ISG56 is involved in CXCL10 expression induced by TLR3 signaling in BEAS-2B bronchial epithelial cells

（BEAS-2B　気道上皮細胞における TLR3 シグナルによる CXCL10 発現誘導への ISG56 の関与について）

申請者　　弘前大学大学院医学研究科　病態制御科学領域
呼吸病態内科学教育研究分野　4 年

氏名　　白鳥　俊博

指導教授　　田坂　定智

ISG56 is involved in CXCL10 expression induced by TLR3 signalling in BEAS-2B bronchial epithelial cells

ABSTRACT

Purpose and Aim of the study: Bronchial epithelial cells play an important role in immune response against viral infections. Toll-like receptor 3 (TLR3) is a pathogen recognition receptor that recognizes viral double-stranded RNA (dsRNA). Activation of TLR3 induces the expression of interferon (IFN)-β, and newly synthesized IFN-β exhibits anti-viral activity by upregulating the expression of IFN-stimulated genes (ISGs). ISG56 encodes a multifunctional protein with tetratricopeptide motifs and is involved in anti-viral reactions through various mechanisms. Expression of chemokines such as CXCL10, which induces leukocyte chemotaxis, is essential for defense against airway microbes. However, regulation of chemokine expression by ISG56 in bronchial epithelial cells has not been fully investigated. The aim of this study was to examine the expression of ISG56 and its role in CXCL10 production in BEAS-2B bronchial epithelial cells treated with dsRNA. **Materials and Methods:** BEAS-2B bronchial epithelial cells were treated with polyinosinic-polycytidylic acid (poly IC), a synthetic TLR3 ligand. The mRNA and protein expression levels of ISG 56 were analysed by quantitative reverse transcription polymerase chain reaction and western blotting. The effect of knocking down TLR3, IFN-β and ISG56 was examined using RNA interference. The protein expression of CXCL10 in culture medium was measured using an enzyme-linked immunosorbent assay. **Results:** Poly IC induced ISG56 expression in a concentration- and time- dependent manner. RNA interference showed that ISG56 induction was inhibited by knockdown of TLR3 or IFN-β and that ISG 56 knockdown decreased CXCL10 expression. **Conclusions:** ISG56 was induced by poly IC through TLR3/IFN-β axis, and ISG56 may positively regulated CXCL10 expression in BEAS-2B cells. ISG56 may modulate anti-viral innate immunity, at least in part, by regulating the expression of CXCL10 in bronchial epithelial cells.

keywords: bronchial epithelial cells; CXCL10; IFN-β; ISG56; TLR3

Introduction

The bronchial epithelium is constantly exposed to various microbes, and innate immune reactions in bronchial epithelial cells are the first line prevention against infectious lung diseases. A wide variety of factors produced by bronchial epithelial cells are involved in host defense against microbial infections and inflammatory reactions.[1] When microbial pathogens invade the airway, pattern recognition receptors in respiratory epithelial cells recognize the pathogen-associated molecular patterns (PAMPs) derived from infected microorganisms, and innate immune reactions are initiated. Toll-like receptors (TLRs) function as pattern recognition receptors and are key molecules in the initial step of innate immune system. Binding of PAMPs to TLRs triggers a cascade of intracellular signaling pathways, leading to the induction of molecules that regulate immune and inflammatory reactions.[2] TLR3 is a member of TLR family that recognizes viral double-stranded RNA (ds RNA) as a PAMP. TLR3 is reportedly expressed in human bronchial epithelial cells[3] and plays an essential role in anti-viral innate immune reactions. Binding of viral dsRNA to TLR3 is followed by the induction of type I interferons (IFNs), which are key cytokines in host defense against viral infections. Type I IFNs exert antiviral effects by inducing IFN-stimulated genes (ISGs). Among type I IFNs, IFN-β plays a critical role in reactions against viral infection in bronchial epithelial cells. [4]

ISG56, also known as IFN-induced protein with tetratricopeptide repeats 1, is a member of ISG family.[5] ISG56 exerts a wide variety of biological functions including regulation of translation, inhibition of viral replication, cell migration, cell proliferation, and cell death.[5] Expression of ISG56 is induced in airway epithelial cells by viral infections such as influenza,[6] human metapneumovirus,[7] and H9N2.[8] Reports have

suggested that ISG56 is involved in antiviral immune and inflammatory reactions in bronchial epithelial cells. However, the mechanisms through which ISG56 exerts anti-viral functions in respiratory tract are not fully investigated.

