ORIGINAL ARTICLE

Diamidobenzimidazole, an agonist of STING, induces the expression of retinoic acid-inducible gene-I and interferon-induced transmembrane protein 1 in BEAS-2B cells

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Abstract

The bronchial epithelium is the site of initial exposure in lungs to viruses, including severe acute respiratory syndrome coronavirus 2. Stimulator of interferon genes (STING) is a molecule activated by virus-derived DNA. STING activation leads to the induction of type I interferon (IFN) expression, followed by increased expression of IFN-stimulated genes (ISGs). Retinoic acid-inducible gene-I (RIG-I) and IFN-induced transmembrane protein 1 (IFITM1) are ISGs important in antiviral immunity. However, the interaction between STING and RIG-I/ IFITM1 in bronchial epithelium is not fully understood. We found that the STING agonist diamidobenzimidazole (diABZI) upregulated RIG-I and IFITM1 expression in BEAS-2B. Knockdown of STING decreased diABZI-induced IFN-βexpression and the phosphorylation of signal transducer and activator of transcription (STAT) 1, while treatment with recombinant human IFN-β upregulated the expression level of RIG-I and IFITM1. These data indicate that activation of STING/IFN-β axis upregulates the levels of RIG-I and IFITM1 in bronchial epithelium, activating RNA virus sensing pathway, inhibiting virus entry into cells. This study contributes to the discovery of new therapeutic targets for upper airway infections caused by viruses.

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Key words: STING; RIG-I; IFITM1; BEAS-2B.

Introduction

The respiratory tract interacts permanently with foreign microorganisms, such as viruses, potentially resulting into the infection of the bronchial epithelium. A number of viruses including respiratory syncytial virus can produce respiratory tract infections[1]. Although most respiratory viral infections are produced by viruses with common features and mainly affect the upper respiratory tract causing a variety of symptoms, occasionally, a more severe infection, pneumonia, may occur. Recently, the pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a global issue. Therefore, the molecular mechanisms that protect humans against airway viral infections must be elucidated.

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The immune system consists of innate and acquired immunity, which are essential for host defense against invading microbes. Innate immunity is characterized by rapidly induced non-clonal immune responses. Bronchial epithelial cells are important for initiating innate immune reactions for viral infections. On the other hand, acquired immunity is characterized by clonal immune reactions against specific pathogens, with lymphocytes and antibodies playing a crucial role in this system. Acquired immunity is triggered following the activation of innate immunity after cells are exposed to various viruses, including unknown viruses. Recognition of pathogen-associated molecular patterns by pattern recognition receptors (PRRs) triggers an innate immune reaction and activates cascades that induce type I interferon (IFN) expression.

Stimulator of IFN genes (STING) is a key molecule involved in antiviral innate immunity[2]. Cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase (cGAS) is a PRR that recognizes virus-derived double-stranded DNA. Upon infection with a DNA virus, cGAS is activated to produce cyclic GMP-AMP (cGAMP). The binding of cGAMP, a secondary messenger protein, to STING activates the latter, resulting in a series of reactions whose final end product is type I IFN induction. Furthermore, type I IFN induces the transcriptional activation of hundreds of IFN-stimulated genes (ISGs) in an autocrine or paracrine manner[3] via the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) system; the products of these ISGs present diverse antiviral activities. Thus, the cGAS-STING-type I IFN-ISGs axis plays an essential role in antiviral innate immune reactions. STING also contributes to innate immunity against RNA virus infection[4].

Besides biological cyclic dinucleotide (CDNs) -cGAS pathway, synthetic small molecules belonging to amidobenzimidazole (ABZI) family function as STING ligands[5]. Among ABZI family members, diaminobenzimidazole (diABZI) is a potent STING agonist. DiABZI possesses antiviral activity against viruses including SARS-CoV-2[6]. Although its antiviral activity seems to be exerted via STING-type I IFN-ISGs axis, the mechanism underlying the activation of STING by its agonist, diABZI, in the bronchial epithelium remains unclear.

