In vivo and in vitro studies on the antiviral activities of viperin against influenza H1N1 virus infection

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Influenza A virus has caused a number of pandemics in past decades, including the recent H1N1-2009 pandemic. Viperin is an interferon (IFN)-inducible protein of innate immunity, and acts as a broad-spectrum antiviral protein. We explored the antiviral activities and mechanisms of viperin during influenza virus (IFV) infection in vitro and in vivo. Wild-type (WT) HeLa and viperin-expressing HeLa cells were infected with influenza A/WSN/33/H1N1 (WSN33) virus, and subjected to virological, light and electron microscopic analyses. Viperin expression reduced virus replication and titres, and restricted viral budding. Young and old viperin-knockout (KO) mice and WT control animals were challenged with influenza WSN33 at lethal doses of 10^3 and 10^4 p.f.u. via the intratracheal route. Lungs were subjected to histopathological, virological and molecular studies. Upon lethal IFV challenge, both WT and KO mice revealed similar trends of infection and recovery with similar mortality rates. Viral quantification assay and histopathological evaluation of lungs from different time points showed no significant difference in viral loads and lung damage scores between the two groups of mice. Although the in vitro studies demonstrated the ability of viperin to restrict influenza H1N1 virus replication, the viperin-deficient mouse model indicated that absence of viperin enhanced neither the viral load nor pulmonary damage in the lungs of infected mice. This may be due to the compensation of IFN-stimulated genes in the lungs and/or the influenza non-structural protein 1-mediated IFN antagonism dampening the IFN response, thereby rendering the loss of viperin insignificant. Nevertheless, further investigations that exploit the antiviral mechanisms of viperin as prophylaxis are still warranted.

INTRODUCTION

Epidemics and pandemics caused by the influenza virus (IFV) have resulted in large numbers of vaccine-preventable deaths worldwide. The Spanish influenza pandemic in 1918 alone is estimated to have resulted in up to 40 million fatalities (de Wit & Fouchier, 2008). Furthermore, with the emergence of the highly infectious H1N1-2009 strain, the continuing threat of H5N1 avian influenza, as well as the rapid evolution of the virus via mutation to evade the current control measures of vaccination and antiviral drugs, influenza remains one of the major health threats to humankind (Memoli et al., 2008).

Two supplementary figures are available with the online version of this paper.

One of the new strategies in the field of antiviral discovery is to exploit host innate antiviral factors and mechanisms to counter viral infections. This approach allows cross-strain targeting of the virus, and is independent of viral mutation as it does not interact directly with viral proteins (Karlas et al., 2010; König et al., 2010; Stertz & Shaw, 2011; Watanabe et al., 2010). The host innate immune factors, especially the interferon (IFN)-stimulated genes (ISGs), are pivotal as they are the first line of defence against viral infection, and often indiscriminately eliminate viruses that invade the host (Sanders et al., 2011; Wu et al., 2011). Therefore, it is interesting to investigate whether these ISGs play critical roles in host antiviral defences, with the view of harnessing their mechanisms to identify novel anti-influenza therapies. Viperin (or virus inhibitory protein, endoplasmic reticulum-associated, interferon inducible), is one such ISG that is
induced by multiple viruses such as IFV, hepatitis C virus, cytomegalovirus, vesicular stomatitis virus and Sindbis virus (SINV) (Boudinot et al., 2000; Chin & Cresswell, 2001; Fitzgerald, 2011; Helbig et al., 2005; Waheed & Freed, 2007; Wang et al., 2007). It serves as a broad-spectrum antiviral protein that inhibits viruses that utilize lipid rafts or lipid bodies in their replication cycle (Chin & Cresswell, 2001; Hinson & Cresswell, 2009; Jiang et al., 2008; Wang et al., 2007). Viperin also acts as a radical S-adenosyl-L-methionine enzyme (Shaveta et al., 2010), and is also involved in T-cell activation and T-cell receptor-mediated activation of NF-κB (nuclear factor kappa B) and AP-1 (activating protein-1) (Qiu et al., 2009), although the detailed roles of viperin require further investigation.

