Original Research *Highlight article*

Toll-like receptor 3 signaling induces interferon-induced transmembrane protein 1 in BEAS-2B cells

Masaki Dobashi[1], Toshihiro Shiratori[1], Tadaatsu Imaizumi[2], Shun Hashimoto[3], Shogo Kawaguchi[2], Kazuhiko Seya[2] and Sadatomo Tasaka[1]

[1]Department of Respiratory Medicine, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan; [2]Department of Vascular Biology, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan; [3]Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan
Corresponding author: Masaki Dobashi. Email: h19gm143@hirosaki-u.ac.jp

Impact Statement

Interferon-induced transmembrane protein 1 (IFITM1) exerts antiviral properties by blocking the virus entry into host cells, but IFITM1 expression in human bronchial epithelium is not well known. We examined IFITM1 expression in cultured BEAS-2B cells incubated with poly I:C, an agonist for TLR3. Poly I:C-upregulated IFITM1 expression was time- and concentration-dependent. Using specific siRNAs, we also revealed that knockdown of interferon-β (IFN-β) or interferon-stimulated gene 56 (ISG56) decreased the expression level of IFITM1. TLR3, IFN-β, and ISG56 may contribute to poly I:C-mediated IFITM1 induction. The findings of this study help elucidate the mechanism of antiviral reactions in bronchial epithelial cells.

Abstract

The human bronchial epithelium plays a crucial role in mediating antiviral immune reactions. When double-stranded RNA (dsRNA) binds to the receptor named Toll-like receptor (TLR) 3, activation of antiviral innate immune reactions is initiated by producing interferon (IFN) type I. Then, type I IFN promotes the transcription of IFN-stimulated genes (ISGs). Proteins encoded by ISGs reveal antiviral effects. The IFN-induced transmembrane protein 1 (IFITM1) is an ISG family member that inhibits viral infection by preventing the entry of viruses with a cell membrane. However, IFITM1 expression in human bronchial epithelium remains largely undetermined. Here, we investigated whether IFITM1 is expressed in cultured BEAS-2B bronchial epithelial cells. Polyinosinic:polycytidylic acid (poly I:C) was used for treatment of BEAS-2B as a TLR3 ligand. IFITM1 expression levels were measured using reverse transcription–quantitative PCR and Western blotting. Using RNA interference, we determined the significance of IFN-β and ISG56 on IFITM1 upregulation. Poly I:C treatment significantly upregulated IFITM1 expression in BEAS-2B cells, and it was concentration- and time-dependent. Knockdown of IFN-β or ISG56 decreased poly I:C-induced IFITM1 expression levels. Recombinant IFN-β also increased expression levels of IFITM1. In BEAS-2B cells, IFITM1 expression is upregulated by poly I:C, at least partly, via the TLR3/IFN-β/ISG56 axis. Thus, IFITM1 may contribute to antiviral innate immunity in bronchial epithelium.

Keywords: BEAS-2B cells, TLR3, IFN-β, ISG56, IFITM1

***Experimental Biology and Medicine* 2022; 247: 1917–1922. DOI: 10.1177/15353702221121609**

Introduction

Toll-like receptor 3 (TLR3), an antiviral member of the TLR family, is a receptor which recognize double-stranded RNAs (dsRNAs). dsRNA is a molecular pattern associated with viruses. TLR3 plays crucial roles in defense against viral infections. Viral dsRNA binding to TLR3 triggers the expression of interferon (IFN) type I. These are fundamental cytokines contribute to host defense, inducing production of IFN-stimulated genes (ISGs). It is well known that human bronchial epithelial cells express TLR3,[1] and IFN-β is the primary type I IFN that contributes to antiviral responses in these cells.[2]

IFN-induced transmembrane proteins (IFITMs) are ISGs family proteins bound to the plasma membrane that have been reported to inhibit infection of HCV,[3] influenza A virus,[4] dengue virus, West Nile virus,[5] and HIV.[6] IFITM1 is one of the IFITM proteins, which was shown to inhibit various types of viral infections by preventing entry of viruses with cell membrane.[7] ISG56, another member of ISG family, is an IFN-induced protein with tetratricopeptide repeats.[8] ISG56 exerts various functions against viral infection, including the regulation of gene expression and translation, as well as cell migration, proliferation and death.[8] In human bronchial epithelial cells, ISG56 expression is stimulated by viral infections notably with human metapneumovirus and

influenza.[9–11] ISG56 was also found to function for defense against infection of viruses in bronchial epithelial cells.[12]

