Expression of ISG60 is induced by TLR3 signaling in BEAS-2B bronchial epithelial cells: Possible involvement in CXCL10 expression

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Abstract. Viral infections in the respiratory tract are common, and, in recent years, severe acute respiratory syndrome coronavirus 2 outbreaks have highlighted the effect of viral infections on antiviral innate immune and inflammatory reactions. Specific treatments for numerous viral respiratory infections have not yet been established and they are mainly treated symptomatically. Therefore, understanding the details of the innate immune system underlying the airway epithelium is crucial for the development of new therapies. The present study aimed to investigate the function and expression of interferon (IFN)-stimulated gene (ISG)60 in non-cancerous bronchial epithelial BEAS-2B cells exposed to a Toll-like receptor 3 agonist. BEAS-2B cells were treated with a synthetic TLR3 ligand, polyinosinic-polycytidylic acid (poly IC). The mRNA and protein expression levels of ISG60 were analyzed using reverse transcription-quantitative PCR and western blotting, respectively. The levels of C-X-C motif chemokine ligand 10 (CXCL10) were examined using an enzyme-linked immunosorbent assay, and the effects of knockdown of IFN-β, ISG60 and ISG56 were examined using specific small interfering RNAs. Notably, ISG60 expression was increased in proportion to poly IC concentration, and recombinant human IFN-β also induced ISG60 expression. By contrast, knockdown of IFN-β and ISG56 decreased ISG60 expression, and ISG60 knockdown reduced CXCL10 and ISG56 expression. These findings suggested that ISG60 is partly implicated in CXCL10 expression and that ISG60 may serve a role in the innate immune response of bronchial epithelial cells. The present study highlights ISG60 as a potential target for new therapeutic strategies against viral infections in the airway.

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Abbreviations: CXCL10, C-X-C motif chemokine ligand 10; dsRNA, double-stranded RNA; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IFIT1, IFN-induced protein with tetratricopeptide repeats 1; ISG, IFN-stimulated gene; MDA5, melanoma differentiation-associated gene 5; P-STAT1, phosphorylated-signal transducer and activating transcription-1; poly IC; polyinosinic-polycytidylic acid; RT-qPCR, reverse transcription-quantitative PCR; r(h)IFN-β, recombinant human IFN-β; siRNA, small interfering RNA; TLR3, Toll-like receptor 3

Key words: bronchial epithelial cells, CXCL10, IFN-β, ISG60, TLR3

Introduction

Respiratory infections caused by various viruses, such as influenza, respiratory syncytial virus, parainfluenza, rhinovirus and human coronavirus (including severe acute respiratory syndrome coronavirus 2), pose significant public health challenges (1). Currently, specific antiviral treatments for a number of these viruses are lacking, often leaving symptomatic therapy as the primary approach for managing viral respiratory infections. Furthermore, the ongoing threat of potential pandemics underscores the need to explore novel approaches to combat pulmonary viral infections. Because epithelial cells in the respiratory tract are vulnerable to various viral pathogens, a detailed understanding of the immune system in the airway epithelium is essential for developing new therapeutic strategies.

The innate immune system is a frontline defense against invading microbes, orchestrating subsequent adaptive immune responses (2). Recognition of pathogen-associated molecular patterns by pattern-recognition receptors triggers the initiation of innate immune responses. Among these receptors, Toll-like receptor 3 (TLR3) serves as a key receptor for virus-derived double-stranded RNA (dsRNA) (3), and is prominently expressed in airway epithelial cells. Activation of TLR3 by dsRNA leads to the activation of NF-κB and interferon regulatory factor 3, resulting in the production of

cytokines and chemokines that are pivotal in orchestrating immune and inflammatory responses (4-6). Central to this antiviral defense mechanism is the induction of type I interferons (IFNs) by TLR3 signaling, with IFN-β emerging as a principal type I IFN in bronchial epithelial cells (7). Following IFN-β production, hundreds of IFN-stimulated genes (ISGs) are upregulated, forming a complex network that modulates both innate and adaptive immune responses (8). For example, IFN-β induces the expression of C-X-C motif chemokine ligand 10 (CXCL10), a chemokine that promotes lymphocyte chemotaxis (9), and melanoma differentiation-associated gene 5 (MDA5), a dsRNA receptor and signaling molecule (10). Furthermore, ISG56 and ISG60 have also been speculated to contribute to antiviral immunity alongside other ISGs (11).

Despite extensive studies (12,13) on the molecular mechanisms underlying inflammatory responses and innate antiviral immunity in airway epithelial cells, the specific roles of ISGs remain incompletely understood. ISG56, also known as IFN-induced protein with tetratricopeptide repeats 1 (IFIT1), encodes a protein with a helix-turn-helix four-repeat motif that mediates various antiviral responses (11). A previous study has shown that ISG56 mediates CXCL10 expression in BEAS-2B bronchial epithelial cells during TLR3 signaling (14). ISG60 is another IFIT protein, also known as retinoic acid-inducible gene G (15) and IFIT3 (16), which induces IFN-β signaling and suppresses adenovirus early gene expression in alveolar adenocarcinoma cell-derived 293FT cells, A549 cells and immortalized normal human diploid fibroblasts (17). These studies suggest that ISG60 may have a crucial role in host defense against adenovirus infection; however, the mechanisms by which ISG60 exerts its antiviral function via TLR3 signaling in airway epithelial cells remain unknown. The present study aimed to investigate the function and expression of ISG60 in BEAS-2B cells primed with a TLR3 ligand, polyinosinic-polycytidylic acid (poly IC).

Materials and methods

Cell culture. Noncancerous bronchial epithelial cell-derived BEAS-2B cells (American Type Culture Collection) were cultured at 37˚C and 5% CO_2 in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Biowest), 100 U/ml penicillin, 100 μg/ml streptomycin B and 250 ng/ml Amphotericin B (Nacalai Tesque, Inc.) as previously described (14). Poly IC (cat. no. P9582; MilliporeSigma) was added to the cell culture medium for treatment at 37˚C. To examine the concentration-dependent effect of poly IC, the cells were treated with 3, 5, 10, 30 and 50 μg/ml poly IC for 16 h (for mRNA expression analysis) or 24 h (for protein expression analysis). In time course experiments, the cells were treated with 30 μg/ml poly IC for 2, 4, 8, 16 and 24 h. For RNA interference, cells at 50-70% density cultured for 24 h in antibiotic-free medium were transfected at 37˚C with either negative control small interfering RNA (siRNA) (cat. no. 1027281; Qiagen GmbH), or siRNAs against IFN-β (custom synthesized by Invitrogen; Thermo Fisher Scientific, Inc.) (18), ISG56 (cat. no. SI02660777; Qiagen GmbH) or ISG60 (cat. no. SI04197788 as ISG60 si-1; cat. no. SI03152737 as ISG60 si-2; Qiagen GmbH) at a concentration of 10 pmol/ml using Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.). After 4 h of transfection, the medium was changed, and the cells were subjected to subsequent experiments 48 h after transfection. The siRNA sequences for IFN-β, ISG56 and ISG60 were as follows. IFN-β siRNA, sense 5'-CCAUGAGCUACAACU UGCUUGGAUU-3', antisense 5'-AAUCCAAGCAAGUUG UAGUCUAUGG-3'; ISG56 siRNA, sense 5'-GGAUCAGAU UGAAUUCCUATT-3', antisense 5'-UAGGAAUUCAAUCUG AUCCAA-3'; ISG60 siRNA-1, sense 5'-AGAUGAUUGAAG CACUAAATT-3', antisense UUUAGUGCUUCAAUCAU CUCT-3'; ISG60 siRNA-2, sense 5'-GUCAUGGACUAUUCG AAUATT-3' and antisense 5'-UAUUCGAAUAGUCCAUAG CAT-3'. The cells were also treated with recombinant human [r(h)]IFN-β (ProSpec-Tany TechnoGene Ltd.) at 37˚C for 16 h.