Viperin expression is highly induced by both type I and II IFNs in response to lipopolysaccharide and dsRNA (Fitzgerald, 2011), and its expression is regulated by the IFN-stimulated gene factor-3 complex (Chin & Cresswell, 2001). Upon synthesis, viperin localizes to the cytoplasmic face of the endoplasmic reticulum via its N-terminal amphipathic α-helix (Hinson & Cresswell, 2009; Wang et al., 2007). Viperin interacts with farnesyl pyrophosphate synthase, a key enzyme that catalyses formation of farnesyl pyrophosphate, an intermediate in host isoprenoid metabolic pathways. Cholesterol formation is thus reduced, ultimately perturbing lipid-raft assembly (Reilly et al., 2002; Wang et al., 2007).

IFV also utilizes lipid rafts in its life cycle, which includes viral entry, protein transport, assembly and budding (Nayak et al., 2009; Ono & Freed, 2005). It was thus hypothesized that IFV is susceptible to viperin activity. In vitro studies with ectopic expression of viperin in cell cultures successfully inhibited IFV budding from cells, leading to reduced virus titres (Wang et al., 2007). Therefore, our study aimed to extend these in vitro findings using an in vivo model, with the hypothesis that viperin deficiency will augment the susceptibility of mice to influenza pneumonitis. Our primary objective was to investigate the significance of viperin in influenza H1N1 virus infection, to determine whether the absence of viperin will culminate in higher morbidity and mortality in a murine model.

RESULTS

**Viperin expression reduces IFV titres in vitro**

Viperin was previously shown to inhibit IFV infection in vitro through the perturbation of lipid rafts in the cellular membrane (Waheed & Freed, 2007; Wang et al., 2007). To verify that human viperin exerts a similar antiviral effect, we infected human HeLaM cells (a HeLa cell subclone) and HeLaV cells (that overexpress viperin) with A/WSN33/H1N1 (WSN33) virus at an m.o.i. of 0.005. Compared with HeLaM cells, HeLaV cells exhibited a consistent ~1 log₁₀ reduction in virus titres across all time points (Fig. 1a). Real-time RT-PCR also confirmed higher viperin mRNA expression in HeLaV cells than in HeLaM cells across all time points (Fig. 1b). This finding is congruent with previous reports on mouse viperin, thus demonstrating that viperin is indeed an ISG product that restricts IFV replication across species in vitro.

**Viperin expression prevents budding of IFV in vitro**

We further conducted transmission electron microscopy (TEM) to ascertain the antiviral characteristics of human viperin, as previous studies were based on murine but not human viperin (Wang et al., 2007). Ultrastructural analysis revealed that viral cores accumulated in the cytoplasm of infected HeLaV cells, with an absence of viral budding. In contrast, the budding of viruses was readily observed in infected HeLaM cells (Fig. 2; Fig. S1, available in JGV Online). However, unlike a previous study using Tet-On murine viperin-expressing cells, daisy-chain formation of progeny viruses on the cellular membrane was not observed. Instead, clumping of viral cores in the cytoplasm below the cell membrane was noted, suggesting that human
viperin may be involved in additional mechanisms to restrict the budding of IFV progeny.

**Age does not significantly influence susceptibility of viperin-deficient mice to influenza pneumonitis**

There have been relatively few reports on infection models of viperin-knockout (KO) animals, even though the mechanism of inhibition of IFV by viperin has been characterized in recent years (Fitzgerald, 2011; Wang et al., 2007). We infected ‘young’ 8–12 week-old wild-type (WT, \( n = 14 \)) and KO (\( n = 13 \)) mice of the C57BL/6 background with a lower lethal dose of \( 10^3 \) p.f.u. WSN33 virus via the intratracheal route. The mortality rates of mice from both groups displayed no significant difference (WT 21.4% versus KO 30.8%, \( P = 0.632 \)) after infection (Fig. 3a). All uninfected-control mice survived. This implies that absence of viperin alone does not abrogate innate host defence against influenza pneumonitis. To ascertain whether there was any differential response in both groups of mice under different conditions, we also infected 8–12 week-old mice with a higher, lethal, dose of \( 10^4 \) p.f.u. WSN33, and infected ‘old’ mice aged approximately 8 months (to represent the elderly age group) with \( 10^3 \) p.f.u. WSN33. Although the old mice exhibited enhanced susceptibility to the infection, no significant difference was observed between the fatality rates of WT (\( n = 7 \)) and KO (\( n = 5 \)) groups (71.4 versus 100%, respectively, \( P = 0.632 \)) (Fig. 3b). In addition, all ‘young’
WT (n=6) and KO (n=6) mice succumbed equally to the higher challenge dose of \(10^4\) p.f.u., from 8 to 10 days post-infection (p.i.) with no significant difference in survival (\(P=0.830\)) (Fig. 3c). Moreover, preliminary findings involving challenge with 500 p.f.u. of another H1N1 virus (influenza A/PR/8/34) revealed no significant difference in survival (\(P=0.124\)) between 8–12 week-old WT (n=10) and KO (n=7) mice (data not shown), indicating a similar effect of viperin between two different H1N1 strains.