BEAS-2B are cell line of non-cancerous human bronchial epithelial cells,[13] which provides a proper model for investigating innate immunity. ISG56 is induced by polyinosinic–polycytidylic acid (poly I:C), an activator of TLR3, and enhances CXCL10 expression in BEAS-2B.[12] However, it remains unclear whether IFITM1 is expressed in bronchial epithelial cells via TLR3 activation. In this study, IFITM1 expression was investigated in BEAS-2B cells incubated with poly I:C. Moreover, it was examined whether IFN-β and/or ISG56 are associated with inducing poly I:C-mediated IFITM1 expression.

Materials and methods

Cell culture

We purchased BEAS-2B cells from ATCC (Manassas, VA, USA) and this cell line is verified to be human bronchial epithelium. The cells were cultured with Dulbecco's Modified Eagle Medium (DMEM)–10% fetal bovine serum (FBS) (Invitrogen, Frederick, MD, USA), and were incubated with 0, 10, 30, and 50 μg/mL of poly I:C (Sigma, St. Louis, MO, USA) in the first experiment. In second experiment, we treated the cells with 30 μg/mL poly I:C to examine time course. We also treated the cells for 16 h with r(h) IFN-β (ProSpec-Tany, Rehovot, Israel) at concentration of 1 ng/mL. In the experiments for RNA interference, BEAS-2B were incubated for 24 in antibiotics-free culture medium. Then, using a Lipofectamine RNAiMAX kit (Invitrogen), transfection was performed using siRNAs (a control siRNA (QIGGEN, Hilden, Germany), IFN-β siRNA (Invitrogen), or ISG56 siRNA (QIAGEN). The cells were incubated for additional 48 h, followed by addition of 30 μg/mL poly I:C to the medium. Subsequently, the cells were incubated as indicated.

Reverse transcription–quantitative real-time PCR (qRT-PCR)

Using the illustraRNA spin kit (GE healthcare, Buckinghamshire, England), RNA was isolated from BEAS-2B. Single-stranded cDNA was transcribed from the RNA template. Oligo$(dT)_{18}$ primer (FASMAC, Kanagawa, Japan) and MMLV reverse transcriptase (Invitrogen) were used. The expression of IFITM1 and ISG56 was examined by qRT-PCR using Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). As a control, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers for the amplification of cDNAs were as follows:

IFITM1-F: 5′-TCGCCTACTCCGTGAAGTCTA-3′,
IFITM1-R: 5′-TGTCACAGAGCCGAATACCAG-3′,
GAPDH-F: 5′-GCACCGTCAAGGCTGAGAAC-3′, and
GAPDH-R: 5′-ATGGTGGTGAAGACGCCAGT-3′.

Western blot analysis

IFITM1, ISG56, and actin protein expression was examined using Western blotting as described.[14] Shortly, after washing the cells with PBS, BEAS-2B were lysed in Laemmli buffer. Lysates of cells were applied to a 5% to 20% gradient PAGE. Proteins were transferred onto a PVDF (Millipore, Darmstadt, Germany). The membrane was treated with anti-IFITM1 (1:2000) (GeneTex, Irvine, CA, USA), anti-ISG56 (1:3000) (GeneTex), or anti-actin (1:3000) (Sigma). The bands for target proteins were visualized using an anti-rabbit IgG antibody, HRP-labeled (MBL, Nagoya, Japan) and a HRP substrate for chemiluminescence (Millipore).

Data analysis

The assays were done at least two times, and qRT-PCR was done in triplicate. The data of qRT-PCR were shown as mean ± standard deviations (SD). For estimation of statistical differences, Student's *t*-test was used.

Results

IFITM1 is upregulated by poly I:C in human BEAS-2B

In unstimulated human BEAS-2B, IFITM1 mRNA expression level was low, whereas IFITM1 protein was not detected in the condition we tested. Poly I:C treatment of BEAS-2B lead to the increased expression of IFITM1 mRNA and protein. IFITM1 expression levels were dependent on concentration and time (Figures 1 and 2). IFITM1 mRNA expression was gradually upregulated after poly I:C treatment until 16 h, followed by partial reduction at 24 h (Figure 2(a)). IFITM1 protein increase was behind that of mRNA and increased up to 24 h (Figure 2(b)).