C-X-C motif chemokine ligand 10 (CXCL10) is a member of CXC chemokine family. It was originally identified as an IFN-γ-inducible protein, and is also a type of ISGs. CXCR3 is the receptor for CXCL10, and binding of CXCL10 to CXCR3 induces chemotaxis of $CXCR3^+$ cells including T cells and NK cells.[9] CXCL10 is involved in the protection against various viral infections and the pathogenesis of virus-induced infectious diseases.[10] We have previously reported that polyinosinic-polycytidylic acid (poly IC), an authentic dsRNA ligand for TLR3, induces the expression of CXCL10 in BEAS-2B cells, a cell line derived from normal human bronchial epithelial cells.[11] In the present study, we examined the expression of ISG56 in the downstream of TLR3 signalling in BEAS-2B cells, and investigated the role of ISG56 in the induction of CXCL10 expression.

Materials and methods

Reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), M-MLV reverse transcriptase and Lipofectamine RNAiMAX were purchased from Invitrogen (Frederick, MD, USA). Poly IC and rabbit anti-actin IgG were acquired from Sigma (St. Louis, MO, USA). Molecular weight of this poly IC is heterogeneous, and distributes in a wide range from low to high molecular weight. Small interfering RNAs (siRNAs) against TLR3 (SI02655156), ISG56 (SI02660777) and non-silencing negative control siRNA (1027281) were from Qiagen (Hilden, Germany). An siRNA against IFN-β was described previously,[12] and recombinant human (r(h)) IFN-β was obtained

from ProSpec-Tany Technogene (Rehovot, Israel). Rabbit antibody against ISG56 (N2C3) was from GeneTex (Irvine, CA, USA). The illustra RNAspin kit was from GE Healthcare (Buckinghamshire, England), and dNTP mix was obtained from Thermo Fisher Scientific (Asheville, MA, USA). SsoAdvanced Universal SYBR Green Supermix was obtained from Bio-Rad (Hercules, CA, USA). Oligo $(dT)_{18}$ primer for reverse transcription and oligonucleotide primers for polymerase chain reaction (PCR) were synthesized by Greiner Japan (Atsugi, Japan). Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was purchased from Rockland Immunochemicals (Limerick, PA, USA). Polyvinylidene difluoride (PVDF) membranes and Luminata Crescendo Western HRP Substrate were from Merck Millipore (Darmstadt, Germany). Enzyme-linked immunosorbent assay (ELISA) kit for CXCL10 was obtained from R&D systems (Minneapolis, MN, USA).

Cell culture

BEAS-2B is a cell line derived from human bronchial epithelial cells,[12] and has been used as a good model for the study of innate immune reactions in human bronchial epithelial cells.[8,14] BEAS-2B cells were cultured in DMEM supplemented with 10% FBS as previously described.[11] The cells were treated with 10-50 μg/mL poly IC in the initial experiment, and with 30 μg/mL poly IC for up to 24 h in the subsequent experiments. The cells were also treated with 1 ng/mL r(h) IFN-β for 16 h. For RNA interference, the cells were cultured in medium without antibiotics for 24 h before the transfection. The cells were then transfected with non-silencing control siRNA or siRNAs against TLR3, IFN-β, or ISG56 using a Lipofectamine RNAiMAX reagent according to the manufacture's protocol. After 24 h incubation, the cells were treated with 30 μg/mL poly IC for the indicated period of time.

Quantitative real-time reverse transcription (RT)-PCR analysis

Total RNA was extracted from cells using the illustra RNAspin kit. Single-strand cDNA as a PCR template was synthesized from the RNA using oligo$(dT)_{18}$ primer and M-MLV reverse transcriptase. The mRNA expression of CXCL1, CXCL10, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IFN-β and ISG56 were examined with a quantitative real-time PCR system using SsoAdvanced Universal SYBR Green Supermix. The following primers were used.