ISGs-encoded molecules include a wide variety of enzymes, cytokines and other signaling molecules, adhesion molecules, receptors, adapter molecules, transcriptional factors, regulators of translation, channels, chaperones, ubiquitin-like molecules, etc[3]. These ISGs, in the downstream of diABZI-activated STING, may form networks and control complex, complicated antiviral responses. In this study, we focused on two molecules among ISGs; retinoic acid-inducible gene-I (RIG-I) and IFN-induced transmembrane protein 1 (IFITM1). RIG-I, also named DDX58, is involved in vascular inflammation[7] and in differentiation of myeloid leukemia cells[8]. RIG-I is a PRR that acts against virus-derived double-stranded RNA, and its activation promotes antiviral immune responses[9]. STING has been widely known to be involved in the DNA virus infection pathway, but recently it was reported to be involved also in the RNA virus infection pathway[4]. Because RIG-I is a key molecule in RNA virus infection pathway, we focused on RIG-I to investigate the interaction between the pathways responding to infection with DNA virus and RNA virus in bronchial epithelium. IFITM1 is an IFN-inducible membrane protein that restricts the entry of viruses into the cells[10]. Therefore, we hypothesized that IFITM1 is an important molecule for elucidating the role of STING in innate immunity in bronchial epithelium. Although both RIG-I and IFITM1 are important ISGs-encoded molecules responsible for innate immune response against viruses, the effects of diABZI, a STING agonist,

on RIG-I and IFITM1 expression has not been well explored. We researched the effectiveness of diABZI on the expression level of RIG-I and IFITM1 using BEAS-2B, a model of human bronchial epithelial cells.

Material and Methods

1) Cell culture

Human bronchial epithelium-derived BEAS-2B cells were obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Frederick, MD, USA) with antibiotics penicillin/streptomycin. To examine the effect of the STING agonist diABZI (Selleck Chemicals, Houston, TX, USA) on RIG-I and IFITM1 expression, the cells at 80–90 % confluence were incubated with diABZI (0, 0.08, 0.4, 2, 10, and 50 µM). The temporal pattern of RIG-I and IFITM1 expression was examined by exposing the cells to diABZI 10 µM for 0, 2, 4, 8, 16, and 24 h. Additionally, knockdown experiments using RNAi were also performed. BEAS-2B cells were incubated in antibiotic-free DMEM with 10% FBS for 24 h before siRNA transfection. The cells at approximately 40–50 % confluence were transfected with a control siRNA (QIAGEN, Hilden, Germany), two different STING siRNAs (Thermo Fisher Scientific, Waltham, MA, USA), or an IFN-β siRNA (Thermo Fisher Scientific) using Lipofectamine RNAiMAX kit (Invitrogen, Frederick, MD, USA). After the cells were incubated for 48 h, 10 µM diABZI was added to the medium. RNA and proteins were collected at the indicated time points.

2) Quantitative RT-PCR (RT-qPCR)

The Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, England) was used to extract and purify total RNA from BEAS-2B cells. cDNA was synthesized as follows: RNA templates were denatured with random primers at 70 °C for 10 min, and reverse transcription was conducted using Moloney Murine Leukemia Virus reverse transcriptase in the presence of recombinant porcine RNase inhibitor (Takara Bio, Kusatsu, Japan) at 37 °C for 60 min. The levels of expression of RIG-I, IFITM1, and IFN-β mRNA were quantified by RT-qPCR using the THUNDERBIRD qPCR Mix (TOYOBO, Osaka, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The following primers were used to amplify cDNA:

GAPDH-F: 5'- GCACCGTCAAGGCTGAGAAC -3',
GAPDH-R: 5'- ATGGTGGTGAAGACGCCAGT -3',
IFITM1-F: 5'- TCGCCTACTCCGTGAAGTCTA -3',
IFITM1-R: 5'- TGTCACAGAGCCGAATACCAG -3',
IFN-β-F: 5'- CCTGTGGCAATTGAATGGGAGGC -3',
IFN-β-R: 5'- CCAGGCACAGTGACTGTACTCCTT -3',
RIG-I-F : 5'- GTGCAAAGCCTTGGCATGT -3',
RIG-I-R: 5'- TGGCTTGGGATGTGGTCTACTC -3',
STING-F: 5'- GAGAGCCACCAGAGCACAC -3',
and STING-R: 5'- CGCACAGTCCTCCAGTAGC -3'.