**Innate expression of viperin in vivo does not decrease IFV titres in lungs**

Although no significant difference in mortality rates was observed in the survival experiments, we noted a slight, albeit non-significant, increase in mortality of infected KO mice. Hence, we conducted a further time-course analysis of infection to ascertain whether viperin deficiency culminated in any specific temporal differences during the infection process. Thus, 8–12 week-old WT and KO mice were infected with \(10^3\) p.f.u. WSN33 virus, and euthanized on 3, 5, 7 and 9 days p.i. Plaque assays were performed to measure the virus titres in lung homogenates, while real-time RT-PCR was performed concurrently to ensure that only the WT mice expressed viperin. The lung virus titres peaked at 5 days p.i., and then declined (Fig. 4a), but no significant difference in titres was observed between the two groups of mice across all time points. Real-time RT-PCR showed that viperin mRNA expression in infected WT mice peaked at 5 days p.i., declined at 7 days p.i., but was virtually absent at 9 days p.i., while no viperin expression was detected in the KO counterparts (Fig. 4b). Despite the differential viperin expression between infected WT and KO mice, no significant difference in virus titres was evident between the two groups at all time points, thus coinciding with the survival analysis. Viperin deficiency of mice did not significantly influence the viral load in and viral clearance from the lungs, thus supporting the lack of differential susceptibility to influenza pneumonitis. On the other hand, IFN-\(\beta\) mRNA expression was detected in both infected KO and WT mice (with no significant difference), indicating intact IFN-\(\beta\) responses after influenza challenge even in the absence of viperin (Fig. 4c).

**Viperin deficiency does not significantly alter pulmonary damage induced by IFV infection**

To investigate further whether viperin deficiency contributes to differential susceptibility during pulmonary IFV infection, histopathological analyses were conducted on the lungs at 3, 5, 7 and 9 days p.i. (Fig. 5). The lung pathology was then scored according to four criteria based on features of lung damage. Uninfected-control mice exhibited negligible pulmonary pathology. The calculated damage scores of infected mice increased steadily with time, mainly due to the higher percentages of the worst-affected areas in the lungs p.i. (Fig. 6; Fig. S2). No significant difference in pulmonary damage scores was observed in infected WT and KO mice across all time points, suggesting negligible
differential responses to IFV infection in the absence of viperin to critically impact host susceptibility and survival.

**Deficiency of viperin does not affect production of neutralizing antibody against IFV**

After showing that, during an innate response, the absence of viperin did not influence the susceptibility of mice to influenza pneumonitis significantly, we assessed further whether viperin is involved in the adaptive humoral response. Neutralizing antibodies against WSN33 virus were absent in sera from uninfected-control mice. Sera from infected mice that survived up to 14 days p.i. were tested for neutralizing antibody, which would allude indirectly to the presence of helper T-cell and B-cell responses. Neutralizing antibodies were present in sera of both infected WT and KO mice at 14 days p.i., suggesting that the adaptive humoral response against IFV in KO mice was intact (Fig. 7). There was no significant difference in mean antibody titres between infected WT and KO mice. This signifies that, even in the absence of viperin, the adaptive humoral immune response is not affected and protective antibody production against IFV is occurring normally.