Reverse transcription-quantitative PCR (RT-qPCR). RNA was extracted from the cultured cells using an Illustra RNAspin kit (GE Healthcare). To denature RNA, the RNA solution was heated at 70˚C for 5 min and was then cooled immediately on ice. Subsequently, RT was performed at 37˚C for 1 h using oligo$(dT)_{18}$ primer (FASMAC), M-MLV reverse transcriptase (Thermo Fisher Scientific, Inc.) and dNTP mix (Thermo Fisher Scientific, Inc.), followed by inactivation of the enzyme at 70˚C for 10 min. The cDNA for 18S, CXCL10, IFN-β, ISG56, ISG60 and MDA5 were amplified using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.) with a qPCR system (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: Pre-denaturation at 95˚C for 30 sec; followed by 40 cycles at 95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 20 sec; and final extension at 72˚C for 5 min. 18S ribosomal RNA was utilized as an internal control. The $2^{-\Delta\Delta Cq}$ method (19) was used for quantification. CXCL10 and IFN-β mRNA expression levels are presented in arbitrary units, as these genes were not detectable in the cells not stimulated with poly IC. The mRNA expression levels of the other genes are expressed as a fold increase compared with unstimulated cells. The following primers (custom composites purchased from FASMAC) were used: 18S (NR_146146.1), forward (F) 5'-ACT CAACACGGGAAACCTCA-3', reverse (R) 5'-AACCAGACA AATCGCTCCAC-3'; CXCL10 (NM_001565.4), F 5'-TTCAAG GAGTACCTCTCTCTAG-3', R 5'-CTGGATTCAGACATC TCTTCTC-3'; IFN-β (NM_002176.4), F 5'-CCTGTGGCA ATTGAATGGGAGGC-3', R 5'-CCAGGCACAGTGACTGTA CTCCTT-3'; ISG56 (NM_001270930.2), F 5'-TAGCCAACA TGTCCTCACAGAC-3', R 5'-TCTTCTACCACTGGTTTC ATGC-3'; ISG60 (NM_001031683.4), F: 5'-GAACATGCT GACCAAGCAGA-3', R 5'-CAGTTGTGTCCACCCTTCCT-3'; MDA5 (XM_047445407.1), F 5'-GTTGAAAAGGCTGGC TGAAAAC-3', R 5'-TCGATAACTCCTGAACCACTG-3'.

Western blotting. After cultivation, the cells were washed twice using phosphate-buffered saline, scraped from the dish and Laemmli sample buffer (Cell Signaling Technologies, Inc.) was used for protein extraction. The BCA protein assay kit (FUJIFILM Wako Pure Chemical Corporation) was used for protein quantification. Proteins (20 mg/lane) in the lysates were resolved using SDS-PAGE on 5-20% gels (ATTO Corporation) and were transferred to polyvinylidene difluoride membranes (Merck KGaA). The membranes were blocked with Tris-buffered saline containing 5% nonfat