3) Western blotting

Western blotting was undertaken using standard techniques as previously described[11]. BEAS-2B cells were washed twice with ice-cold PBS, scraped, collected, and lysed in Laemmli reducing buffer. Proteins from cell lysates were separated using electrophoresis on 5–20 % polyacrylamide gels, and were transferred to polyvinylidene difluoride membranes. The membrane was blocked with 5 % non-fat dry milk in Tris-buffered saline containing 0.5 % Tween 20 and, treated with anti-STING rabbit antibody (Cell Signaling Technologies, 1:1500 dilution), anti-RIG-I rabbit antibody[7] (1:10,000 dilution), anti-IFITM1 rabbit antibody (GeneTex, Irvine, CA, USA, 1:3000 dilution), anti-STAT1 rabbit antibody (Santa Cruz Biotechnology, Dallas, TX, USA, 1:7500 dilution), anti-phosphorylated STAT1 (P-STAT1) mouse

antibody（Santa Cruz Biotechnology, 1:5000 dilution）, and anti-actin rabbit antibody（Sigma, St. Louis, MO, USA, 1:5000 dilution）. Horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG was added as a secondary antibody, and proteins were identified using Luminata Crescendo substrate（Millipore, Burlington, MA, USA）.

4）Evaluation of IFN-β using an enzyme-linked immunosorbent assay（ELISA）

Cell culture medium was collected, chilled on ice and centrifuged at 10000 x g and 4 ℃ for 10 minutes. The concentration of IFN-β in the cell culture medium was measured using VeriKine Human IFN-β ELISA kit（PBL Assay Science, Piscataway, NJ, USA）according to the manufacturer's protocol.

Results

1）DiABZI induces RIG-I and IFITM1 expression in BEAS-2B cells

In BEAS-2B cells treated with diABZI, a STING agonist, the expression levels of RIG-I and IFITM1 mRNA（Figure 1A）and protein（Figure 1B）were elevated concentration-dependently. Furthermore, the diABZI-induced changes in RIG-I and IFITM1 mRNA（Figure 1C）and protein（Figure 1D）level of expression were time-dependent. RIG-I mRNA expression increased markedly at 8 h and slightly at 16–24 h compared to 0 h. RIG-I protein expression gradually increased at 2–24 h and peaked at 16–24 h compared to 0 h. Furthermore, IFITM1 mRNA expression increased markedly at 4–24 h, whereas protein expression of IFITM1 was detected at 16–24 h.

2）DiABZI-induced expression of IFN-β, RIG-I, IFITM1 and upregulation of STAT1 phosphorylation is mediated by STING

RNA interference（RNAi）was performed using two STING siRNAs. In BEAS-2B cells

incubated with diABZI, RIG-I and IFITM1 mRNA expression were partially suppressed by STING siRNA transfection（Figure 2A）. Transfection of BEAS-2B with STING siRNA led to significant knockdown of STING protein expression（Figure 2B）. DiABZI-induced RIG-I and IFITM1 level of protein expression was partially suppressed by STING knockdown （Figure 2B）, which is compatible with the results of mRNA shown in Figure 2A. Furthermore, treatment of BEAS-2B with diABZI increased the mRNA（Figure 2C）and protein（Figure 2D）levels of IFN-β; this effect was suppressed by STING-siRNA treatment. Phosphorylated STAT1（P-STAT1）was not detected in BEAS-2B without diABZI treatment, while its expression was upregulated following treatment with diABZI. P-STAT1 level of expression decreased as a result of transfection with STING siRNAs（Figure 2E）.

3）IFN-β is associated with diABZI-induced increase in the levels of RIG-I and IFITM1

Next, we investigated whether IFN-β knockdown affected diABZI-induced increase in the expression level of RIG-I and IFITM1 in BEAS-2B. RNAi against IFN-β suppressed diABZI-induced increase in RIG-I and IFITM1 expression at mRNA（Figure 3A）and protein （Figure 3B）levels. Transfection with a specific siRNA against IFN-β effectively reduced diABZI-induced IFN-β protein expression （Figure 3C）. Addition of recombinant human IFN-β（r(h)IFN-β）to BEAS-2B cells increased RIG-I and IFITM1 expression at mRNA（Figure 3D）and protein（Figure 3E）levels. These data revealed that diABZI upregulated the expression level of RIG-I and IFITM1 in BEAS-2B by upregulating IFN-β expression.