IFN-β and ISG56 contribute to inducing IFITM1 expression

In BEAS-2B cells, transfection with IFN-β siRNA decreased poly I:C-mediated IFITM1 mRNA/protein upregulation (Figure 3(a) and (b)). Furthermore, stimulating BEAS-2B with r(h)IFN-β increased their IFITM1 mRNA/protein level (Figure 3(c) and (d)). Transfection of the BEAS-2B with a specific ISG56 siRNA also inhibited IFITM1 mRNA/protein expression increase (Figure 4(a) and (b)). ISG56 protein expression was not found in unstimulated BEAS-2B, and poly I:C treatment significantly upregulated ISG56 protein expression as previously reported.[12] Effective knockdown of ISG56 protein upon siRNA transfection was further confirmed in Western blot analysis (Figure 4(b)).

Discussion

We report here that TLR3 signaling leads to IFITM1 upregulation in human BEAS-2B cells. This is the first report showing that IFITM1 is upregulated by a TLR3 ligand in BEAS-2B bronchial epithelial cells. The IFITM1 protein is primarily located on the plasma membrane,[15] where it prevents the entry of viruses into the host cell cytoplasm by changing its plasma membrane conformation. TLR3-mediated IFITM1 expression thus appears crucial to prevent the initial step of viral infection within the airway. IFITM1 is also expressed by glomerular and cerebral microvascular endothelial cells.[16] Therefore, IFITM1 may function as an antiviral molecule across multiple organs.

