.
TABLE 1 Cytokine levels in joint tissue homogenates after CHIKV infection

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Genotype</th>
<th>Day 1</th>
<th>P</th>
<th>Day 2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean pg/ml (SEM)</td>
<td></td>
<td>Mean pg/ml (SEM)</td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>WT</td>
<td>7.6 (± 1.0) 0.2</td>
<td>9.8 (± 0.6) 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>12 (± 2.4) 14 (± 1.5)</td>
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<tr>
<td>IL-1β</td>
<td>WT</td>
<td>52 (± 11) 0.3</td>
<td>144 (± 18) 0.2</td>
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<td></td>
<td>Ifitm3&lt;−/−</td>
<td>71 (± 14) 181 (± 9.2)</td>
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</tr>
<tr>
<td>IL-2</td>
<td>WT</td>
<td>11 (± 1.9) 0.03</td>
<td>15 (± 1.9) 0.09</td>
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</tr>
<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>16 (± 1.8) 21 (± 3.3)</td>
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<tr>
<td>IL-3</td>
<td>WT</td>
<td>0.33 (± 0.06) 0.9</td>
<td>0.39 (± 0.08) 0.006</td>
<td></td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>0.33 (± 0.06) 0.76 (± 0.06)</td>
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<tr>
<td>IL-4</td>
<td>WT</td>
<td>3.2 (± 0.2) 0.3</td>
<td>4.2 (± 0.5) 0.1</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>3.2 (± 0.5) 6.1 (± 1.0)</td>
<td></td>
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<tr>
<td>IL-5</td>
<td>WT</td>
<td>0.6 (± 0.2) 0.9</td>
<td>3.0 (± 1.0) 0.7</td>
<td></td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>0.8 (± 0.4) 3.4 (± 1.0)</td>
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<tr>
<td>IL-6</td>
<td>WT</td>
<td>1.5 (± 0.4) 0.8</td>
<td>8.8 (± 1.1) 0.4</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>1.9 (± 1.0) 11 (± 1.9)</td>
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<tr>
<td>IL-9</td>
<td>WT</td>
<td>22 (± 7.1) 0.9</td>
<td>31 (± 9.1) 0.006</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>28 (± 16) 107 (± 24)</td>
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<tr>
<td>IL-10</td>
<td>WT</td>
<td>1.1 (± 0.06) 0.3</td>
<td>3.1 (± 0.9) 0.02</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>1.3 (± 0.2) 5.3 (± 0.8)</td>
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<tr>
<td>IL-12p40</td>
<td>WT</td>
<td>1.1 (± 0.2) 0.8</td>
<td>10 (± 1.6) 0.02</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>1.4 (± 0.3) 15 (± 0.8)</td>
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<tr>
<td>IL-12p70</td>
<td>WT</td>
<td>2.8 (± 0.2) 0.1</td>
<td>6.2 (± 0.7) 0.5</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>3.8 (± 0.5) 7.0 (± 0.6)</td>
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<tr>
<td>IL-13</td>
<td>WT</td>
<td>LOD (38.7) 0.9</td>
<td>LOD (38.7) 0.9</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>44 (± 5.7) 39 (± 0.8)</td>
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<tr>
<td>IL-17</td>
<td>WT</td>
<td>0.3 (± 0.09) 0.9</td>
<td>0.3 (± 0.08) 0.8</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>0.3 (± 0.1) 0.2 (± 0.06)</td>
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<tr>
<td>Eotaxin</td>
<td>WT</td>
<td>151 (± 3.8) 0.5</td>
<td>176 (± 11) 0.9</td>
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<tr>
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<td>Ifitm3&lt;−/−</td>
<td>162 (± 8.2) 176 (± 1.2)</td>
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<tr>
<td>G-CSF</td>
<td>WT</td>
<td>0.7 (± 0.1) 0.9</td>
<td>4.2 (± 1.2) 0.007</td>
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<tr>
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<td>Ifitm3&lt;−/−</td>
<td>0.9 (± 0.3) 8.5 (± 0.8)</td>
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<tr>
<td>GM-CSF</td>
<td>WT</td>
<td>43 (± 4.9) 0.14</td>
<td>59 (± 6.4) 0.04</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>55 (± 5.0) 77 (± 5.7)</td>
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<tr>
<td>IFN-γ</td>
<td>WT</td>
<td>LOD (1.2) &gt;0.9</td>
<td>1.8 (± 0.3) 0.7</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>LOD (1.2) 1.5 (± 0.14)</td>
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<tr>
<td>KC</td>
<td>WT</td>
<td>16 (± 2.6) 0.6</td>
<td>81 (± 15) 0.8</td>
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<td>Ifitm3&lt;−/−</td>
<td>23 (± 5.6) 83 (± 13)</td>
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<tr>
<td>MCP-1</td>
<td>WT</td>
<td>52 (± 15) 0.01</td>
<td>706 (± 119) 0.4</td>
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<td>Ifitm3&lt;−/−</td>
<td>118.5 (± 25.44) 833 (± 70)</td>
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<tr>
<td>MIP-1α</td>
<td>WT</td>
<td>38 (± 1.4) 0.3</td>
<td>168 (± 29) 0.8</td>
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<td>Ifitm3&lt;−/−</td>
<td>50 (± 7.8) 151 (± 13)</td>
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<tr>
<td>MIP-1β</td>
<td>WT</td>
<td>20 (± 3.9) 0.3</td>
<td>150 (± 33) 0.2</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>34 (± 8.5) 89 (± 23)</td>
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<tr>
<td>RANTES</td>
<td>WT</td>
<td>13 (± 2.4) 0.8</td>
<td>109 (± 28) 0.8</td>
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<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>12 (± 3.1) 88 (± 24)</td>
<td></td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>WT</td>
<td>17 (± 3.0) 0.004</td>
<td>48 (± 7.5) 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ifitm3&lt;−/−</td>
<td>41 (± 6.4) 56 (± 7.4)</td>
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</table>