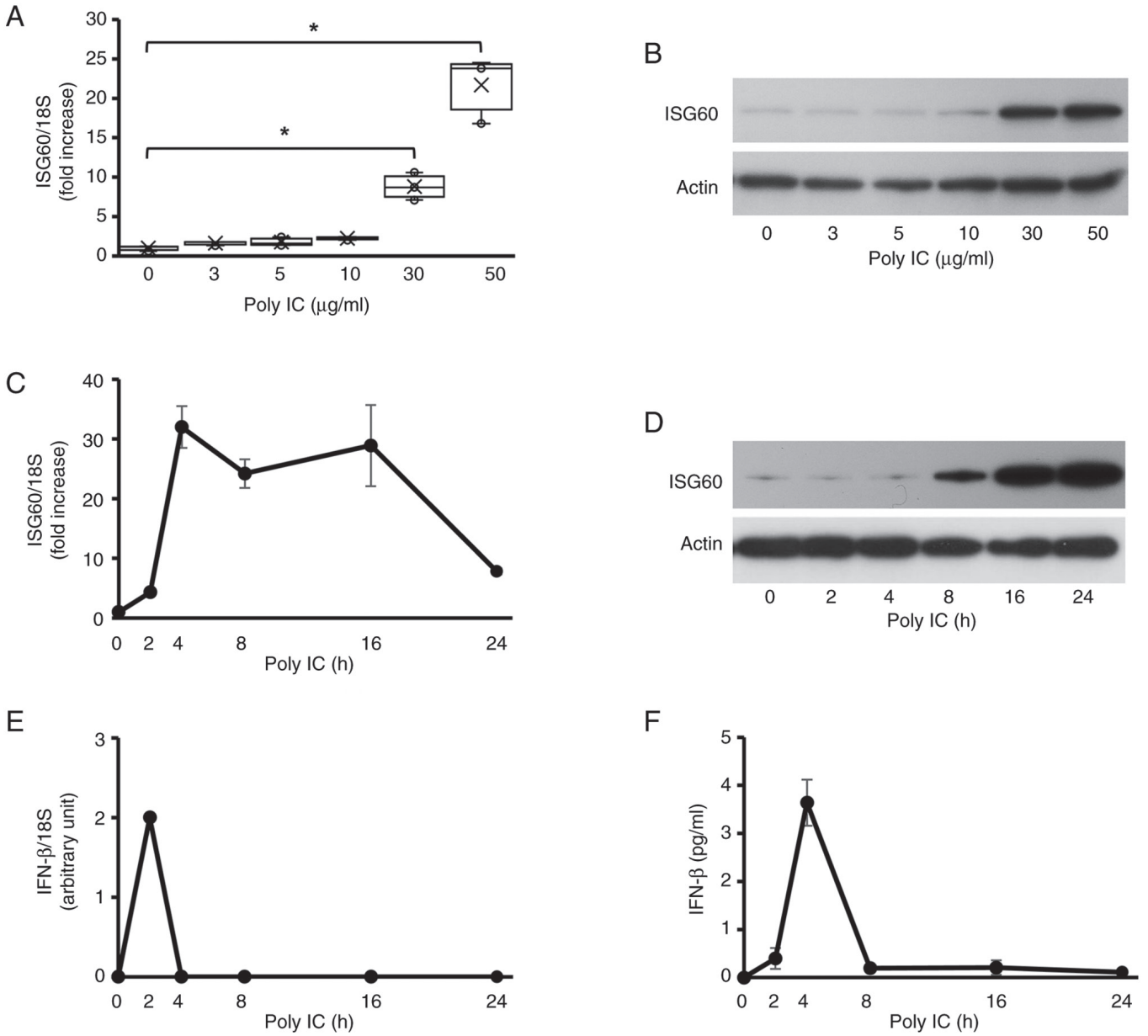

Figure 1. Poly IC induces the expression of ISG60 in BEAS-2B bronchial epithelial cells. (A and B) BEAS-2B cells were cultured and stimulated with different concentrations of the Toll-like receptor 3 agonist poly IC. (A) After 16 h, RNA was extracted from the cells, and RT-qPCR analysis of ISG60 mRNA was performed. Data are presented as box plots showing the median (center of the box), 25th percentile (bottom of the box), 75th percentile (top of the box) and range (minimum and maximum), and were analyzed using one-way ANOVA and Dunnett's test, n=3. *P<0.05. (B) Cellular proteins were extracted after 24 h, and western blotting was performed for ISG60 and actin. (C-F) Cells were stimulated with 30 μg/ml poly IC for up to 24 h. (C) ISG60 mRNA expression levels were detected using RT-qPCR analysis. (D) ISG60 protein expression levels in cell lysates were detected using western blotting. (E) IFN-b mRNA expression was examined using RT-qPCR analysis. (F) IFN-β protein concentration in the conditioned medium was measured using enzyme-linked immunosorbent assay. (C, E and F) Data are presented as the mean ± SD (n=3). IFN-β, interferon β; ISG60, IFN-stimulated gene 60; poly IC, polyinosinic-polycytidylic acid; RT-qPCR, reverse transcription-quantitative PCR.

dry milk at 25˚C for 2 h, and were then incubated with the following primary antibodies: Rabbit anti-ISG56 (1:3,000; cat. no. GTX118713; GeneTex, Inc.), rabbit anti-ISG60 (1:5,000; cat. no. GTX112442; GeneTex, Inc.), rabbit anti-signal transducer and activator transcription 1 (STAT1) (1:10,000; cat. no. sc-592; Santa Cruz Biotechnology, Inc.), mouse anti-phosphorylated (P)-STAT1 (1:5,000; cat. no. sc-136229; Santa Cruz Biotechnology, Inc.) and rabbit anti-actin IgG (1:5,000; cat. no. A5060; MilliporeSigma) at 4˚C overnight. After washing, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary anti-mouse (1:10,000; cat. no. A28177; Thermo Fisher Scientific, Inc.) or anti-rabbit (1:10,000; cat. no. 458; Medical & Biological Laboratories Co., Ltd.) IgG at 25˚C for 1 h. The Luminata Crescendo Western HRP Substrate (Merck KGaA) was utilized for detection.

Enzyme-linked immunosorbent assay (ELISA). After incubation, the conditioned medium was recovered and centrifuged at 10,000 x g at 4˚C for 10 min. IFN-β and CXCL10 protein concentrations in the collected supernatant were assessed using commercial sandwich ELISA kits from PBL Assay Science (cat. no. 41435-1) and R&D Systems, Inc. (cat. no. DIP100),

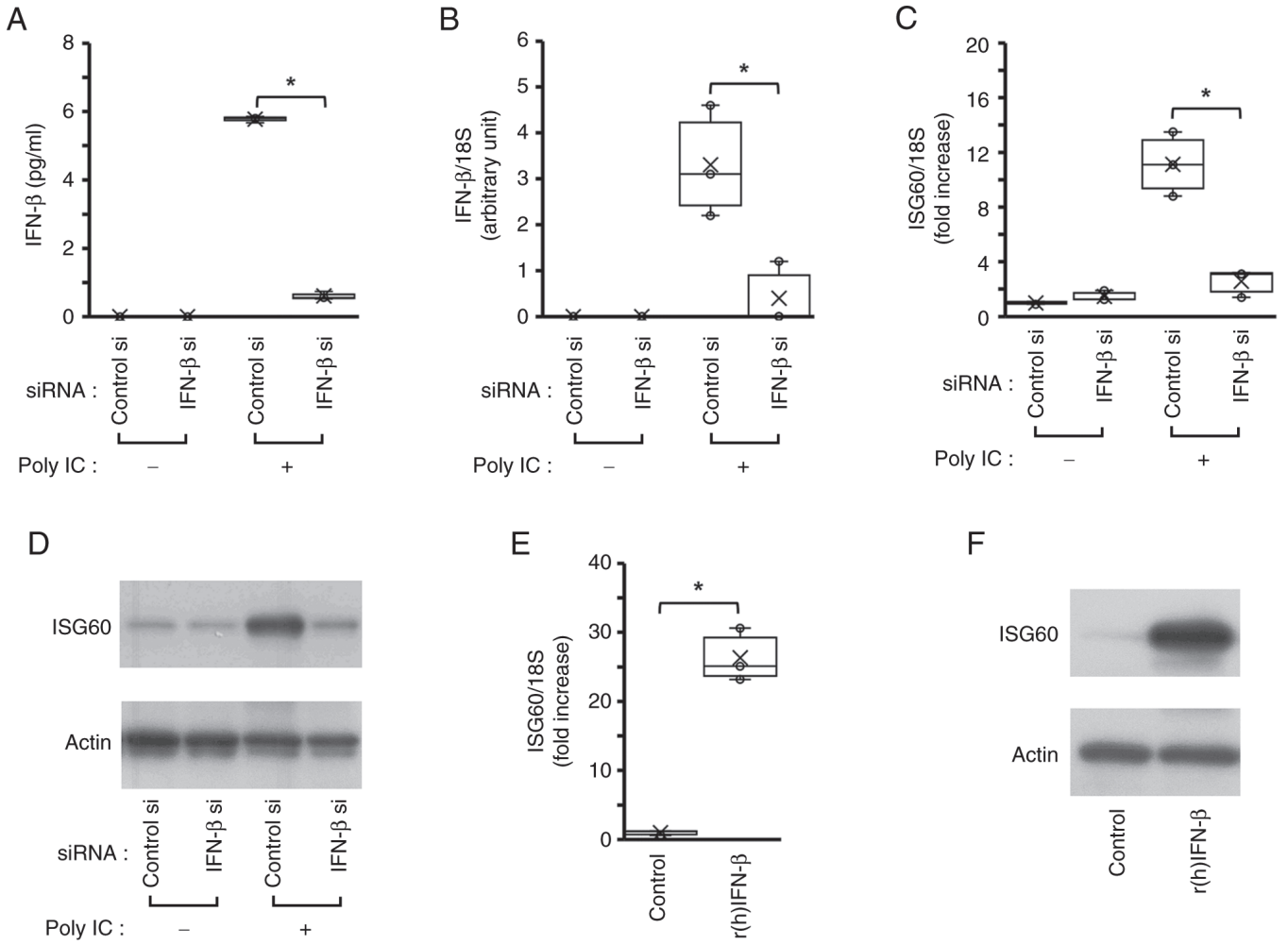

Figure 2. IFN-β is involved in poly IC-induced ISG60 expression. The cells were transfected with a specific siRNA against IFN-β and were incubated for 24 h. Then, the cells were treated with 30 μg/ml poly IC. (A) After 4 h incubation, the medium was collected and IFN-β concentration was measured using an enzyme-linked immunosorbent assay. (B) After 2 h incubation, RNA was extracted and IFN-β mRNA expression was examined using RT-qPCR. (C) After 16 h incubation, RNA was extracted, and ISG60 mRNA expression was examined. (D) ISG60 protein expression was analyzed using western blotting after 24 h. The cells were treated with r(h)IFN-β for 16 h and were subjected to (E) RT-qPCR and (F) western blotting. *P<0.05 using Mann-Whitney U-test; n=3. IFN-β, interferon β; ISG, IFN-stimulated gene; poly IC, polyinosinic-polycytidylic acid; r(h), recombinant human; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering.

respectively. The assays were performed according to the manufacturers' protocols.