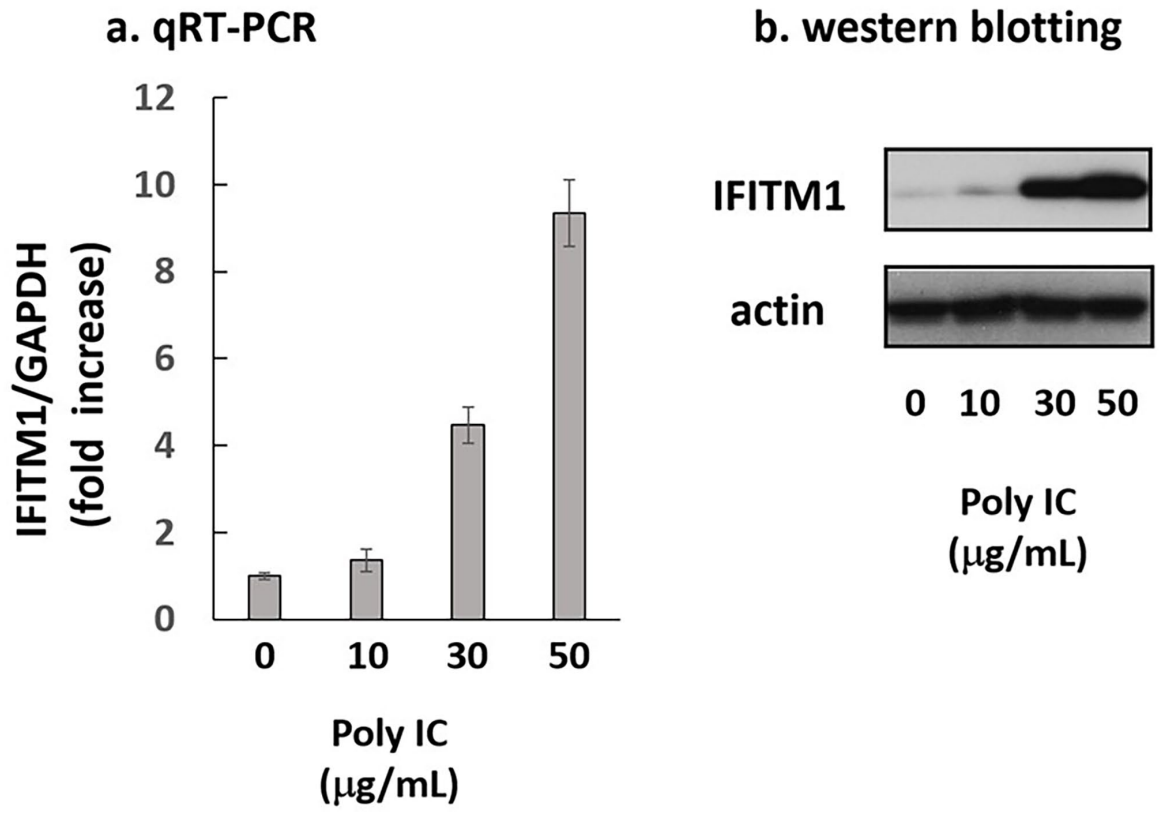

Figure 1. Concentration-dependent IFITM1 upregulation in BEAS-2B treated with poly I:C. (a) Cultured BEAS-2B were incubated in the presence of poly I:C (0, 10, 30, and 50 μg/mL) for 16 h, and RNA extraction was performed. From the RNA extracted, cDNA was reverse-transcribed, and qRT-PCR analysis for IFITM1 as well as GAPDH mRNA was performed. The results are expressed as mean ± standard deviations (SD) (n = 3). (b) BEAS-2B were incubated in the presence of poly I:C as above for 24 h, and the cell were lysed. The lysates were analyzed using Western blotting to measure IFITM1 and actin proteins.

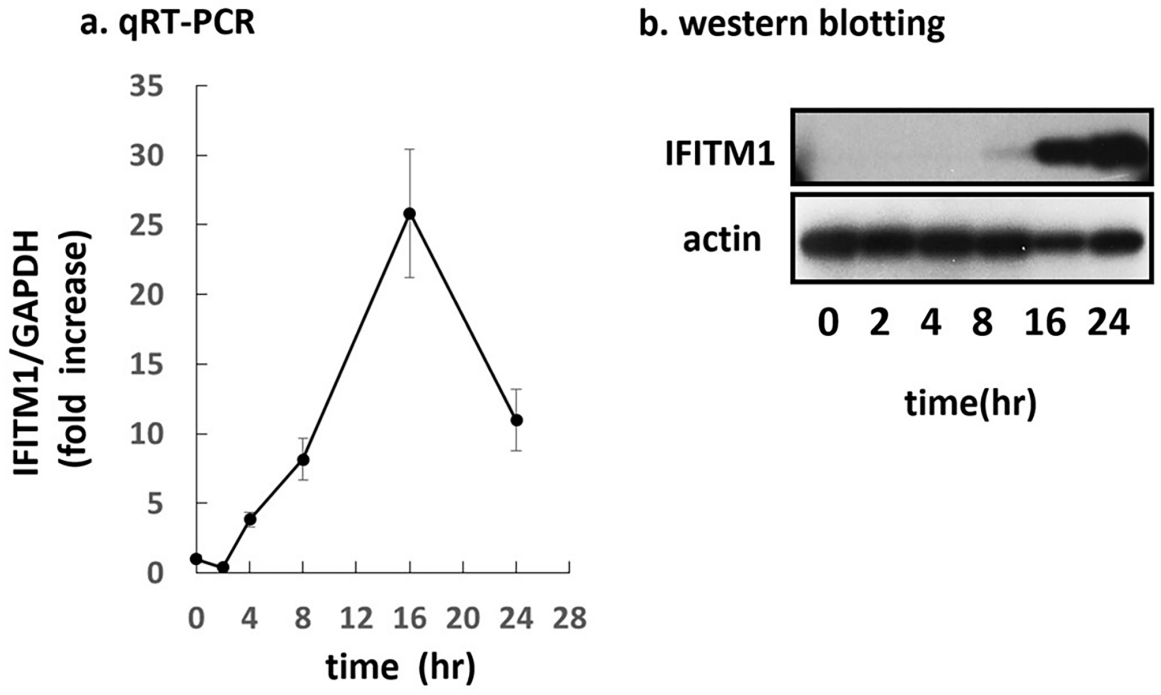

Figure 2. IFITM1 expression in BEAS-2B is increased with poly I:C treatment in a time-dependent fashion. BEAS-2B were incubated for up to 24 h with poly I:C (30 μg/mL). (a) From the cells, RNA was isolated and used for qRT-PCR in order to measure IFITM1 and GAPDH mRNA. (b) After cell lysis, proteins of IFITM1 and actin were analyzed using Western blot technique.

