Human respiratory syncytial virus infection is inhibited by IFN-induced transmembrane proteins

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The IFN immune system plays an essential role in protecting the host against most viral infections. In order to explore the interactions between the IFN pathway and human respiratory syncytial virus (RSV) infection, and to identify potential IFN-stimulated genes (ISGs) that may be involved in suppressing the replication of RSV, we utilized an IFN pathway-specific microarray to study the effects of RSV infection on the IFN pathway in HeLa cells. We showed that RSV infection enhanced the expression of a series of ISGs, including oligoadenylate synthetase 2, IFITM1 (IFN-induced transmembrane protein 1) and myxovirus resistance 2. Our results also showed that the IFITM proteins potently inhibited RSV infection mainly by interfering with both virus entry and the subsequent replication steps, but not the attachment process. The antiviral effect of IFITM3 was not affected by ubiquitination modification. Furthermore, knocking down the endogenous and IFN-induced expression of IFITM1 and 3 facilitated RSV infection. Expression of the IFITM proteins was found to delay the phosphorylation of IFN regulatory factor 3 through interfering with the detection of viral RNA by MDA5 (melanoma differentiation-associated gene 5) and RIG-I (retinoic acid-inducible gene I). These results demonstrated that the restriction of RSV infection by the IFITM proteins was achieved through the inhibition of virus entry and replication, and they provided further insight for exploring the mechanism of IFITM-protein-mediated virus restriction.

INTRODUCTION

As the first line of defence against virus infection, the innate immune system in hosts utilizes multiple cellular receptors to discern different kinds of virus-specific factors (Garcia-Sastre & Biron, 2006). The recognition of viral infection further triggers downstream signalling pathways that ultimately lead to the production of type I IFNs (Sen, 2001). Type I IFNs constitute a class of secretory cytokines that demonstrate multiple functions, including antiviral defence, antitumour activity, cell growth regulation and immunomodulation (Sadler & Williams, 2008). Once synthesized and secreted, IFNs bind to IFN receptors present on the cell surface through autocrine or paracrine mechanisms and subsequently stimulate the Jak–STAT (Janus kinase–signal transducer and activator of transcription) signalling pathway, leading to the upregulation of hundreds of IFN-stimulated genes (ISGs). These ISGs set up an immune response against the invading pathogens (Jewell et al., 2007). Only a few of the ISGs possessing a viral restriction function have been well characterized in terms of their antiviral mechanisms. These include the 2’-5’-OAS (2’-5’-oligoadenylate synthetase)/RNaseL system, protein kinase R and Mx (myxovirus resistance) genes (Sadler & Williams, 2008). Recently, one family of ISGs, called IFN-inducible transmembrane (IFITM) proteins, and known to play diverse roles in cell signalling, adhesion, tumour genesis and immune regulation, was found to have the ability to restrict the cell entry and replication of a number of viruses. Most of the viruses restricted by the IFITM proteins are enveloped viruses, such as influenza A, dengue virus and West Nile virus (Brass et al., 2009; Weidner et al., 2010; Huang et al., 2011; Lu et al., 2011; Raychoudhuri et al., 2011; Mudhasani et al., 2013), but reovirus, which is a non-enveloped dsRNA virus, was also recently reported to be sensitive to IFITM3 repression (Anafu et al., 2013). IFITM3 has been reported to restrict cell infection of RSV and control disease pathogenesis in the Ifitm3 knockout mouse model (Everitt et al., 2013). The complete nature of the antiviral mechanisms of the IFITM proteins has not been elucidated. Reports have demonstrated that IFITM proteins restrict virus infection independently of receptor usage, but occur in late endosomes or influence virus trafficking from endosomes to lysosomes to reduce virus infection indirectly (Diamond & Farzan, 2013). IFITM3 was also shown to reduce the pathogenesis
of influenza in mice and humans (Everitt et al., 2012). Ubiquitination of IFITM3 can occur in both Lys48-linked and Lys63-linked polyubiquitin, and further impact the restriction effect of IFITM3 on influenza virus (Yount et al., 2012).

*Human respiratory syncytial virus* (RSV) belongs to the family Paramyxoviridae and is an enveloped virus with a negative-sense ssRNA genome (McCarthy & Hall, 2003). As a major viral cause of lower respiratory tract infection in infants and children worldwide, RSV infection often leads to mild, cold-like symptoms. Severe symptoms are usually restricted to the very young and elderly (Falsey et al., 1995; Handforth et al., 2000). Currently, there is no effective vaccine against RSV infection. The ability of RSV to induce IFN was shown to be weak compared with that of other RNA viruses (Lo et al., 2005). However, RSV was also found to induce high levels of IFN-α expression in different subsets of dendritic cells, and of IFN-β in cultures of various human respiratory epithelial cells and fibroblasts (Guerrero-Plata et al., 2005). Thus, the roles of type I IFNs in RSV infection, and the interaction between RSV and the IFN pathway, are complex processes that have not been elucidated fully.