Statistical analysis. The results of RT-qPCR and ELISA are shown using box plots, which are presented as the median (center of the box), 25th percentile (bottom of the box), 75th percentile (top of the box) and range (minimum and maximum), or as the mean ± standard deviation. The Mann-Whitney U-test, or one-way ANOVA and Dunnett's post hoc test were utilized for statistical analyses using EZR software version 1.65 (20) (Saitama Medical Center, Jichi Medical University, Saitama, Japan). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of ISG60 in BEAS-2B cells treated with poly IC. In unstimulated BEAS-2B cells, only small amounts of ISG60 protein and mRNA were expressed. The protein and mRNA expression levels of ISG60 increased in cells supplemented with poly IC. At ≤10 μg/ml, poly IC did not affect the protein and mRNA expression levels of ISG60, whereas ISG60 expression was markedly upregulated in response to 30-50 μg/ml poly IC (Fig. 1A and B). Therefore, the cells were supplemented with 30 μg/ml poly IC in subsequent experiments. The mRNA expression levels of ISG60 were markedly increased at 4-16 h and declined at 24 h in cells treated with poly IC (Fig. 1C). Furthermore, IFN-β mRNA was not detected in unstimulated cells. Increased expression levels of ISG60 protein were observed in cells treated with poly IC at 8 h, which progressively increased until 24 h (Fig. 1D). Transient expression of IFN-β mRNA was detected 2 h after poly IC treatment (Fig. 1E). IFN-β protein levels were transiently detected in the conditioned medium from cells treated with poly IC; they peaked at 4 h and quickly decreased thereafter (Fig. 1F).

IFN-β serves a role in poly IC-induced expression of ISG60 in BEAS-2B cells. As shown in Fig. 1E and F, IFN-β mRNA and protein was not detected in cells without poly IC stimulation, thus suggesting that no IFN-β mRNA and protein are

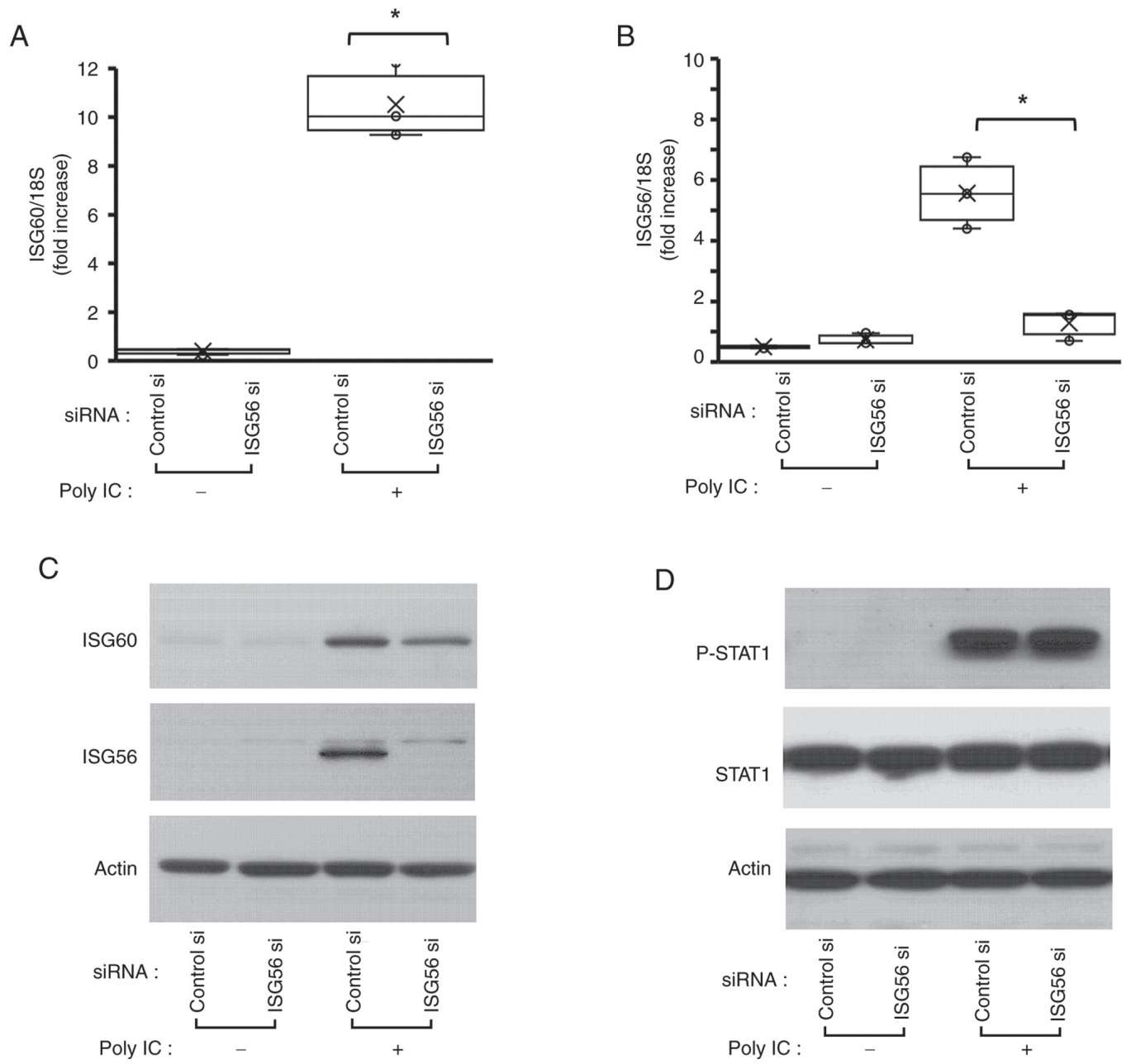

Figure 3. ISG56 is involved in poly IC-induced ISG60 expression. The cells were transfected with a specific siRNA against ISG56 and were incubated with poly IC for 16 h (for ISG60 and ISG56 mRNA analysis), 24 h (for ISG60 and ISG56 protein analysis) and 6 h (for P-STAT1 and STAT1 protein analysis). (A) ISG60 and (B) ISG56 mRNA expression was examined using reverse transcription-quantitative PCR. *P<0.05 using Mann-Whitney U-test; n=3. (C) Protein expression levels of ISG60, ISG56, and (D) P-STAT1 and STAT1 were investigated using western blotting. ISG, IFN-stimulated gene; P-, phosphorylated; poly IC, polyinosinic-polycytidylic acid; si, small interfering; STAT1, signal transducer and activator transcription 1.

expressed in unstimulated BEAS-2B cells. In the medium collected from cells transfected with control siRNA or IFN-β siRNA without poly IC-stimulation, IFN-β protein was not detected (Fig. 2A), and IFN-β mRNA expression was also not detected in those cells (Fig. 2B). Transfection of BEAS-2B cells with siRNA against IFN-β resulted in almost complete knockdown of IFN-β protein and mRNA expression in poly IC-stimulated cells, suggesting that transfection of IFN-β siRNA was successful (Fig. 2A and B). In addition, knockdown of IFN-β almost completely suppressed ISG60 mRNA (Fig. 2C) and protein (Fig. 2D) expression upon poly IC treatment. By contrast, treatment of BEAS-2B cells with 1 ng/ml r(h)IFN-β increased the expression levels of ISG60 mRNA (Fig. 2E) and protein (Fig. 2F).