**DISCUSSION**

The discovery of viperin, a member of the antiviral ISG family, has shed light on its broad inhibitory effects on a range of viral pathogens. Furthermore, its antiviral mechanism does not require direct interaction with the virus, thus making it a potential candidate for developing novel treatment strategies that are independent of viral mutations (Stertz & Shaw, 2011; Watanabe *et al.*, 2010). *In vitro*, the ectopic expression of viperin is able to restrict the replication of several viruses by altering the cholesterol level or the lipid-droplet structure of the cell (Fitzgerald, 2011). However, it is necessary to verify the promising *in vitro* findings using *in vivo* models to elucidate the functional significance of viperin during influenza viral infections.

Recent *in vivo* studies to investigate viperin include a viperin-expression model against SINV (Zhang *et al.*, 2007)
and a viperin-deficient model against West Nile virus (WNV) (Szretter et al., 2011). Both studies demonstrated that viperin conferred protection against these viruses that cause acute inflammation in the central nervous system (CNS). While there have been in vitro studies demonstrating specifically that IFV replication can be inhibited by viperin expression, hitherto there have been no animal models (Fitzgerald, 2011; Wang et al., 2007). Hence, it is important to embark on a detailed in vivo study to assess the roles of viperin as an IFN-stimulated, anti-influenza factor in an animal model.

Our in vivo experimental data revealed an outcome contrary to those of the SINV and WNV mouse models, as well as the in vitro influenza model, revealing that influenza H1N1 virus-infected C57BL/6 mice with or without viperin expression displayed similar susceptibility to influenza pneumonitis. Even at the peak of viperin expression at 5 days p.i., no significant difference was observed in the virus titres or histopathological damage scores in the lungs of infected mice from both WT and KO groups. These results correlated with the survival rates when mice were challenged with different viral doses and H1N1 strains.

There are important differences between the influenza model compared with the SINV and WNV infection models. First, the latter models focus on viruses that primarily infect the CNS, a localized and relatively sterile environment with limited immune responses mediated mainly by the resident immune cells (Kaushik et al., 2011; Lim et al., 2011). In contrast, IFV mainly infects the upper and lower respiratory tracts, an area constantly exposed to the exterior environment, that is highly vascularized and accessible to large numbers of immune cells and components (Sanders et al., 2011; Whitsett, 2002). There is thus likely to be redundancy in terms of innate antiviral responses, whereby the absence of viperin can be compensated by other ISGs activated in the lungs, thus allowing infected mice to still combat the infection.

Secondly, type I IFN-independent expression of viperin may be possible via direct induction by interferon regulatory factor 1 (IRF-1) (Stirnweiss et al., 2010), and viperin itself can maintain its own expression via a positive-feedback loop (Saitoh et al., 2011). IFVs possess non-structural protein 1 (NS1), which acts as a highly potent antagonist of the IFN pathway. Influenza NS1 blocks the dsRNA sensors and Toll-like receptors (Hale et al., 2010; Schmolke & García-Sastre, 2010), and thus delays the upstream activation of IFR-1 and type I IFN, and subsequently of viperin downstream. It is notable that viperin expression only peaked at 5 days p.i., coinciding with the peak in viral load. Hence, the delayed IFN and viperin expression of WT mice may have resulted in the non-significant difference to IFV challenge. In addition, NS1 activity may also have rendered the absence of viperin in KO mice to be negligible (Moltedo et al., 2009).

Thirdly, unlike WNV and SINV infections, IFV infections are relatively acute, with short incubation periods. The virus replicates once it reaches the upper respiratory tract, and symptoms appear as early as 24–48 h p.i. (Patrozou & Mermel, 2009). As a result, the natural induction of the ISGs such as viperin may not be early enough to counter acute influenza, whereas viperin induction may be sufficiently timely during SINV and WNV infection of the CNS, which may explain the varying responses in different infections. The SINV infection model was based on ectopic expression of viperin that confers a protective effect (Zhang et al., 2007). However, our influenza infection model utilizes an approach similar to the WNV model, i.e. based on viperin-deficient mice, in order to assess the role of natural viperin expression by the innate immune system. Therefore, it still remains to be elucidated whether prophylactic ectopic expression of viperin or administration of entities that share a similar mechanism of action to viperin, will be able to confer a protective effect against IFV infection. Such artificial intervention that exploits viperin’s mechanism other than its natural expression may warrant further exploration as a novel anti-influenza therapy.