*Mice were infected with 10^3 FFU of CHIKV-LR in the footpad. Ipsilateral joint tissues were collected at 1 and 2 days after infection, homogenates were prepared, and the indicated cytokines were measured by Bio-Plex array. Data represent the means (±standard errors of the means) in picograms per milliliter from 9 to 11 mice per group. Statistical significance was determined by the Mann-Whitney test. LOD, limit of detection.
tified mechanisms, including host transcriptional shutoff (69) or antagonism of IFN signaling (70). To assess possible effects of Ifitm3 on cellular tropism, we assessed CHIKV antigen staining using flow cytometric analysis of splenocytes at day 1 postinfection. These cells were chosen because they were easily profiled and exhibited a substantial (160-fold) difference in viral yield at this time point. Although the overall percentages of CHIKV-positive myeloid cells were similar in the spleens of Ifitm3−/− and WT mice, a higher number of macrophages were positive for CHIKV antigen, suggesting a possible role for Ifitm3 in controlling viral growth in these cell types. One limitation of the flow cytometry experiments is that we cannot be certain that CHIKV antigen-positive staining defines bona fide infection, as it remains possible that we are detecting bound/opsonized virus on the surface of cells rather than E1 and E2 proteins prior to budding. To address this issue, we tried infection studies in WT and Ifitm3−/− mice with double subgenomic reporter gene viruses (e.g., CHIKV-GFP); however, the fluorescence staining was too dim for conclusive results, possibly because of the attenuation of these viruses. Nonetheless, our studies with bone marrow-derived macrophages support a role for Ifitm3 restriction of CHIKV infection in this cell type, as increased titers were observed in cells from Ifitm3−/− mice.

Our in vivo findings were not limited to CHIKV, as we also observed greater mortality, weight loss, and viral burden following VEEV infection of Ifitm3−/− mice. These data suggest an important role for Ifitm3 in restricting alphavirus pathogenesis in vivo, by limiting replication and dissemination early during infection. Future studies using analogous flow cytometric approaches and conditional gene deletions are planned to define the cell-
type-specific antiviral effect of Ifitm3 in the context of VEEV pathogenesis.

A possible antiviral role of IFITM proteins against alphaviruses has not been extensively analyzed. Studies with pseudotyped virions (alphavirus structural proteins and retroviral RNA) initially suggested that IFITMs had little antiviral activity against CHIKV, SINV, and VEEV (reference 28; M. Farzan, unpublished observations). It remains uncertain why Ifitm3 would not inhibit pseudotyped alphavirus virions although the icosahedral display of E1 and E2 may be altered in these viruses, which could affect entry and fusion of virus particles. Ifitm3 has been implicated, although not definitively demonstrated, as a restriction factor for alphaviruses. Karki et al. identified IFITM3 as one of 31 human ISGs that functioned synergistically with zinc finger antiviral protein (ZAP) to enhance restriction of SINV infection (50). Schoggins et al. reported that IFITM3 moderately reduced CHIKV and ONNV infection in human cells ectopically expressing IFITM3 (49, 71). Consistent with these observations, a recent paper reported an inhibitory effect of IFITM3 and IFITM1 against SFV and SINV when ectopically expressed in human A549 cells (47). These data support our findings of an antiviral activity of Ifitm3 against multiple alphaviruses.

The characterization of Ifitm3 as an antiviral ISG against alphaviruses adds to the known host defense genes that block alphavirus infection. ISG15 protects against SINV in vivo, likely via conjugation (ISGylation) to viral proteins (40–42); ZAP restricts SINV, Ross River virus, SFV, and VEEV by blocking the accumulation of viral genomes in the cytoplasm (72); and BST-2 (tetherin) prevents CHIKV egress by retaining budding virus on the plasma membrane (43). SINV also is strongly inhibited by protein kinase R (PKR) in the context of replication in dendritic cells (DCs) (44). Finally, a separate genetic screen revealed several unique ISGs with possible antiviral activity against SINV, including Isg20, Ifit1, Ifit2, Ifit3, and Rsad2 (viperin) (45).

In studies with other viruses, IFITM3 appears to restrict early steps in the viral life cycle, particularly fusion into the cytoplasm (24–26). This is supported by data from our FFWO experiments in the context of CHIKV infection and by recent studies with SFV (47). However, it remains possible that IFITM3, akin to effects on HIV, could restrict alphavirus infection in a pH-insensitive manner by integrating into the viral membrane, which we are currently exploring using mass spectrometric analysis of alphavirus virions derived from cells expressing or lacking Ifitm3. An additional mechanism that warrants investigation is the possible role of Ifitm3 in preventing viral budding and/or egress. IFITM3 can be detected at the plasma membrane, and its expression and localization are enhanced upon IFN stimulation (18, 65, 66).

In summary, we have shown that Ifitm3 can restrict several alphaviruses both in vitro and in vivo. Our data in mice suggest...
that Ifitm3 may function to restrict early replication and dissemination of aliphavirus, thereby preventing pathogenesis. Further investigation into additional mechanisms of Ifitm3-mediated restriction of aliphavirus is warranted as well as effects of gene polymorphisms, which could contribute to relative disease susceptibility in humans. Indeed, a common human allelic IFITM3 variant, rs12252-C, encodes a 21-amino-acid deletion of the N-terminal part of the protein that appears to be associated with susceptibility to IAV infection (19–21). It remains to be determined whether this or other polymorphisms in the IFITM3 gene can be linked to more severe or persistent aliphavirus infection.

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