ISG56 is associated with ISG60 expression. The present study assessed the function of ISG56 on ISG60 expression. ISG56 mRNA and protein knockdown was confirmed using RT-qPCR (Fig. 3B) and western blotting in poly IC-treated cells, respectively (Fig. 3C). Notably, there was no significant difference between groups in unstimulated cells, because ISG56 protein expression was not detectable in unstimulated cells (Fig. 3C). ISG56 knockdown markedly reduced poly IC-induced expression of ISG60 mRNA (Fig. 3A) and slightly

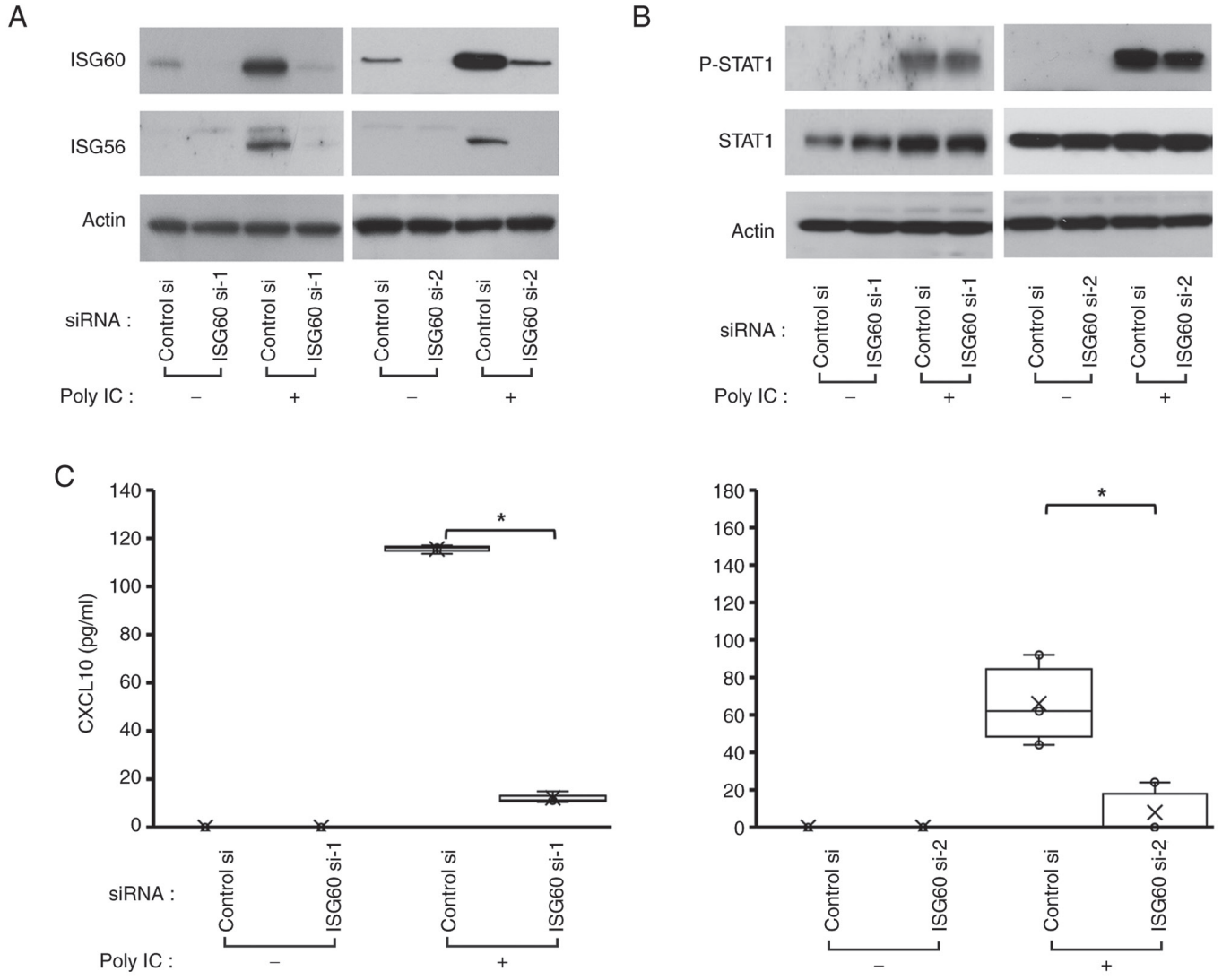

Figure 4. Continued.

reduced poly IC-induced ISG60 protein (Fig. 3C). However, P-STAT1 expression was not affected by ISG56 knockdown (Fig. 3D).

ISG60 is implicated in poly IC-mediated expression of ISG56 and CXCL10, but does not affect IFN-β expression and STAT1 phosphorylation. Transfection of the cells with ISG60 si-1 and ISG60 si-2, followed by western blotting, demonstrated that these two siRNAs markedly decreased poly IC-induced ISG60 expression, suggesting that ISG60 expression was successfully knocked down (Fig. 4A). Furthermore, ISG60 knockdown decreased poly IC-induced protein and mRNA expression levels of ISG56 (Fig. 4A and D), and protein concentration and mRNA expression levels of CXCL10 (Fig. 4C and D), whereas no notable change was observed in P-STAT1 or STAT1 protein expression (Fig. 4B). Furthermore, knockdown of ISG60 using ISG60 si-1 did not affect the mRNA expression levels of IFN-β (Fig. 4D). In order to examine whether ISG60 selectively inhibits poly IC-induced expression of ISG56 and CXCL10, or inhibits the expression of all ISGs, the present study next examined the effect of ISG60 knockdown on the poly IC-induced expression of another ISG, MDA5. MDA5 was chosen because it is one of the key ISGs in antiviral innate immune reactions (21). The results revealed that poly IC-induced MDA5 mRNA expression was not affected by transfection with ISG60 si-1 (Fig. 4D). These results suggested that ISG56 and CXCL10 were selectively regulated by ISG60. Transfection of cells with ISG60 si-2 slightly decreased the mRNA expression levels of IFN-β and MDA5, and this may be due to weak non-specific effects.