In this study, an IFN-α/β response PCR array analysis was utilized to study the effects of RSV infection on 84 genes related to the type I IFN pathway and it was shown that RSV infection elevated the expression of multiple ISGs. The potential antiviral effects of IFITM proteins on RSV were investigated and the results revealed that IFITM proteins could suppress RSV infection at an early stage after virus entry, with the replication process being the most probable main target of restriction. Furthermore, IFITM3 expression delayed the phosphorylation of IFN regulatory factor 3 (IRF3) caused by RSV infection. This study identified new ISGs that could restrict RSV infection and offered a direction for discovering the mechanisms of IFITM protein control of virus infection.

**RESULTS**

**Type I IFNs inhibit RSV infection**

The effects of type I IFNs on RSV infection were investigated by preincubating cells with different concentrations of type I IFNs followed by RSV infection. As shown in Fig. 1(a), infection of RSV was partially repressed by low concentrations of IFNs (100 and 500 IU ml\(^{-1}\)) and the repression was increased with increasing levels of IFNs. At 2000 IU ml\(^{-1}\), RSV was unable to replicate normally in both HeLa and Hep2 cells. However, Hep2 cells pretreated at the lower doses of IFNs produced less inhibitory effect than the corresponding HeLa cells (Fig. 1b). These results showed that RSV replication was inhibited by type I IFN pretreatment in a dosage-dependent pattern.

When virus infection and IFN treatment were conducted simultaneously, the viral RNA levels showed that IFNs produced an inhibitory effect on RSV at nearly all concentrations of IFN-β used in both cell lines (Fig. 1c, d). However, compared with cells that were infected after IFN pretreatment, low doses of IFN-x2b produced lower inhibitory effects, but high doses still inhibited strongly. Similar to the pretreated cells, the restriction effect of IFN-β was more pronounced than IFN-x2b at the same concentrations in both cell lines. These results suggested that cell infection accompanied by IFN treatment generally reduced the inhibitory effects of IFN. Next, an MTS [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] cytotoxicity assay was performed to evaluate the potential cytotoxic effects of the IFNs on cells. The results indicated that IFNs had no obvious cytotoxic effects on cells (data not shown). These results suggested that the inhibitory effect of IFN treatment could be the result of certain ISGs induced by the IFN and functioning as antiviral proteins acting on RSV infection.

**Effects of type I IFNs and RSV on the IFN pathway**

An IFN-α/β response microarray analysis was conducted to obtain a comprehensive monitor of the changes of 84 type I IFN pathway-related genes upon IFN treatment and RSV infection in HeLa cells. All array data are listed in Table S1 (available in the online Supplementary Material).

The majority of genes detected were upregulated in the IFN-treated non-infection group as compared with the control group, with 10 out of the 84 genes showing significant changes, including several ISGs with known antiviral functions. Only SLC1A2 [solute carrier family 1 (glial high affinity glutamate transporter), member 2] was downregulated. In contrast, only a small number of genes were upregulated in cells infected with RSV alone. Interestingly, again only SLC1A2 was downregulated. This result indicated that RSV infection entailed certain repression effects on the IFN pathway (Fig. 2a, Table S1). The gene regulation pattern in IFN-pretreated RSV-infected cells was similar to that in the cells treated with IFN alone except for the downregulation of OAS2 and strong induction of STAT1 (Fig. 2b, Table S1). Compared with IFN-treated cells, the upregulation of genes induced by IFN (IFN-treated group compared with control group) was mostly abolished in IFN-pretreated RSV-infected cells, with only STAT1 being enhanced (Fig. 2c, Table S1). The array results confirmed that several ISGs with well-known antiviral function could be regulated by IFN treatment and RSV infection, including the broad-spectrum antiviral gene products OAS2 and IFITM1. The restriction effect of the OAS family on RSV replication was reported previously (Lin et al., 2009). Thus, the IFITM family was selected here for further studies.

**Induction of IFN-induced Ifitm gene expression by RSV**

The effects of RSV infection on *Ifitm* genes was further explored in HeLa and Hep2 cells. Consistent with the array...
results, mRNA levels of Ifitm1 and Ifitm2/3 increased upon IFN treatment in both cell lines (Fig. 3a, b). RSV infection without IFN treatment also enhanced the mRNA of Ifitm1 and Ifitm2/3 compared with those in mock-infected control cells in both cell types (Fig. 3a, b). The protein level of IFITM1 increased significantly upon RSV infection.