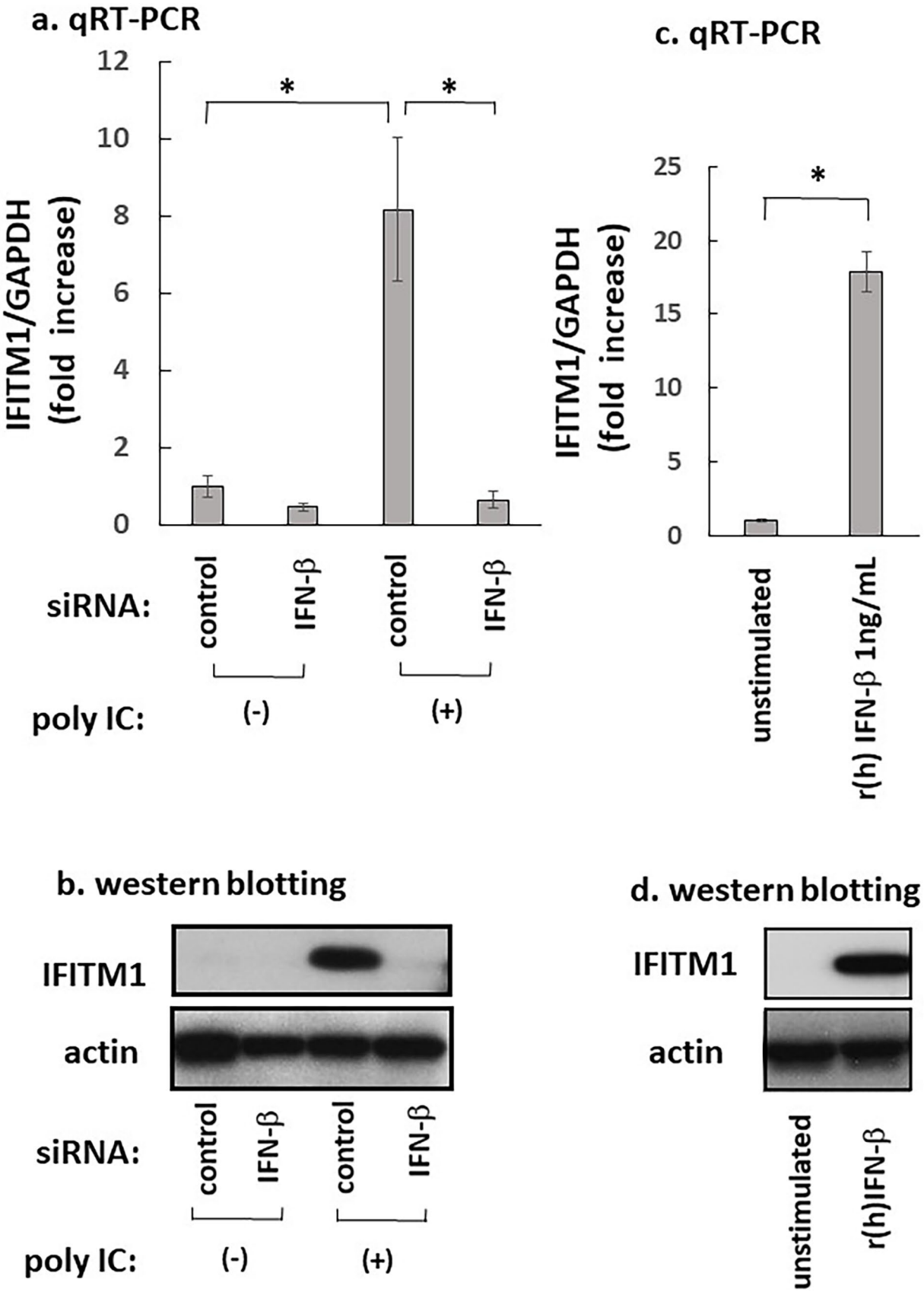

Figure 3. IFN-β contributes to poly I:C-induced increase of IFITM1. (a, b) BEAS-2B cells, transfected with IFN-β-siRNA or non-silencing siRNA, were incubated for 48 h, followed by incubation in the medium containing 30 μg/mL poly I:C. (a) After 16 h incubation, RNA was extracted. Subsequently, qRT-PCR was conducted to measure IFITM1 mRNA expression ($n=3$; *$p<0.01$ by t-test). (b) BEAS-2B cell lysates were obtained after 24 h, and Western blotting was used to estimate IFITM1 protein. (c, d) Effect of addition r(h)IFN-β (1 ng/mL, for 16 h) to the culture medium on IFITM1 level was examined by qRT-PCR (c) and Western blotting (d).