Discussion

Previous studies have shown that TLR3 is expressed in various cell types, including dendritic cells (22), fibroblasts (23), intestinal epithelial cells (24) and airway epithelial cells (25). TLR3 serves a physiological function in antiviral innate immunity and is involved in various inflammatory conditions (26); for example, TLR3-deficient mice have been reported to be susceptible to encephalomyocarditis virus compared with wild-type mice, whereas they were resistant to influenza virus (27). TLR3 activates the signal transduction cascade after recognizing virus-derived dsRNA to facilitate the transcription of type I IFNs. IFN-β is a type I IFN mainly

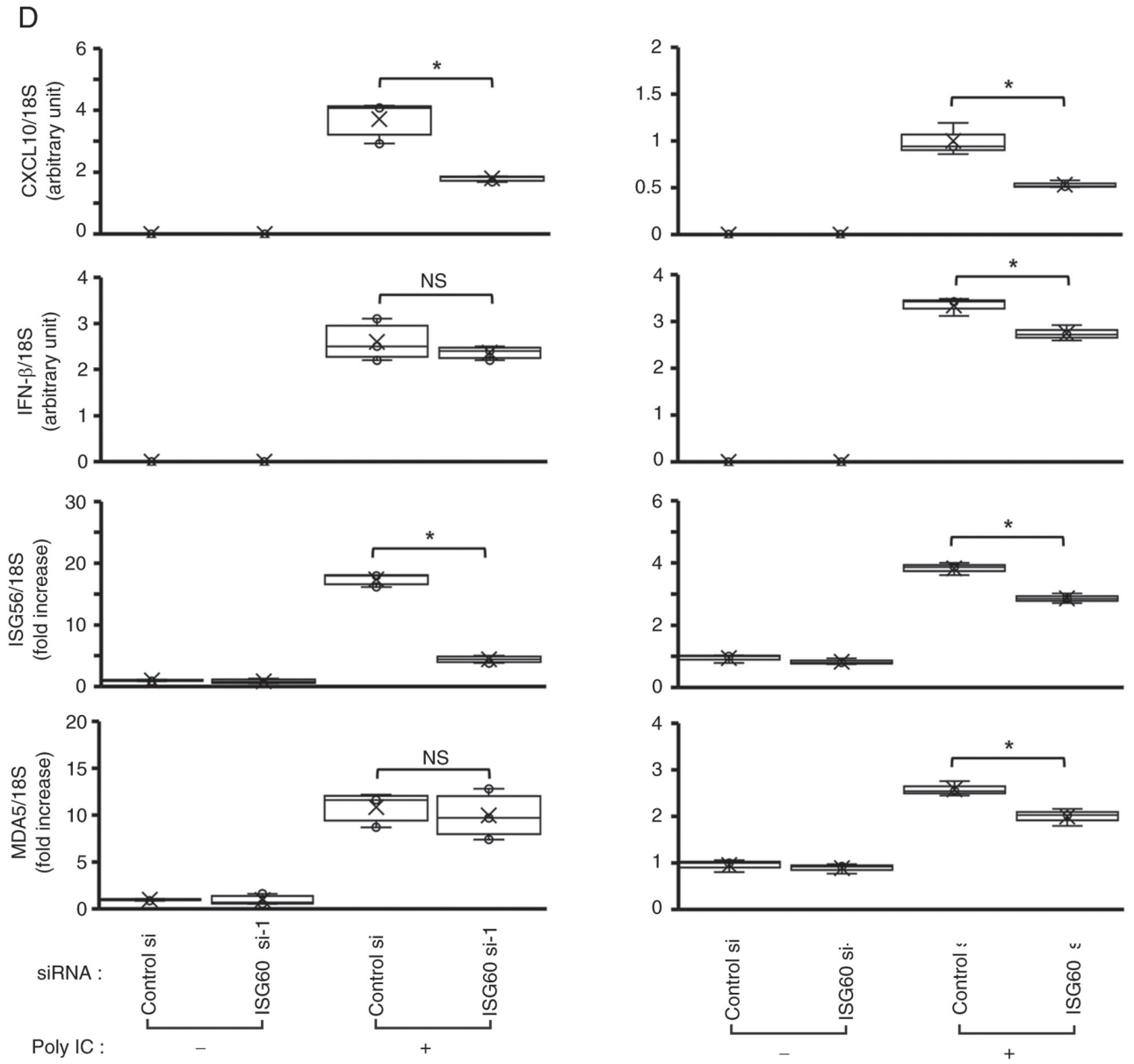

Figure 4. ISG60 is partially involved in poly IC-induced expression of CXCL10 and ISG56. The cells transfected with two different ISG60 siRNAs were treated with poly IC. (A) After incubating for 24 h, the cells were lysed and subjected to western blotting for ISG56, ISG60 and actin. (B) After incubating for 6 h, the cells were lysed and subjected to western blotting for P-STAT1, STAT1 and actin, (C) After 24 h incubation, the cell-conditioned medium was collected, and the concentration of CXCL10 protein was measured using an enzyme-linked immunosorbent assay. (D) After incubating for 2 h (for IFN-β mRNA analysis) or 16 h (for CXCL10, ISG56 and MDA5 mRNA analysis), RNA was extracted from the cells, and reverse transcription-quantitative PCR was performed. *P<0.05 using Mann-Whitney U-test; n=3. CXCL10, C-X-C motif chemokine ligand 10; IFN-β, interferon β; ISG, IFN-stimulated gene; MDA, melanoma differentiation-associated gene 5; NS, not significant; P-, phosphorylated; poly IC, polyinosinic-polycytidylic acid; si, small interfering; STAT1, signal transducer and activator transcription 1.

expressed in bronchial epithelial cells, which induces the expression of ISGs that function in antiviral host defense reactions.

The present study confirmed that the expression of ISG60 in BEAS-2B cells was time- and concentration-dependent when stimulated by poly IC. This finding is consistent with those of previous studies showing poly IC-induced ISG60 expression in U373MG astrocytoma cells (28) and hCMEC/D3 human brain microvascular endothelial cells (29). These findings suggested that ISG60 may have a role in congenital antiviral immune responses in various tissues, including bronchial epithelial cells. Because ISG60 is a member of the ISG family, the present study investigated whether *de novo*-produced IFN-β contributes to poly IC-induced ISG60 expression using RNA interference against IFN-b. As a result, IFN-β knockdown largely suppressed poly IC-induced ISG60 expression, and the induction of ISG60 protein and mRNA expression was observed in BEAS-2B cells treated with r(h)IFN-β. These results indicated that poly IC-induced ISG60 expression may be dependent on newly synthesized IFN-β, and ISG60 was implicated in TLR3/IFN-β-mediated antiviral reactions in BEAS-2B cells.