Fig. 1. The inhibitory effect of type I IFNs on RSV infection. (a) HeLa and (b) Hep2 cells were pretreated with 100, 500, 1000 and 2000 IU IFN-α2b/β/ml for 24 h without culture medium substitution. Cells were then infected with RSV (m.o.i. 0.1) for another 48 h. (c) HeLa and (d) Hep2 cells were treated with the same concentrations of IFNs as in (a, b), and simultaneously infected with RSV (m.o.i. 0.1) for 48 h. Cells and culture supernatants were collected for RNA extraction and viral RNA levels were measured using absolute quantitative real-time (qRT)-PCR. Copies represent the load of virus RNA in 100 ng RNA. Significant difference from control cells: *P<0.05; **P<0.01. Numbers below columns indicate dose of IFN added to medium (IU ml⁻¹). n=3.
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and IFN treatment, whereas levels of IFITM2/3 showed no obvious change due to the basal high levels of both proteins in HeLa (Fig. 3c) and Hep2 (Fig. 3d) cells. The influence of RSV infection on IFITM protein expression was examined by immunofluorescence in HeLa cells, which confirmed the significant upregulation of IFITM1 expression induced by RSV infection, whilst IFITM2/3 displayed a slight increase upon RSV infection (Fig. 3e). These results demonstrated that RSV infection enhanced the status of IFITM protein expression.

IFITM proteins suppress RSV infection

Based on these results, the restriction effect of IFITM proteins on RSV infection was explored further. To this end, cells were transfected to express IFITM1, 2 or 3 followed by RSV infection. Quantitative real-time (qRT)-PCR results demonstrated that transient expression of IFITM proteins was significantly inhibited on RSV infection in both HeLa and Hep2 cells, suggesting that the inhibitory effect observed was independent of cell type. At both m.o.i. tested, the restriction effect of IFITM proteins on RSV was similar in HeLa cells, whereas increasing the viral load weakened the restriction effect of all IFITM proteins in Hep2 cells (Fig. 4a–d). To confirm the inhibition effects and facilitate further work on exploring the anti-RSV mechanism of IFITM proteins, doxycycline-inducible IFITM-protein-expressing HeLa cell lines were established (Fig. 4e). The inhibition of RSV infection by IFITM proteins was verified by flow cytometry. All of the IFITM proteins shifted the mean fluorescence intensity peak to the left, which indicated the percentage of RSV-positive cells decreased compared with the control group (Fig. 4f). Thus, these data suggested that expression of IFITM1, 2 or 3 could suppress RSV infection.

Restriction of RSV entry and replication by IFITM proteins

To further study which stages of the RSV life cycle were affected by IFITM proteins, we focused first on the attachment process. The qRT-PCR results demonstrated that there was no significant difference in the amount of virus RNA between control and IFITM-overexpressing cells (Fig. 5a). Thus, the attachment of RSV to cells could not be affected by IFITM proteins, we focused first on the inducible IFITM-protein-expressing HeLa cell lines were established (Fig. 4e). The inhibition of RSV infection by IFITM proteins was verified by flow cytometry. All of the IFITM proteins shifted the mean fluorescence intensity peak to the left, which indicated the percentage of RSV-positive cells decreased compared with the control group (Fig. 4f). Thus, these data suggested that expression of IFITM1, 2 or 3 could suppress RSV infection.

Fig. 2. Effect of RSV infection on the IFN-α/β response array. HeLa cells were divided into four groups for type I IFN and/or virus infection as described in Methods. RNA was extracted from each group for reverse transcription and the expression of 84 genes was assessed using an IFN PCR array based on the relative qRT-PCR method. Fold changes of each gene were compared between two groups: (a) IFN-treated group and RSV-infected group versus control group and (b) IFN-pretreated RSV-infected group versus IFN-treated group. The results of fold changes for all genes are listed in Table S1.
Fig. 3. RSV infection enhanced IFN-induced *Ifitm* gene expression. (a) HeLa and (b) Hep2 cells were differently treated with IFN or RSV as described in the array. Total RNA was extracted at 72 h p.i. from half of the cells, and the mRNA levels of *Ifitm1* and *Ifitm2/3* were measured by relative qRT-PCR. (c, d) The other half of the cells was used for protein extraction and Western blotting was conducted to detect the change of protein level of each IFITM protein. GAPDH was used as control. (e) HeLa cells
investigated in IFITM-expressing cells infected with RSV at 0, 15 and 30 min post-incubation (p.i.) using qRT-PCR. The viral RNA was only decreased in IFITM3-expressing cells at 30 min p.i. at 37 °C (Fig. 5b). These results indicated that the entry of RSV was partially repressed by IFITM3. When cells were infected by RSV (m.o.i. 1) for 2 h prior to the induction of IFITM proteins for another 24 h to bypass the entry process, the amounts of RSV viral RNA were still evidently affected by all of the induced IFITM proteins (Fig. 5c). However, the inhibition effect was weakened compared with pre-expression of IFITM proteins (Fig. 4a). These results indicated that the restriction effect of IFITM proteins likely targeted an early step in RSV infection.