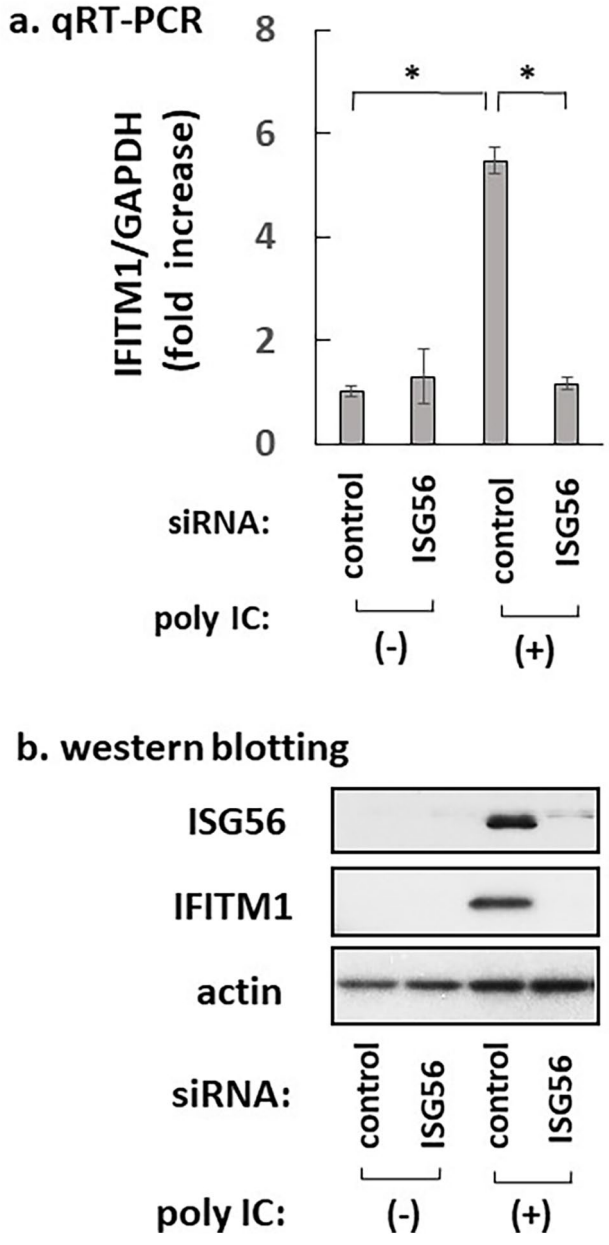

Figure 4. ISG56 plays a role in poly I:C-mediated IFITM1 upregulation. BEAS-2B cells were cultured, and transfection with ISG56 siRNA or control siRNA was performed. After 48 h, BEAS-2B were incubated with poly I:C as in Figure 3. Then, RNA and lysates were extracted from the cells. qRT-PCR (a) ($n = 3$; $^*p < 0.01$ by *t*-test) and Western blotting (b) was used for analysis.

IFN-β, a major type I IFN, induces ISGs including IFITM1, which in turn contributes to the defense against viral infection.[1] In this study, IFN-β knockdown decreased poly I:C-induced IFITM1 upregulation. These results suggest that IFITM1 expression was triggered by the TLR3/IFN-β axis.