CXCL10, a CXC chemokine, is a robust chemoattractant of T lymphocytes and natural killer cells, which

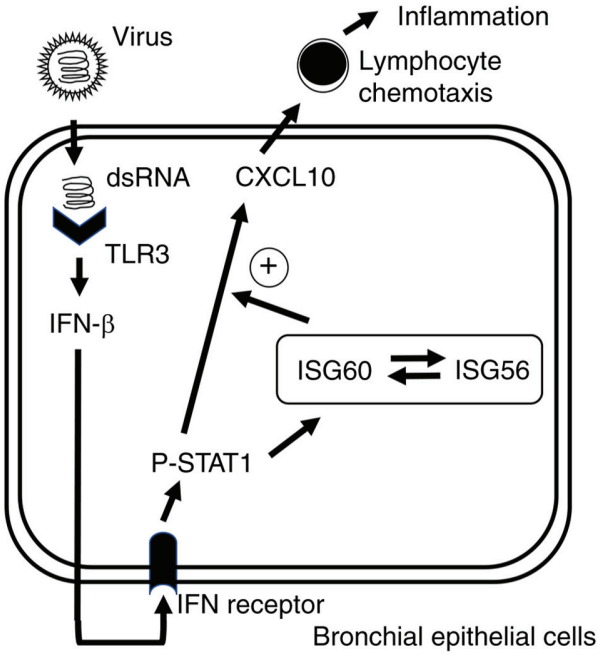

Figure 5. Schematic model of ISG60 expression downstream of TLR3 signaling in bronchial epithelial cells. CXCL10, C-X-C motif chemokine ligand 10; dsRNA, double-stranded RNA; IFN, interferon; ISG, IFN-stimulated gene; P-STAT1, phosphorylated-signal transducer and activator transcription 1; si, small interfering; TLR3, Toll-like receptor 3.

serves various roles in the pathogenesis of inflammatory diseases and infections (30). The expression of CXCL10 must be tightly regulated to induce chemotaxis of appropriate numbers of lymphocytes to the site of infection (31). Insufficient CXCL10 expression can exacerbate viral infections, while excessive CXCL10 can lead to excessive lymphocyte infiltration, resulting in tissue injury (32). Moreover, CXCL10 protein levels in serum and the severity of acute respiratory virus infection have been shown to be correlated (33). Therefore, 'fine-tuning' CXCL10 expression is crucial for appropriate immune responses in the airway. In the current study, ISG60 knockdown using two different siRNAs partially decreased poly IC-induced CXCL10 mRNA expression and protein levels. These findings indicated that ISG60 may not be the primary regulator of CXCL10 expression, but may be implicated in the 'fine-tuning' of TLR3-mediated CXCL10 expression in BEAS-2B cells. This finding is consistent with that of a previous study in U373 astrocytoma cells (28).

Phosphorylation of STAT1 is an important step in the signaling pathway induced by IFN-β. In the present study, poly IC-induced IFN-β expression and subsequent STAT1 phosphorylation were not affected by ISG60 knockdown in BEAS-2B cells. This finding suggested that ISG60 may be involved in a reaction downstream of the IFN-β/STAT1 axis. However, this finding is in contrast to the results of a previous study in U373 cells, in which ISG60 positively regulated STAT1 phosphorylation (28). Additionally, in hCMEC/D3 brain endothelial cells, ISG60 negatively regulated TLR3-induced signaling (29). These differences indicated that ISG60 may function differently in various cell types.

ISG60, also known as IFIT3 (34), is a tetratricopeptide repeat protein that participates in various biological processes, including protein-protein and protein-RNA interactions within multi-molecule complexes. These proteins regulate chaperones, transcription, splicing, cell cycle control, protein transport, and phosphate turnover (35). In addition, ISG56/IFIT1 is known to be linked to innate immunity (11). In a previous study, we reported that ISG56 may be implicated in poly IC-induced CXCL10 expression in BEAS-2B cells (14). ISG56 is considered a partner molecule of ISG60 (11); therefore, this relationship was further assessed in the present study. ISG60 knockdown decreased poly IC-induced ISG56 expression, whereas poly IC-induced expression of MDA5, another innate immunity-related ISG, remained unchanged upon ISG60 knockdown. These findings suggested that ISG60 may selectively regulate ISG56 expression. By contrast, ISG56 knockdown decreased ISG60 expression, whereas the P-STAT1 expression did not change. These findings indicated that ISG60 and ISG56 are mutually regulated downstream of P-STAT1 in poly IC-treated BEAS-2B cells.

Because IFIT proteins, including ISG60 and ISG56, are known to have broad-spectrum antiviral functions (36), the results of the present study suggested that ISG60 and ISG56 may serve crucial roles in antiviral innate immune reactions in airway epithelial cells. Gene expression is regulated by protein complexes containing transcriptional factors, transcriptional regulators, coactivators and RNA polymerase. In addition, other components, including non-coding RNAs, are involved in the precise control of the gene expression. It has been reported that IFIT proteins regulate antiviral immune reactions by mediating a variety of protein-protein and protein-RNA interactions (11). Therefore, it was hypothesized that ISG60, as a component of a multiple protein complex, may regulate the expression of ISG56 and CXCL10 via protein-protein and/or protein-RNA interactions. However, the present study has some limitations. First, the study did not clarify the specific molecular mechanisms by which ISG60 regulates the poly IC-induced expression of CXCL10, or by which ISG60 and ISG56 regulate each other. Future studies should assess the detailed pathways and interactions involved. Second, the present study did not examine an experimental model with ISG60 upregulation. Third, this study only used an *in vitro* cell culture system. Future studies using lentiviral transfection to upregulate ISG60 or animal models may enhance the relevance and applicability of the present findings.

In conclusion, the present study demonstrated that ISG60 was upregulated by TLR3 signaling and may have a role in the 'fine-tuning' of CXCL10 expression in BEAS-2B cells. The present study reported that the TLR3/IFN-β/ISG60/CXCL10 axis (Fig. 5) may be a novel mechanism to enhance antiviral innate immune responses in bronchial epithelial cells. This axis may be important in antiviral immunity in the respiratory tract and appropriate activation of this axis could contribute to the host defense against viral infection. However, excess or dysregulated activation of this axis may lead to excess accumulation of lymphocytes; since excess accumulation of lymphocytes induces the excess activation of cytokine networks (37),