To investigate whether the replication process was affected by IFITM proteins, a time-course experiment was performed. As shown in Fig. 5(d), the replication of RSV started rapidly at 1 h p.i. with the peak beginning at 9 h p.i. All IFITM proteins demonstrated a strong repressive effect at the beginning of replication, which indicated that the inhibition pattern of IFITM proteins on RSV was rapid after infection. Amongst the three IFITM proteins, IFITM3-mediated restriction was more apparent than IFITM1 and 2. Therefore, it was suggested that IFITM proteins could inhibit RSV replication and target early events of the viral life cycle following viral internalization until viral replication, and the replication process was the main target.

Knockdown of IFITM proteins restores RSV infection

To confirm the restriction results of IFITM proteins on RSV infection, the effect of IFITM protein knockdown on RSV infection was investigated. Cells stably transfected with small hairpin RNA (shRNA) targeting IFITM1 and 3 significantly reduced the expression of these proteins with or without IFN treatment, whilst shRNA targeting IFITM2 had no obvious knockdown effect (Fig. 6a). Each knockdown cell line was infected with RSV, and the qRT-PCR results showed that RSV infection was enhanced in IFITM3-depleted cells by 4.5-fold but in IFITM1-depleted HeLa cells had no obvious impact as only a 10% increase of viral load was observed, which might have been due to the low basal level of IFITM1 in HeLa cells. Knockdown of the basal level of IFITM2 had no effect on RSV replication (Fig. 6b). When knockdown cell lines were pretreated with IFN-β for 24 h prior to RSV infection, the results showed that IFITM3 knockdown resulted in ~5.5-fold increase of RSV viral load and the increase of RSV was extended to 12.5-fold in IFITM1-depleted cells. However, the shRNA targeting IFITM2 only increased replication of RSV slightly (Fig. 6c). Collectively, these results suggested that downregulating the expression of IFITM proteins, especially IFITM1 and 3, permitted the enhancement of RSV infection, and the inhibition of replication by type I IFNs was due in part to an increase in the expression of IFITM proteins.

Impact of ubiquitination on anti-RSV activity of IFITM3

As the ubiquitination modification impacts the restriction effect of IFITM3 on influenza virus, ubiquitin points of IFITM3 were mutated and the impact on RSV replication was investigated. The complete loss of ubiquitinated bands was achieved when all four lysine residues (Lys24, Lys83, Lys88 and Lys104) were mutated to alanine (UbΔIFITM3); however, the protein level of ubiquitination-deficient IFITM3 was not increased compared with WT IFITM3 (Fig. 7a, b), which indicated ubiquitination of IFITM3 did not promote the degradation of proteins. The influence of ubiquitination on the anti-RSV activity of IFITM3 was investigated. Expression of both WT and UbΔIFITM3 resulted in an apparent reduction of RSV infection; however, the restriction effects of WT and UbΔIFITM3 were similar at both m.o.i. and in both cell lines (Fig. 7c, d). These results suggested that ubiquitination on IFITM3 affected neither the protein stability nor its anti-RSV function.

IFITM3 delays the phosphorylation of IRF3 upon RSV infection

The RLR [RIG-I (retinoic acid-inducible gene I)-like receptor] pathway, characterized by the cytoplasmic receptors RIG-I and MDA5 (melanoma differentiation-associated gene 5), plays significant roles in defending against RSV infection (Aaron et al., 2012; Ling et al., 2009). Could obstruction by IFITM proteins on RSV further delay the whole infection cycle of RSV, and reduce the probability of RSV RNA contact with MDA5 and RIG-I receptors? As shown in Fig. 8(a), RSV infection induced the phosphorylation of IRF3 in HeLa cells. The increased phosphorylation level began at 6 h in the control group, but was delayed to 8 h upon IFITM3 overexpression. The results indicated that IRF3 phosphorylation induced by RSV infection was delayed by IFITM3 expression. Next, the viral RNA captured by MDA5 and RIG-I receptors after RSV infection was investigated. As shown in Fig. 8(b, c), RSV RNA levels showed no obvious difference in MDA5 and RIG-I immunoprecipitates at 2 h p.i. in the IFITM3-overexpressing cells and the control cells. However, viral RNA levels captured by both MDA5 and RIG-I were reduced in IFITM3-expressing cells at 6, 12 and 24 h p.i. compared with the control cells. Results
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![Graphs and diagrams showing RNA virus replication and IFITM expression levels.](https://example.com/figure1.png)

**Figure 1:**

(a) Copy numbers of RSV (m.o.i. 0.1) in HeLa cells. **(b)** Copy numbers of RSV (m.o.i. 0.1) in Hep2 cells. **(c)** Western blotting of IFITM expression. **(d)** Western blotting of GAPDH expression. **(e)** Western blotting of IFITM expression with Doxycycline treatment. **(f)** FACS analysis of IFITM expression and virus counts.