Discussion

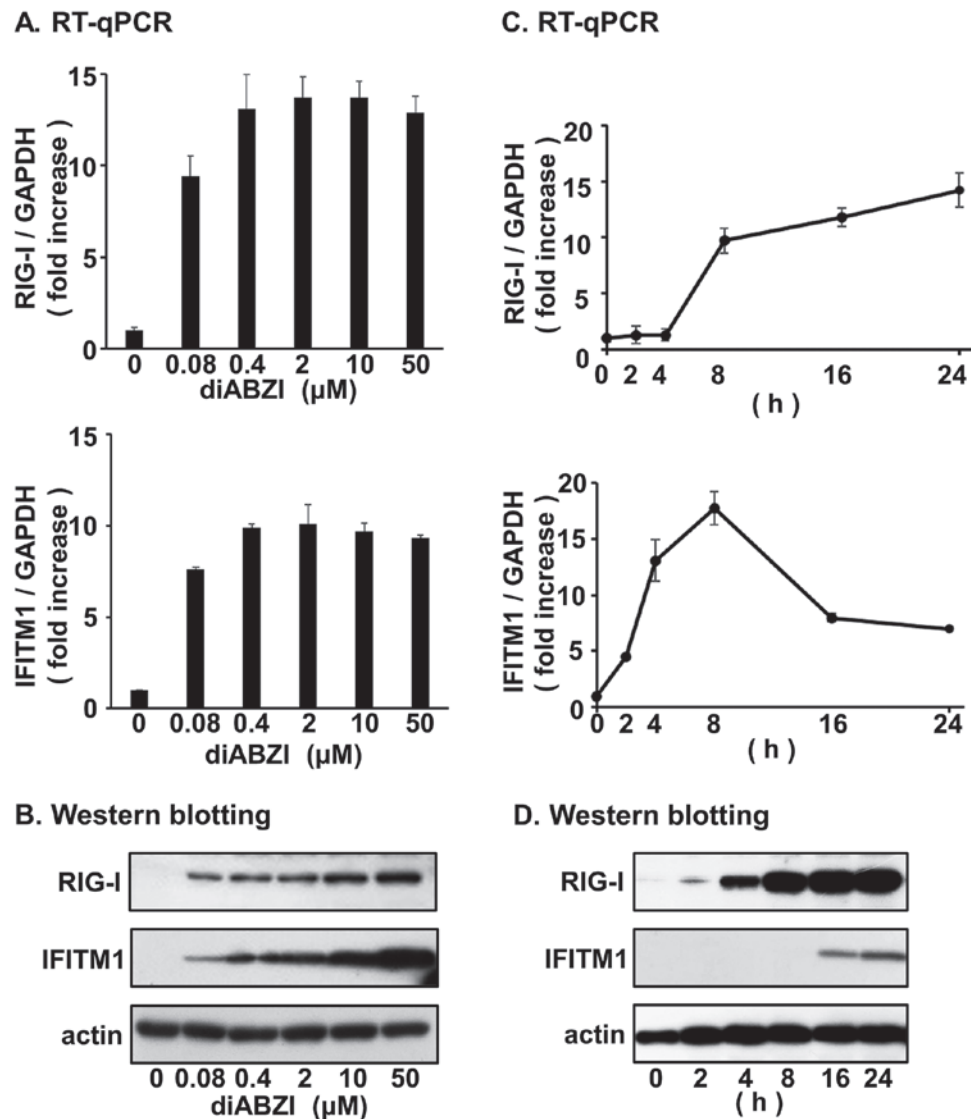The expression of STING at mRNA level was