dysregulated activation of this axis may exacerbate inflammation and airway injury induced by viral infection. Therefore, this axis may be involved in both physiological antiviral innate immune reaction and pathological inflammation induced by viral infection in the airways. The present findings suggested that ISG60 may serve as a potential target for developing new therapeutic strategies against viral respiratory infections.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YT and TI contributed to all experiments and prepared the manuscript. YK performed the ELISA. MT performed the western blotting. TS, MD and MS performed the RNA extraction and RT-qPCR analyses. SK and KS performed the cell culturing. TI and ST designed the study. All authors have read and approved the final version of the manuscript. YT and TI confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bakaletz LO: Viral-bacterial co-infections in the respiratory tract. Curr Opin Microbiol 35: 30-35, 2017.
2. Medzhitov R and Janeway C Jr: Innate immunity: Impact on the adaptive immune response. Curr Opin Immunol 9: 4-9, 1997.
3. Okahira S, Nishikawa F, Nishikawa S, Akazawa T, Seya T and Matsumoto M: Interferon-beta induction through toll-like receptor 3 depends on double-stranded RNA structure. DNA Cell Biol 24: 614-623, 2005.
4. Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M and 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 280: 5571-5580, 2005.
5. Yu M and Levine SJ: Toll-like receptor, RIG-I-like receptors and the NLRP3 inflammasome: Key modulators of innate immune responses to double-stranded RNA viruses. Cytokine Growth Factor Rev 22: 63-72, 2011.
6. Kawai T and Akira S: The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors. Nat Immunol 11: 373-384, 2010.
7. Hsu ACY, Parsons K, Barr I, Lowther S, Middleton D, Hansbro PM and Wark PAB: Critical role of constitutive type I interferon response in bronchial epithelial cell to influenza infection. PLoS One 7: e32947, 2012.
8. Majde JA: Viral double-stranded RNA, cytokines, and the flu. J Interferon Cytokine Res 20: 259-272, 2000.
9. Hamamdzic D, Phillips-Dorsett T, Altman-Hamamdzic S, London SD and London L: Reovirus triggers cell type-specific proinflammatory responses dependent on the autocrine action of IFN-beta. Am J Physiol Lung Cell Mol Physiol 280: L18-L29, 2001.
10. Imaizumi T, Aizawa-Yashiro T, Tsuruga K, Tanaka H, Matsumiya T, Yoshida H, Tatsuta T, Xing F, Hayakari R and Satoh K: Melanoma differentiation-associated gene 5 regulates the expression of a chemokine CXCL10 in human mesangial cells: Implications for chronic inflammatory renal diseases. Tohoku J Exp Med 228: 17-61, 2012.
11. Fensterl V and Sen GC: The ISG56/IFIT1 gene family. J Interferon Cytokine Res 31: 71-78, 2011.
12. Vareille M, Kieninger E, Edwards MR and Regamey N: The airway epithelium: Soldier in the fight against respiratory viruses. Clin Microbiol Rev 24: 210-219, 2011.
13. Sanders CJ, Doherty PC and Thomas PG: Respiratory epithelial cells in innate immunity to influenza virus infection. Cell Tissue Res 343: 13-21, 2011.
14. Shiratori T, Imaizumi T, Hirono K, Kawaguchi S, Matsumiya T, Seya K and Tasaka S: ISG56 is involved in CXCL10 expression induced by TLR3 signaling in BEAS-2B bronchial epithelial cells. Exp Lung Res 46: 195-202, 2020.
15. Yu M, Tong JH, Mao M, Kan LX, Liu MM, Sun YW, Fu G, Jing YK, Yu L, Lepaslier D, *et al*: Cloning of a gene (RIG-G) associated with retinoic acid-induced differentiation of acute promyelocytic leukemia cells and representing a new member of a family of interferon-stimulated genes. Proc Natl Acad Sci USA 94: 7406-7411, 1997.
16. Zhou X, Michal JJ, Zhang L, Ding B, Lunney JK, Liu B and Jiang Z: Interferon induced IFIT family genes in host antiviral defense. Int J Biol Sci 9: 200-208, 2013.
17. Chikhalya A, Dittmann M, Zheng Y, Sohn SC, Rice CM and Hearing P: Human IFIT3 protein induced interferon signaling and inhibits adenovirus immediate early gene expression. mBio 12: e0282921, 2021.
18. Imaizumi T, Tanaka H, Matsumiya T, Yoshida H, Tanji K, Tsuruga K, Oki E, Aizawa-Yashiro T, Ito E and Satoh K: Retinoic acid-inducible gene-I is induced by double-stranded RNA and regulates the expression of CC chemokine ligand (CCL) 5 in human mesangial cells. Nephrol Dial Transplant 25: 3534-3539, 2010.
19. Schmittgen TD and Livak KJ: Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101-1108, 2008.
20. Kanda Y: Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant 48: 452-458, 2013.
21. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P and Rice CM: A diverse range of gene products are effectors of the type I interferon antiviral response. Nature 472: 481-485, 2011.
22. Muzio M, Bosisio D, Polentarutti N, D'amico G, Stoppacciaro A, Mancinelli R, van't Veer C, Penton-Rol G, Ruco LP, Allavena P and Mantovani A: Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: Selective expression of TLR3 in dendritic cells. J Immunol 164: 5998-6004, 2000.
23. Matsumoto M, Kikkawa S, Kohase M, Miyake K and Seya T: Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. Biochem Biophys Res Commun 293: 1364-1369, 2002.
24. Cario E and Podolsky DK: Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 68: 7010-7017, 2000.
25. Lai Y, Yi G, Chen A, Bhardwaj K, Tragesser BJ, Valverde RA, Zlotnick A, Mukhopadhyay S, Ranjith-Kumar CT and Kao CC: Viral double-strand RNA-binding proteins can enhance innate immune signaling by toll-like Receptor 3. PLoS One 6: e25837, 2011.
26. Matsumoto M and Seya T: TLR3: Interferon induction by double-stranded RNA including poly(I:C). Adv Drug Deliv Rev 60: 805-812, 2008.

27. Zhang SY, Herman M, Ciancanelli MJ, Pérez de Diego R, Sancho-Shimizu V, Abel L and Casanova JL: TLR3 immunity to infection inmice and humans. Curr Opin Immunol 25: 19-33, 2013.
28. Imaizumi T, Yoshida H, Hayakari R, Xing F, Wang L, Matsumiya T, Tanji K, Kawaguchi S, Murakami M and Tanaka H: Interferon-stimulated gene (ISG) 60, as well as ISG56 and ISG54, positively regulates TLR3/IFN-β/STAT1 axis in U373MG human astrocytoma cells. Neurosci Res 105: 35-41, 2016.
29. Imaizumi T, Sassa N, Kawaguchi S, Matsumiya T, Yoshida H, Seya K, Shiratori T, Hirono K and Tanaka H: Interferon-stimulated gene 60 (ISG60) constitutes a negative feedback loop in the downstream of TLR3 signaling in hCMEC/D3 cells. J Neuroimmunol 324: 16-21, 2018.
30. Liu M, Guo S, Hibbert JM, Jain V, Singh N, Wilson NO and Stiles JK: CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. Cytokine Growth Factor Rev 22: 121-130, 2011.
31. Dwinell MB, Lugering N, Eckmann L and Kangnoff MF: Regulated production of interferon-inducible T-cell chemoattractants by human intestinal epithelial cells. Gastroenterology 120: 49-59, 2001.
32. Frangogiannis NG, Mendoza LH, Smith CW, Michael LH and Entman ML: Induction of the synthesis of the C-X-C chemokine interferon-gamma-inducible protein-10 in experimental canine endotoxemia. Cell Tissue Res 302: 365-376, 2000.
33. Hayney MS, Henriquez KM, Barnet JH, Ewers T, Champion HM, Flannery S and Barrett B: Serum IFN-γ-induced protein 10 (IP-10) as a biomarker for severity of acute respiratory infection in healthy adults. J Clin Virol 90: 32-37, 2017.
34. Schmeisser H, Mejido J, Balinsky CA, Morrow AN, Clark CR, Zhao T and Zoon KC: Identification of alpha interferon-induced genes associated with antiviral activity in Daudi cells and characterization of IFIT3 as a novel antiviral gene. J Virol 84: 10671-10680, 2010.
35. Blatch GL and Lässle M: The tetratricopeptide repeat: A structural motif mediating protein-protein interactions. Bioessays 21: 932-939, 1999.
36. Diamond MS and Farzan M: The broad-spectrum antiviral functions of IFIT and IFITM proteins. Nat Rev Immunol 13: 46-57, 2013.
37. Tang Y, Liu J, Zhang D, Xu Z, Ji J and Wen C: Cytokine storm in COVID-19: The current evidence and treatment strategies. Front Immunol 11: 1708, 2020.