**Graphs**: The graphs show the copy numbers of RNA virus replication in HeLa and Hep2 cells with different IFITM expression levels. The x-axis represents the different treatments (Control, IFITM1, IFITM2, IFITM3, Mock), and the y-axis represents the copy numbers.

**Western Blotting**: The western blotting images show the expression levels of IFITM and GAPDH proteins under different conditions.

**FACS Analysis**: FACS analysis shows the distribution of virus counts and IFITM expression levels in different groups.
of Western blotting (Fig. 8d) indicated expression of IFITM3, MDA5 and RIG-I in the immunoprecipitates and inputs. These results demonstrated that IFITM3 hindered virus RNA detected by receptors, which further contributed to postponing IRF3 phosphorylation after RSV infection.

**Fig. 4.** IFITM proteins restricted RSV infection. (a) HeLa and (b) Hep2 cells transfected to express HA-tagged IFITM proteins were infected with RSV at different m.o.i. for 2 h and then maintained in growth medium for 72 h. Culture supernatants and cells were harvested, and the viral level was assayed using absolute qRT-PCR targeting the N gene of RSV. Significant difference from control cells: **P<0.01. GAPDH was used as control. n=3. (c, d) IFITM protein expression in cells was assayed by Western blotting using anti-HA antibody. (e) HeLa cells were treated with doxycycline (1 μg ml⁻¹) for 24 h and levels of IFITM protein expression were confirmed by Western blotting. GAPDH was used as control. (f) HeLa cells expressing IFITM proteins were treated with doxycycline (1 μg ml⁻¹) for 24 h prior to infection with RSV at m.o.i. 1. At 24 h p.i., RSV-positive cells were detected by flow cytometry.

**Fig. 5.** IFITM proteins suppressed RSV entry and replication. (a) HeLa cells were transduced with doxycycline (1 μg ml⁻¹) for 24 h. The cells were then incubated with RSV (m.o.i. 5) for 1 h on ice to allow attachment without endocytosis. After extensive washing with cold PBS, cells were collected for RNA extraction and qRT-PCR. (b) To investigate the entry process, HeLa cells were transduced with doxycycline (1 μg ml⁻¹) for 24 h, and then incubated with RSV for 0, 15 and 30 min at 37 °C. After washing with PBS to remove any virus that had not entered into the cytoplasm, intracellular viral RNA was extracted and measured using qRT-PCR. (c) HeLa cells were infected with RSV (m.o.i. 1) for 2 h followed by the addition of doxycycline (1 μg ml⁻¹) for another 48 h. Cells and supernatants were collected for RNA extraction, and viral RNA was detected by absolute qRT-PCR. (d) HeLa cells were transduced with doxycycline (1 μg ml⁻¹) for 24 h and then incubated with RSV (m.o.i. 5). Cells were collected hourly from 0 to 11 h p.i. and intracellular viral RNA was measured by absolute qRT-PCR. The results are expressed as copies per 100 ng RNA. Significant difference from control cells: *P<0.05; **P<0.01. n=3.
DISCUSSION

In this study, an IFN-α/β response microarray analysis demonstrated that IFN treatment and RSV infection in HeLa cells could stimulate expression of multiple ISGs by different degrees, which could explain the sensitivity of RSV to IFN pretreatment. The IFITM protein family was chosen, and its profound restriction effects on RSV entry and replication were discovered. IFITM proteins were expressed ubiquitously in both the membrane and cytoplasm in many tissue and cell types, and were strongly induced by type I IFNs (Lu et al., 2011). Compared with other viruses inhibited by IFITM proteins, the restriction of RSV was more profound than for human immunodeficiency virus, hepatitis C virus and influenza virus A at the same m.o.i. (Huang et al., 2011; Lu et al., 2011; Raychoudhuri et al., 2011). However, Rift Valley fever virus infection also displayed intensive inhibition by IFITM3 (Mudhasani et al., 2013). The different replication properties and IFN sensitivity of these viruses might contribute to their different sensitivity to the resistance of IFITM proteins. The knockdown results confirmed the vital role of IFITM proteins on the IFN-mediated antiviral effect and also suggested that other ISGs might participate in the restriction of IFNs in RSV infection. Therefore, IFITM inhibition might be important under natural infection conditions in hosts.