Fig. 1 RIG-I and IFITM1 were upregulated by diABZI in concentration- and time- dependent manner
A. Quantification of RIG-I and IFITM1 mRNA level after exposure to various concentration of diABZI. BEAS-2B cells were treated with various concentrations of diABZI (0, 0.08, 0.4, 2, 10, and 50 μM) for 8 h; RT-qPCR was performed. The data are expressed as fold increase vs. unstimulated cells and shown as mean ± standard deviation (SD) (n=3). B. Direction of the expression of RIG-I, IFITM1, and actin at protein level in experimental conditions detailed in A. Cells were treated with diABZI as in A, then incubated for 24 h. Western blotting was performed. C. Quantification of RIG-I and IFITM1 mRNA level after exposure to diABZI (10 μM) for 0, 2, 4, 8, 16, and 24 h. RT-qPCR testing was performed as in A. D. Direction of the expression of RIG-I, IFITM1, and actin at protein level in experimental conditions detailed in C using Western blotting.

upregulated in BEAS-2B bronchial epithelial cells by human H1N1 influenza A virus infection[12]. Additionally, knockdown of STING downregulated the expression of suppressor of cytokine signaling 1 (SOCS1) in BEAS-2B cells[13]. However, the function of STING in innate immunity of BEAS-2B cells has not been well characterized. We showed in the present study
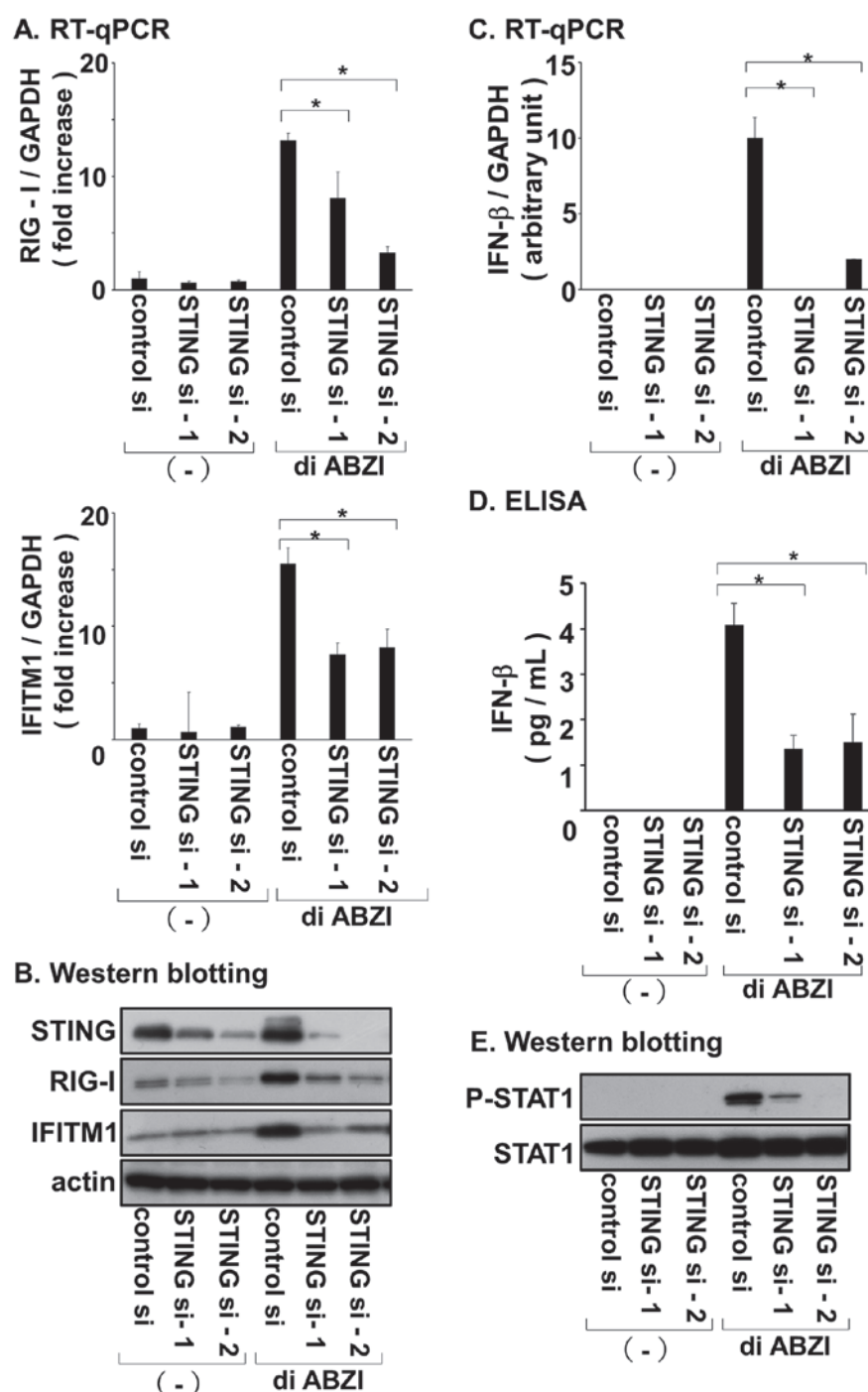
Fig. 2 RIG-I and IFITM1 expression were suppressed by RNA interference of STING
BEAS-2B cells at approximately 40–50 % confluence were transfected with control siRNA or two different
STING siRNAs to knockdown STING expression. The cells were incubated for 48 h, then cultured with