In summary, our findings reveal that the natural in vivo expression of viperin does not confer significant protection against influenza pneumonitis, possibly due to the compensation and redundancy of ISGs in the lungs that mask the effect of viperin deficiency. Another explanation may be that the influenza-mediated IFN antagonism may dampen the IFN response, thereby rendering the loss of viperin insignificant. Notwithstanding this, there may still be other potential mechanisms of viperin that can be harnessed, such as artificial manipulation to mimic viperin activity in vivo to provide prophylactic protection against influenza.
METHODS

Cell cultures and generation of HeLaV cells. Madin–Darby canine kidney (MDCK) cells were cultured in Eagle's minimal essential medium (MEM; ATCC) with 10% FBS (Biowest). HeLaM (a subclone of HeLa) and HeLaV cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biowest) with 10% FBS. To derive HeLaV cells, human viperin cDNA was initially subcloned into the pLNCX2 expression vector with a neomycin-resistance gene (Clontech). The recombinant pLNCX2-viperin plasmid was transfected into HeLaM cells, and positive clones were selected in the presence of G418 (Invitrogen). The expression of viperin in HeLaV was then confirmed by Western blot analysis using a rabbit antibody to viperin (data not shown).

Virus strains and infection of cells. The influenza H1N1 virus strains A/WSN/33 (WSN33) and A/PR/8/34 were used for in vitro and/or in vivo experiments. Overnight cultures of 5×10⁵ HeLaM and HeLaV cells in 24-well plates were infected with WSN33 virus at an m.o.i. of 0.005, and incubated at 37 °C for 1 h. DMEM (400 μl) was added to each well before incubating at 37 °C for 24, 48 and 72 h. The supernatants in the wells were then harvested, and the virus titres were determined via virus plaque assay.

TEM. Approximately 5×10⁵ HeLaM and HeLaV cells were infected with WSN33 at an m.o.i. of 0.5, and incubated at 37 °C for 1 h. Uninfected cells served as negative controls. Cells were fixed in 4% paraformaldehyde and 4% glutaraldehyde in PBS at 4 °C, post-fixed with 1% osmium tetroxide and 0.8% potassium ferrocyanide (Electron Microscopy Sciences) for 1–2 h, and dehydrated in ascending series of ethanol at room temperature. Cells were then infiltrated with ethanol and London Resin White (LRW; Electron Microscopy Sciences) embedding medium, pelleted, resuspended in LRW, and centrifuged at 4000 r.p.m. using a Jouan S40 rotor at 35 °C for 20–25 min. Gelatin capsules containing the cells and LRW were allowed to polymerize. Hardened blocks were subsequently sectioned to obtain 70–90 nm ultrathin slices, placed onto copper grids, stained with uranyl acetate and lead citrate (Electron Microscopy Sciences), and viewed under a Philips 208S transmission electron microscope (Phoon et al., 2001). Primary mouse mAb against influenza M1 protein (Abcam) and secondary immunogold-labelled antibody were used for the detection of IFV particles.

Animal strains, breeding and maintenance of colonies. Animal usage and breeding were approved by the Institutional Animal Care and Use Committee (IACUC). Breeding of mice of the C57BL/6 background was carried out to produce litters that were categorized as WT mice with intact viperin expression, and as KO mice whose viperin gene was knocked out and replaced with a neomycin-resistance gene. Genotyping of the mice was based on the detection of viperin (WT) or neomycin-resistance (KO) genes. Genomic DNAs were isolated from 0.5 cm tail snips, and subjected to classical PCR (Promega) with primers targeting the viperin gene (5'-CGCCCCCGACACAGACAAGATCAG-3' and 5'-CTGGAGGAGAACCTTCCTTCCAGG-3') and the neomycin-resistance gene (5'-GATGGATTTGCCGAGCTTCTC-3' and 5'-CGGCTTGCCATCCGAGTGC-3'). The amplified PCR products were analysed by agarose gel electrophoresis, with diagnostic amplicon sizes of 282 bp for viperin and 421 bp for neomycin.