The restriction effect of IFITM proteins on RSV was not associated with the attachment process, which is consistent with the results from multiple other viruses restricted by IFITM proteins (Weidner et al., 2010; Raychoudhuri et al., 2011). Previous antiviral research on IFITM proteins also demonstrated that the entry processes of most viruses were sensitive to IFITM proteins, which indicated a similar antiviral role was utilized by IFITM proteins. However, IFITM1 restricted HIV replication without affecting viral entry (Lu et al., 2011). Recent studies indicated that IFITM3 localized to endosomes and lysosomes, leading to alterations in the endosomal compartments, including increased association with clathrin – an important protein utilized by multiple viruses during virus endocytosis (Huang et al., 2011; Yount et al., 2012). However, another report contradicted the results of clathrin-mediated endocytosis of RSV and demonstrated that RSV utilized an actin-dependent, clathrin- and pH-independent macrophagocytosis process to enter cells (Krzyzaniak et al., 2013). The clathrin-dependent manner utilized by RSV is consistent with the finding that the entry process of RSV was one of the targets of IFITM proteins (Kolokoltsov et al., 2007). No direct evidence of IFITM proteins associated with macrophagocytosis has been shown; however, both proteins have a direct association with Rab5, which indicates IFITM proteins may also have the ability to

Fig. 6. Effects of knockdown of IFITM proteins on RSV infection. (a) Ifitm1–3 knockdown cell lines were established as described in Methods. Established cell lines were treated or not treated with 1000 and 2000 IU IFN-β ml⁻¹ for 48 h to assess the expression of each endogenous and IFN-induced expression of IFITM proteins. GAPDH was used as control. (b, c) IFITM protein knockdown cells were incubated without (b) or with (c) 1000 IU IFN-β ml⁻¹ for 24 h and then infected with RSV (m.o.i. 0.1) for an additional 72 h. The viral RNA levels were measured using relative qRT-PCR. n = 3.
influence the macropinocytosis process of RSV (Yount et al., 2012; Krzyzaniak et al., 2013). The deficiency in ubiquitination of IFITM3 enhanced antiviral activity against H1N1 influenza virus through its increased localization on endolysosomal compartments (Yount et al., 2012). However, ubiquitination-deficient IFITM3 showed no enhancement in anti-RSV activity. One possible explanation for this phenomenon is that the ubiquitinated IFITM3 located on the endolysosomal compartments is sufficient for the restriction effect on RSV, so the enhancement of ubiquitin-deficient IFITM3 on endolysosomal compartments had no enhancement effect on the antiviral function (Hsu et al., 2010).

A study on Rift Valley fever virus also proposed that IFITM proteins modulate the biology of the vesicular compartments to affect virus trafficking, which results in the inhibitory effect (Anafu et al., 2013). Our results demonstrated that the phosphorylation of IRF3 was delayed, and viral RNA captured by MDA5 and RIG-I was also reduced upon IFITM3 expression. These results suggest that IFITM proteins imprison or sequester RSV in the endosomes, lysosomes or other membrane vesicular structures during

![Fig. 7. Effects of ubiquitination on IFITM3 antiviral activity. HeLa cells were transfected with WT and UbΔIFITM3 for 24 h. Cells were then lysed for protein extraction. (a) Western blotting was performed using anti-HA (1:3000) and anti-ubiquitin (1:1000) antibodies to detect the expression of WT and UbΔIFITM3, and (b) immunoprecipitation (IP) was performed using anti-HA antibody. (c) HeLa and (d) Hep2 cells transfected to express HA-tagged WT and UbΔIFITM3 for 24 h were infected with RSV at different m.o.i. for 2 h. Culture supernatants and cells were harvested, and the viral RNA level was assayed using absolute qRT-PCR at 72 h p.i. Significant difference from control cells: **P<0.01. n=3.](http://vir.sgmjournals.org)
the entry and endocytosis processes, which further delays the release or migration of viral RNA from the endocytosed vesicles into the cytoplasm or other membrane structures, where replication and immune recognition occurs. However, the impeditive effect of IFITM proteins could not totally explain the inhibition of RSV replication, because RSV infection that bypassed entry and endocytosis during natural infection before IFITM protein induction was also repressed by IFITM proteins. This indicated that other inhibitory mechanisms for the effect of IFITM proteins on the replication process of RSV also exist.

In summary, this study suggested that RSV infection has multiple functions on the type I IFN pathway, including the stimulation of many ISGs, and that this effect was partly due to IFITM proteins which displayed repression during the early stages of RSV infection. This study also discovered the stimulation delay effect on the RLR pathway by IFITM proteins during RSV infection. Further work will be done to explore the exact antiviral mechanism of IFITM proteins and determine whether other ISGs also contribute to type I IFN defence against RSV infection.