There are various antiviral mediators in the ISGs pathway, which exert antiviral effects through their complex interactions.[17] In a prior research, Shiratori *et al.*[12] showed that the TLR3/IFN-β/ISG56/CXCL10 axis in BEAS-2B cells. Therefore, the relationship between ISG56 and IFITM1 was investigated in this study. We found that knockdown of ISG56 decreased poly I:C-induced IFITM1 upregulation, suggesting that ISG56 may contribute to IFITM1 induction

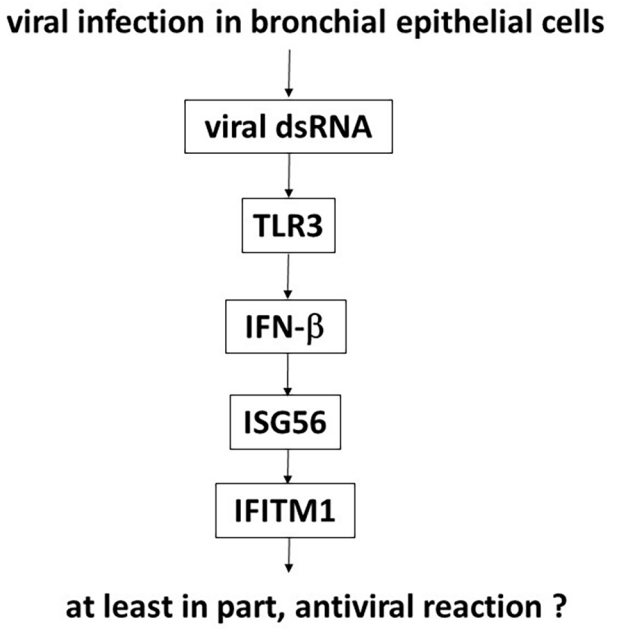

Figure 5. Speculated model of the expression and the significance of IFITM1 in bronchial epithelial cells.

by poly I:C. ISG56 is a multifunctional protein that exerts its activities in a complicated manner. However, molecular mechanisms by which ISG56 regulates IFITM1 expression remain unclear. In contrast, we found that knockdown of IFITM1 had no effect on ISG56 and CXCL10 expression, suggesting that ISG56 functions upstream of IFITM1 in the TLR3/IFN-β/ISG56/CXCL10 axis.

Our results together suggest a novel mechanism by which antiviral innate immune reactions are regulated in bronchial epithelial cells. Recently, Buchrieser *et al.*[18] reported that IFITM1 inhibits the formation of syncytia in SARS-CoV-2-infected cells. This suggests that IFITM1 can restrict SARS-CoV-2-induced lung damage. Therefore, the TLR3/IFN-β/ISG56/IFITM1 axis in bronchial epithelial cells may importantly prevent the development of SARS-CoV-2 infection.

There are some limitations to this study. First, our data were obtained only from *in vitro* studies. To confirm our results, further, experiments using genetically modified mouse models may be useful. Second, we used BEAS-2B, not normal human bronchial epithelial cells (NHBE). Nevertheless, BEAS-2B are widely recognized as a good model of NHBE. Third, we used poly I:C, which mimics instead of inducing viral infection. In our pilot study, BEAS-2B were infected with a virus, but viability of the infected cells was too low to establish the model. Fourth, this study is a descriptive work, and we could not demonstrate any mechanistic pathways by which ISG56 regulate IFITM1 expression in poly I:C-treated BEAS-2B cells are still largely unclear. Fifth, the mechanisms by which IFITM1 exerts its antiviral activity in BEAS-2B cells have not been clarified. Further studies are warranted to overcome these limitations.

We reported that IFITM1 is induced in BEAS-2B by poly I:C. TLR3, IFN-β, and ISG56 may contribute to poly I:C-mediated IFITM1 upregulation. We speculate that IFITM1 induced via this axis may play a part in mediating antiviral reactions among the airway epithelium (Figure 5).

AUTHORS' CONTRIBUTIONS

MD and TI took part in all the experiments and in preparation the manuscript. TS and SH contributed to cell culture and treatment. SK and KS contributed to qRT-PCR and Western blotting. TI and ST carried out designing of the study.

ACKNOWLEDGEMENTS

The authors are thankful to Nakata M and Munakata K who technically assisted the work.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

DATA AVAILABILITY

Upon reasonable request, data obtained in the present study can be available from MD, the corresponding author.