diABZI（10 µM）. A. Quantification of RIG-I and IFITM1 mRNA level using RT-qPCR. RNA was extracted
after incubation for 8 h, and testing was performed. B. Direction of STING, RIG-I, IFITM1, and actin protein
expression using Western blotting. Cells were lysed after incubation for 24 h, and testing was performed.
STING protein expression was effectively decreased by STING siRNA treatment. C. Quantification of
IFN-β mRNA using RT-qPCR. After incubation for 2 h, cells were subjected to testing. D. Measurement of
IFN-β concentration using ELISA. The cell-conditioned medium was collected after incubation for 6 h, and
measurement was performed. E. Detection of the expression of phosphorylated STAT1（P-STAT1）and
STAT1 at protein level using Western blotting. Cells were lysed after incubation for 4 h, and testing was
performed. The data in A, C, and D are shown as mean ± SD（n=3）. *p<0.05 by U-test.
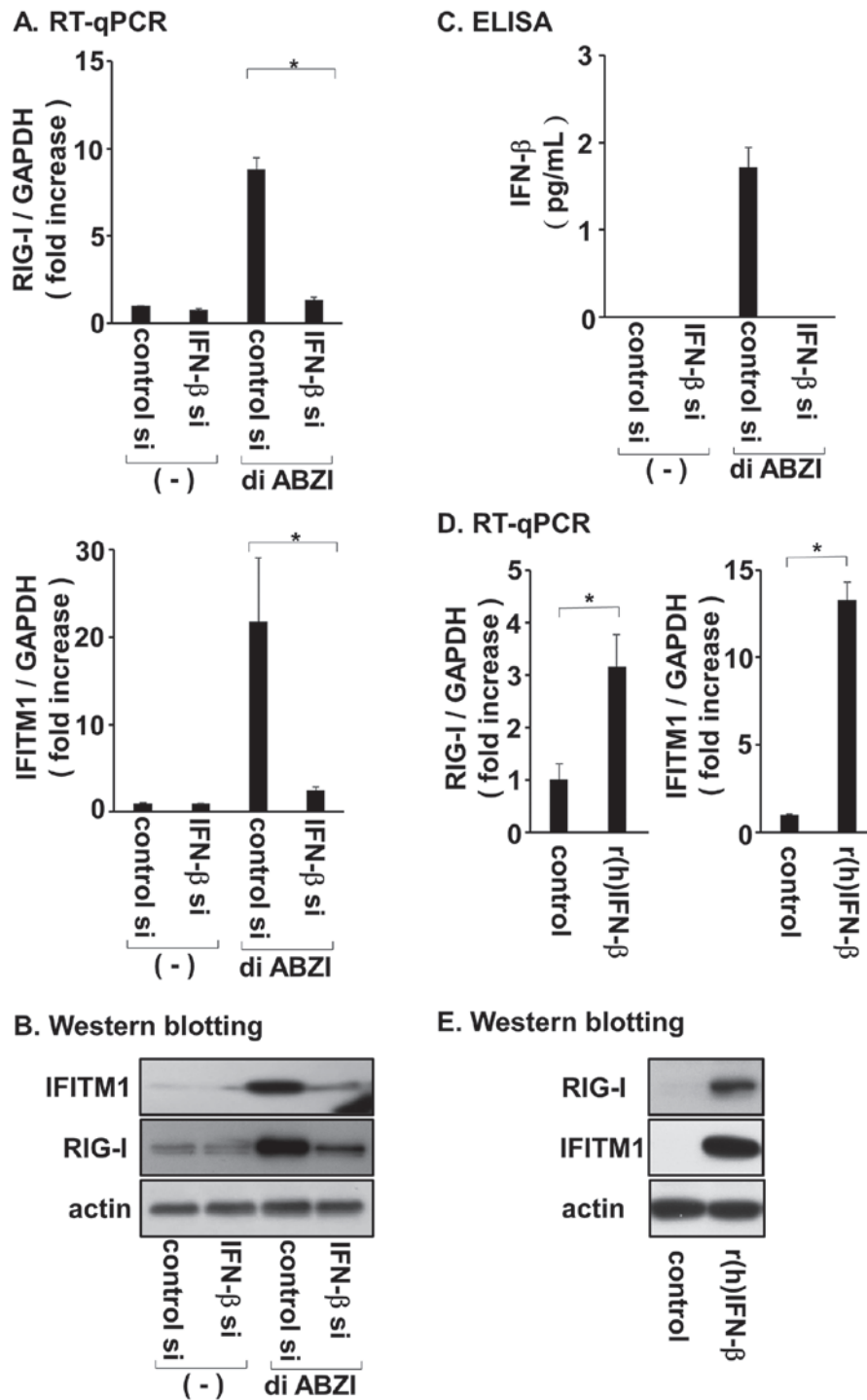
Fig. 3 diABZI-induced RIG-I and IFITM1 expression is mediated by IFN-β
A, B, and C. BEAS-2B cells were transfected with a specific siRNA against IFN-β, followed by treatment with diABZI (10 μM) as in Figure 3. Cells were analyzed by RT-qPCR after 8 h (A) and by Western blotting after 24 h (B) of incubation. IFN-β protein concentration in cell-conditioned medium after 6 h incubation was measured (C). D and E. The cells were incubated with 1 ng/mL r(h)IFN-β for 16 h. After incubation, RT-qPCR (D) and Western blotting (E) were performed, as described above. The data in A, C, and D are shown as mean ± SD (n=3). *p<0.05 by U-test.