**METHODS**

**Cells and virus propagation.** HeLa and Hep2 cells were maintained in Dulbecco’s minimal essential medium (Gibco/Life Technologies) supplemented with 10% FBS (Life Technologies), 100 IU penicillin ml⁻¹, 100 mg streptomycin ml⁻¹ (Life Technologies) and 5% L-glutamine (Gibco/Life Technologies). RSV (ATCC VR-26) was prepared by infecting monolayers of HeLa cells and the virus titre was determined by TCID₅₀.

**Plasmids, antibodies and reagents.** Ifitm genes were amplified from HeLa cells after induction by IFN-α₂b (1000 IU ml⁻¹) for 6 h, and cloned into the pCMV-HA and pTre2-HA vectors. The ubiquitination-deficient HA-Ifitm3 was constructed using a Multi-points mutagenesis kit (TaKaRa). Rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and mouse anti-haemagglutinin (HA)
antibodies were obtained from Sigma-Aldrich. Mouse anti-IFITM1, rabbit anti-IFITM2 and anti-IFITM3, rabbit anti-MDA5, rabbit anti-RIG-I, and rabbit anti-ubiquitin antibodies were all purchased from Proteintech. Rabbit anti-IRF3 and phosphorylated IRF3 (p-IRF3) antibodies were obtained from Cell Signaling Technology. Alexa Fluor 488-conjugated anti-mouse antibody, Alexa Fluor 594-conjugated anti-rabbit antibodies, and HRP-conjugated anti-rabbit and anti-mouse antibodies were all obtained from Santa Cruz Biototechnology. The anti-RSV G-protein antibody was purchased from Millipore. BSA was obtained from Becton Dickinson. Primer sequences used to amplify Ifitm1–3 are available upon request.

**Evaluation of the cytotoxicity effect of type I IFNs by an MTS-based assay.** Cells were grown in a 96-well plate and then incubated with various amounts of IFNs for 24 h, followed by incubation with the MTS labelling reagent for 4 h. (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega). The absorbance of the soluble formazan was measured at 490 nm.

**RNA extraction and qRT-PCR.** Cellular and viral RNAs were isolated using TRIzol reagent (Life Technologies). Total RNA (1 μg) was reverse transcribed with a GoScript Reverse Transcripate Kit (Promega). Two-step qRT-PCR was performed in a SYBR Green assay using an Applied Biosystems Master Mix reagents kit in an ABI 7300 Real-Time PCR system. GAPDH was used as the internal control. All experiments were performed using the following amplification profile: one cycle at 95 °C for 10 min, 40 cycles of 95 °C for 15 s and one cycle at 60 °C for 1 min. Primer sequences used in qRT-PCR are available upon request.

**Evaluation of IFN treatment and RSV infection by IFN PCR array.** HeLa cells were divided into four groups and differentially treated with IFNs and/or RSV (type I IFN-treated group, RSV-infected group, IFN pretreated followed by RSV infection group and mock control group). The total RNA of HeLa cells was extracted 72 h.p.i. using an RNeasy Mini kit (Qiagen) and then treated with RNase-Free DNase (Qiagen) to remove genomic DNA. Reverse transcription using 1 μg total RNA was performed using an RT2 First Strand Kit (Qiagen). Then, a human IFN-α/β response RT2 Profiler PCR array (SuperArray Bioscience) was performed on the ABI 7300 system to analyze the relative expression of 84 key genes involved in the IFN pathway. Data were analyzed using the 2^(-ΔΔCt) method and the SABiosciences RT2 Profiler PCR Array Data Analysis online software. The fold changes of various treatments were compared as described in Table S1.

**Western blotting analysis.** Whole-cell extracts were prepared from cells lysed with lysis buffer containing 50 mM Tris/HisCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% Triton X-100 and Complete Protease Inhibitor Cocktail Tablets (Roche). Cell extracts were subjected to 12% SDS-PAGE, transferred to PVDF membranes (GE) followed by blocking with Tris-buffered saline/Tween 20 containing 5% skimmed milk and then probed with the indicated primary antibody at different dilutions. After three washes, membranes were incubated with 1:5000 HRP-conjugated secondary antibody. The proteins were detected using ECL (GE) reagent.