Infection of mice and processing of lungs. Mice were grouped according to their genotype (WT or KO), age and weight. Mice were anaesthetized with a mixture of 7.5 mg ketamine ml⁻¹ (Parnell Laboratories) and 0.1 mg medetomidine ml⁻¹ (Pfizer Animal Health), and infected with 10⁷ or 10⁹ p.f.u. WSN33 or 500 p.f.u. A/PR/8/34 virus via the intratracheal route. Uninfected-control WT and KO mice were given PBS instead. Mice were monitored daily for weight loss and general status. For the survival study, mice were euthanized in a CO2 chamber when they reached the end point of 30% weight loss, or when they were in moribund condition, or upon attaining day 14, in compliance with IACUC guidelines. For the time-course study, mice were euthanized on 3, 5, 7 and 9 days p.i. Their lungs were harvested, approximately 10% of each lung was stored at –80 °C, and then homogenized with a gentleMACS tissue dissociator (Miltenyi Biotec) to facilitate RNA extraction. The lung homogenates were subjected to virus plaque assay and real-time RT-PCR for mRNA expression.

Histopathological staining of murine lungs. The remaining 90% of each lung was fixed in 4% formalin, dehydrated in ascending ethanol concentrations, embedded in paraffin, sectioned into 4 μm slices, and stained with haematoxylin and eosin (H&E; Thermo Scientific/Merck). The scoring of lung damage was performed by an experienced pulmonary histopathologist (J. E. Seet), based on a modified scoring system (Matute-Bello et al., 2001), i.e. percentage significant lung damage × [alveolar haemorrhage +2(alveolar infiltrates) +3(fibron)+ alveolar septal congestion]. The four criteria were then scored on a scale of 0–3. The scoring was performed based on the worst-affected areas of the lungs, and we determined the percentages of the significantly damaged regions (defined as at least two criteria with a maximum score of 3; the whole lung was defined as 100%).

Viral plaque quantification assay. MDCK cells were seeded into 24-well plates, and incubated at 37 °C for 24 h. Samples were diluted serially from 10⁻¹ to 10⁻⁶ before infecting the cells, incubated at 35 °C for 1 h, and the viral inocula were discarded from the wells. Stock suspension of Avicel (FMC Biopolymer) was prepared by dispersing 6 g Avicel powder in 250 ml water, and autoclaved. MEM, HEPES and NaHCO₃ were then added to the Avicel suspension. Avicel overlay was then added to each well, incubated at 35 °C for 65–72 h, and the cells were fixed with 8% formalin (Sigma-Aldrich) and stained with 1% crystal violet (Merck). The number of p.f.u. was calculated based on the dilution factor. Each virus titre was normalized to the amount of protein present in the lung homogenate as measured by the Bradford protein assay (Narasaraju et al., 2011).

Real-time quantitative RT-PCR. RNAs were isolated from cell cultures and lung homogenates using an RNeasy RNA purification kit (Qiagen), and reverse-transcribed with MMLV reverse transcriptase (Promega). The resultant cDNAs were subjected to real-time RT-PCR analysis with SYBR Green PCR mix in a LightCycler (Roche). Primers targeting viperin (5'-CTTCAACGTTGACGAGACA-3' and 5'-GAGCTCTCAAGAATGTTTCA-3'), IPN-β (5'-CACAGGGCATGAAAGAT-3' and 5'-TTGAGCTGAGCTGATTAT-3'), and GAPDH (5'-ATGAGTGTCTCAACTGCTTC-3' and 5'-GATATTCACTACGATCTCCG-3'), were used for detection of these genes at different time points (Leong & Chow, 2006; Moltedo et al., 2009).

Neutralizing antibody assay. MDCK cells were seeded into 96-well plates, and incubated at 37 °C for 24 h to obtain confluent monolayers. Each serum sample was subjected to twofold serial dilutions with medium starting from 1:8 dilution. An equal volume containing 100 TCID₅₀ WSN33 virus was incubated with each serum dilution at 35 °C for 2 h. Cells were washed three times before adding serum-free medium, inoculated with 50 μl of each virus–serum mixture, and incubated at 35 °C for 72 h. The neutralizing antibody titre was defined as the reciprocal of the highest dilution of the serum at which the infectivity of the virus was neutralized completely in 50% of the wells (Lee et al., 2010).

Statistical analyses. Results are represented as means ± SD. Statistical analyses were performed using spss and GraphPad Prism.
i.e. Student's t-test for comparison of means, and Kaplan–Meier's logrank survival analysis.

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