that treatment of BEAS-2B with diABZI, a STING agonist, upregulated the expression of RIG-I and IFITM1 at mRNA and protein levels. Additionally, STING knockdown decreased the upregulation of these molecules. These data indicate that STING regulates the expression of antiviral genes in BEAS-2B.

RIG-I is an RNA-binding protein that functions as a PRR to detect virus-derived double-stranded RNA. After binding to double-stranded RNA, RIG-I works as an adaptor protein[14]. In airway epithelial cells, RIG-I is upregulated by infection with various viruses, such as respiratory syncytial virus[15] and rhinovirus respiratory syncytial virus[16], and by an authentic Toll-like receptor 3 ligand[17], suggesting that the regulation of RIG-I expression is needed for anti-viral immune responses in the respiratory tract. RIG-I and STING are nucleic acid-sensing receptor/adaptor proteins that are activated by virus-derived RNA and DNA, respectively. However, viral DNA can be transcribed into RNA, whereas viral RNA can be reverse-transcribed into DNA during the life cycle of some viruses, and these nucleic acid products can occasionally be ligands for PRRs. Crosstalk exists between RIG-I and STING pathways[18], and STING pathway activation restricts the infection with various RNA viruses[19]. The degradation of STING is also mediated by RIG-I, suggesting that it could be a negative feedback mechanism that regulates the unregulated innate immune response induced by ds-DNA[20]. The results of this study suggest that STING enhance RIG-I-mediated RNA-sensing pathway in bronchial epithelial cells.