**Detection of IFITM1–3 expression upon RSV infection by immunofluorescence analysis.** HeLa cells were seeded onto slide chambers and then infected with RSV (m.o.i. 1) for 48 h. Uninfected cells treated with IFN-α2b plus IFN-β (500 IU ml⁻¹) were used as positive control. Cells were fixed in 1% paraformaldehyde and then permeabilized with 0.1% Triton X-100 before being incubated with 5% BSA. The cells were then incubated with the respective antibodies against IFITM1 (1:400) or IFITM2/3 (1:200) and incubated with Alexa Fluor 488-conjugated anti-mouse or Alexa Fluor 594-conjugated anti-rabbit IgGs (1:200). The slides were mounted using DAPI reagent (Calbiochem) and images analysed by confocal microscopy (Leica SP8).

**Establishment of stable cell lines.** To establish the doxycycline-inducing IFITM1–3 stable cell lines, HeLa cells were transduced with pTet plasmid using Lipofectamine LTD reagent (Life Technologies) and selected under G418 (400 μg ml⁻¹) to generate the HeLa-pTet cell lines. Next, each of the HA-tagged IFITM1–3 plasmids, combined with the companion plasmid pTK-Hyg (Clonetech) at a ratio of 10:1, was transfected into the HeLa-pTet cell lines and selected under hygromycin (200 μg ml⁻¹) and G418 (400 μg ml⁻¹). Then, the cell lines were exposed to doxycycline (1 μg ml⁻¹) for 24 h followed by Western blotting with anti-HA antibody to detect the expression of IFITM proteins. IFITM protein knockdown cell lines were established by transfecting cells with shRNA plasmids (OriGene) targeting each of the Ifitm1–3 genes and then selected under puromycin (4 μg ml⁻¹) for 2 weeks. The knockdown efficiency was verified by Western blotting. The sequences of the shRNA targeting Ifitm1–3 and control shRNA were: shRNA-IFITM1, TGCCACAGGAGAATCATGAGGT- GCGCTGTG; shRNA-IFITM2, CCGCAGGAGACTCTGCTGCCGT- ACCATG; shRNA-IFITM3, TCCCTAGCAGATCATGCTATCATG- CATC; and shRNA-control, GCACCTACGACGCTAACATCGA- GTACT.

**Evaluation of the inhibitory effect of IFITM on RSV infection using flow cytometry.** HeLa cells were transduced with doxycycline (1 μg ml⁻¹) for 24 h to express IFITM proteins and then infected with RSV (m.o.i. 1) for 24 h. Cells without RSV infection were used as control. The infected cells were fixed in 4% paraformaldehyde and permeabilized with 1% Triton X-100 solution, and then blocked overnight with 5% BSA in PBS. The samples were then incubated with anti-RSV antibody at a dilution of 1:200 and stained with Alexa Fluor 488-conjugated anti-mouse IgG antibody. The stained samples were measured with a flow cytometer (FACSCalibur; BD).

**Immunoprecipitation experiments.** HeLa cells lines were transduced to express IFITM3 for 24 h and infected by RSV (m.o.i. 1). For immunoprecipitation of endogenous MDA5 and RIG-1, cells were collected and lysed in lysis buffer containing 40 U RNase inhibitor ml⁻¹ (Promega) for protein extraction at 2, 6, 12 and 24 h.p.i. The cell extracts were precleared with protein A/G Sepharose beads (Sigma-Aldrich) for 2 h in the presence of 1 μg rabbit IgG and 0.05% BSA. Then, the supernatants were incubated with 0.1 μg anti-MDA5 or anti-RIG-1 antibodies or rabbit IgG for 6 h at 4 °C followed by incubation with protein A/G beads for 1 h. Beads were washed three times with lysis buffer, and an aliquot was eluted in SDS sample buffer for Western blotting analysis to detect the expression of MDA5 and RIG-1. The remaining beads were incubated with 50 μg proteinase K (Sigma-Aldrich) for 30 min at 37 °C. Co-precipitated viral RNA adsorbed on MDA5 or RIG-1 proteins was extracted by TRIzol reagent, and RSV viral load was determined by qRT-PCR. For immunoprecipitation of endogenous p-IRF3, cells infected with RSV were collected and lysed. Endogenous p-IRF3 was immunoprecipitated with 0.1 μg p-IRF3 antibody followed by absorption on protein A/G beads for 6 h at 4 °C. Western blotting was utilized to detect p-IRF3 levels. For immunoprecipitation of ubiquitinated IFITM3, cells were transfected with HA-tagged WT IFITM3 and ubiquitination-deficient IFITM3 (UbAIFITM3) for 24 h, cell extracts were incubated with anti-HA agarose (Sigma) for 6 h at 4 °C, and Western blotting was performed using anti-IRF3 and anti-ubiquitin antibodies.

**Statistical analysis.** Measurements were compared using a one-way ANOVA. Statistical significance comparisons were calculated using Student’s t-test. Values were considered statistically significantly different at P<0.05. Data from all experiments are presented as the mean ± SEM.
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