ORCID ID

Masaki Dobashi https://orcid.org/0000-0002-3505-467X

REFERENCES

1. Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, Si-Tahar M. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem* 2005;**280**:5571–80
2. Hsu AC, Parsons K, Barr I, Lowther S, Middleton D, Hansbro PM, Wark PA. Critical role of constitutive type I interferon response in bronchial epithelial cell to influenza infection. *PLOS ONE* 2012;**7**:e32947
3. Raychoudhuri A, Shrivastava S, Steele R, Kim H, Ray R, Ray RB. ISG56 and IFITM1 proteins inhibit hepatitis C virus replication. *J Virol* 2011;**85**:12881–9
4. Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* 2009;**139**:1243–54
5. Jiang D, Weidner JM, Qing M, Pan XB, Guo H, Xu C, Zhang X, Birk A, Chang J, Shi PY, Block TM, Guo JT. Identification of five interferon-induced cellular proteins that inhibit West Nile virus and dengue virus infections. *J Virol* 2010;**84**:8332–41
6. Lu J, Pan Q, Rong L, He W, Liu SL, Liang C. The IFITM proteins inhibit HIV-1 infection. *J Virol* 2011;**85**:2126–37
7. Smith SE, Busse DC, Binter S, Weston S, Soria CD, Laksono BM, Clare S, Nieuwkoop SV, Hoogen BG, Clement M, Marsden M, Humphreys IR, Marsh M, Swart RL, Wash RS, Tregoning JS, Kellam P. Interferon-induced transmembrane protein 1 restricts replication of viruses that enter cells via the plasma membrane. *J Virol* 2019;**93**:e02003–18
8. Fensterl V, Sen GC. The ISG56/IFIT1 gene family. *J Interferon Cytokine Res* 2011;**31**:71–8
9. Kan-OK, Ramirez R, MacDonald MI, Rolph M, Rudd PA, Spann KM, Mahalingam S, Bardin PG, Thomas BJ. Human metapneumovirus infection in chronic obstructive pulmonary disease: impact of glucocorticosteroids and interferon. *J Infect Dis* 2017;**215**:1536–45
10. Kim S, Kim MJ, Park DY, Chung HJ, Kim CH, Yoon JH, Kim HJ. Mitochondrial reactive oxygen species modulate innate immune response to influenza A virus in human nasal epithelium. *Antiviral Res* 2015;**119**:78–83
11. Feng B, Zhang Q, Wang J, Dong H, Mu X, Hu G, Zhang T. IFIT1 expression patterns induced by H9N2 virus and inactivated viral particle in human umbilical vein endothelial cells and bronchus epithelial cells. *Mol Cells* 2018;**41**:271–81
12. Shiratori T, Imaizumi T, Hirono K, Kawaguchi S, Matsumiya T, Seya K, Tasaka S. ISG56 is involved in CXCL10 expression induced by TLR3 signaling in BEAS-2B bronchial epithelial cells. *Exp Lung Res* 2020;**46**:195–202
13. Amstad P, Reddel RR, Pfeifer A, Shibley LM, Mark GE, Harris CC. Neoplastic transformation of human bronchial epithelial cell line by a recombinant retrovirus encoding viral Harvey ras. *Mol Carcinog* 1988;**1**:151–60
14. Nakamoto K, Watanabe M, Sada M, Inui T, Nakamura M, Honda K, Wada H, shii W, Takizawa H. *Pseudomonas aeruginosa*-derived flagellin stimulates IL-6 and IL-8 production in human bronchial epithelial cells: a potential mechanism for progression and exacerbation of COPD. *Exp Lung Res* 2019;**45**:255–66
15. Deblandre GA, Marinx OP, Evans SS, Majjaj S, Leo O, Caput D, Huez GA, Wathelet MG. Expression cloning of an interferon-inducible 17-kDa membrane protein implicated in the control of cell growth. *J Biol Chem* 1995;**270**:23860–6
16. Hashimoto S, Imaizumi T, Watanabe S, Aizawa T, Tsugawa K, Kawaguchi S, Seya K, Matsumiya T, Tanaka H. Expression of IFN-induced transmembrane protein 1 in glomerular endothelial cells. *Pediatr Int* 2021;**63**:1075–81
17. Schneider WM, Chevillotte MD, Rice CM. Interferon-stimulated genes: a complex web of host defenses. *Annu Rev Immunol* 2014;**32**:513–45
18. Buchrieser J, Dufloo J, Hubert M, Monel B, Planas D, Rajah MM, Planchais C, Porrot F, Benhassine FG, Werf SV, Casartelli N, Mouquet H, Bruel T, Schwartz O. Syncytia formation by SARS-CoV-2-infected cell. *EMBO J* 2021;**40**:e107405

(Received February 24, 2022, Accepted July 29, 2022)