IFITM1 is a membrane protein which enhances innate immune activity by suppressing the entry of various viruses including the Zika virus[21], Marburg and Ebola filoviruses[22] and SARS-CoV-2[23]. Furthermore, porcine IFITM1 is expressed via the cGAS-STING pathway upon exposure to pseudorabies virus (PRV), and its upregulation blocks PRV entry into porcine kidney cells and macrophages[24]. Our results are consistent with this report and indicate that diABZI activates the STING pathway, upregulates the expression of IFITM1 and inhibits the viral entry through the membrane of human bronchial epithelial cells.

Type I IFNs are essential cytokines that activate innate immune responses. An impaired IFN type I response was observed in patients severely infected with SARS-CoV-2[25], while a persistently high level of expression of IFN-β was found in patients with post-acute coronavirus disease (COVID) syndrome (long COVID)[26]. Therefore, the kinetics of type I IFN and ISGs-encoded molecules might be critical factors in the immunopathology of SARS-CoV-2 infection and possibly, in that of other virus-mediated diseases. ISGs-encoded molecules have diverse cellular functions including boosting antiviral host defense[3]. Both RIG-I and IFITM1 are such molecules and are involved in IFN-mediated immune reactions. In BEAS-2B, RIG-I expression is upregulated by IFN-γ treatment[27] and IFITM1 expression is induced by the TLR3/IFN-β axis[11]. In present study, diABZI induced the expression of IFN-β, a major type I IFN, in bronchial epithelial cells[28]. Moreover, diABZI and r(h) IFN-β-induced an increase in the expression of RIG-I and IFITM1 at mRNA and protein levels in BEAS-2B; the effect of diABZI was counteracted by the knockdown of IFN-β. Furthermore, STAT1 phosphorylation, an essential step in the IFN-mediated increase in ISGs-encoded molecules, was inhibited by STING knockdown. Taken together, these results suggest that STING activation by diABZI induces RIG-I and IFITM1 expression in an IFN-dependent manner in BEAS-2B. These data are compatible with previous studies which revealed that single-stranded DNA generated by radiation or etoposide-induced DNA damage, activates STING to upregulate IFN-β expression

in RAW264.7 mouse macrophage[29].

Recently, diABZI-activated STING pathway was reported to block SARS-CoV-2 replication IFN-dependently both in cultured primary human bronchial epithelial cells *in vitro* and in mice model *in vivo*[30]. Additionally, a defective STING is associated with increased infectivity of Zika virus[31]. Suppression of STING expression by persistent IL-13 signaling impairs innate immunity, resulting in the exacerbation of chronic rhinosinusitis associated with nasal polyps[13]. Since the bronchial epithelium is the first site of defense against pathogenic viruses entering the respiratory tract, pharmacological activation of STING and introducing the expression of IFITM1 and RIG-I in bronchial epithelial cells, may be potential therapeutic strategies against respiratory viral infections.

The present study has several limitations. First, we only performed *in vitro* tests using a cell culture system. Second, this study used a synthetic STING agonist rather than an actual virus. Although these results can be applied for a wide spectrum of viruses, the observations may not be applicable to all viruses. Our findings should be verified in future studies addressing these limitations.

Conclusion

We revealed that diABZI induces RIG-I and IFITM1 expression in a STING- and IFN-β-dependent manner in BEAS-2B cells. These findings indicate that STING activation is a crucial reaction in the defense against respiratory viral infections, and its effect is at least partially, mediated through the activation of RIG-I-related antiviral defense and IFITM1-associated inhibition of viral entry into cells.

Conflicts of interest

All authors have no conflicts of interest

directly relevant to content of this article.

Author contributions

M.S. participated in all experiments and the preparation of the manuscript. T.I. contributed to the cell culturing, RT-qPCR, Western blotting, manuscript preparation and study design. Y.T., J.D. and T.M contributed to the cell culture. S.K., J.D., T.M and K.S. contributed to the RT-qPCR and Western blotting. K.M. contributed to the RT-qPCR. A.M. designed and coordinated this study. All authors approved the final draft of this manuscript.

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