CXCL1-F: 5'- ATGGCCCGCGCTGCTCTCTCC -3',

CXCL1-R: 5'- GTTGGATTTGTCACTGTTCAG -3',

CXCL10-F: 5′-TTCAAGGAGTACCTCTCTCTAG-3′,

CXCL10-R: 5′-CTGGATTCAGACATCTCTTCTC-3′,

GAPDH-F: 5′-GCACCGTCAAGGCTGAGAAC-3′,

GAPDH-R: 5′-ATGGTGGTGAAGACGCCAGT-3′,

IFN-β-F: 5'- CCTGTGGCAATTGAATGGGAGGC -3'

IFN-β-R: 5'- CCAGGCACAGTGACTGTACTCCTT -3'

ISG56-F: 5′-GGTCTCTTCAGCATTTATTGGTG-3′,

and ISG56-R: R: 5′-TGCCGTAGGCTGCTCTCCA-3′.

The mRNA expression of CXCL10, CXCL1, and GAPDH is expressed as fold increase compared to that of unstimulated cells. The results of quantitative RT-PCR for IFN-β are shown as arbitrary units because it was not detected in unstimulated cells.

Western blotting

Western blotting was performed as previously described.[14] The cells were washed twice with phosphate-buffered saline (pH 7.4) and were lysed in Laemmli's reducing

sample buffer. The lysate was subjected to electrophoresis on a 10% polyacrylamide gel, and the proteins were transferred to a PVDF membrane. The membrane was incubated with an antibody against ISG56 (1:2000) or actin (1:3000) and subsequently with a HRP-labeled anti-rabbit IgG. Immunodetection was performed using Luminata Crescendo substrate.

ELISA for CXCL10

Cell-conditioned medium was collected and centrifuged for 10 min. The concentration of CXCL10 in the supernatant was determined using a sandwich ELISA kit according to the manufacturer's protocol.

Results

Poly IC induces ISG56expression in BEAS-2B bronchial epithelial cells Treatment of BEAS-2B cells with poly IC induced the mRNA and protein expression of ISG56 in concentration- (Figure 1) and time-dependent manners (Figure 2). The mRNA expression of ISG56 was gradually increased after poly IC treatment, reaching its maximum level at 16 h and decreasing until 24 h (Figure 2a). On the other hand, the mRNA level of IFN-β increased quickly after treatment, reaching maximum level at 2 h and decreased thereafter (Figure 2a). The increase in protein level of ISG56 lagged behind the mRNA expression, peaking at 16 – 24 h after poly IC treatment (Figure 2b). [Figure 1 and 2 near here]

TLR3 and IFN-β are involved in poly IC-induced expression of ISG56 and CXCL10

Transfection of BEAS-2B cells with specific siRNAs against TLR3 inhibited the mRNA expression of ISG56 and CXCL10 (Figure 3a) and protein expression of ISG56 (Figure 3b) induced by poly IC. Knockdown of IFN-β by RNA interfering also decreased the induction of ISG56 and CXCL10 mRNA (Figure 3a) and ISG56 protein (Figure 3b). Moreover, treatment with r(h) IFN-β also induced the mRNA (Figure 3c) and protein (Figure 3d) expression of ISG56.

[Figure 3 near here]

ISG56 is involved in the expression of CXCL10 Transfection of specific siRNA against ISG56 resulted in significant reduction in CXCL10 mRNA (Figure 4a) and protein (Figure 4b) expression induced by poly IC. However, knockdown of ISG56 did not affect the mRNA expression of CXCL1 and IFN-β. Effective silencing of ISG56 was confirmed by western blotting (Figure 4c).

[Figure 4 near here]

Discussion

TLR3 is one of the major pattern recognition receptors against viral dsRNA, and plays an important role in anti-viral innate immune reactions. Binding of viral dsRNA or poly IC to TLR3 triggers the activation of downstream signaling pathways in bronchial epithelial cells, and induces the production of chemokines, cytokines and other various molecules. These include C-C motif chemokine ligand 5 (CCL5),[16] CCL2,[17] CXCL1,[18] CXCL8,[19] CXCL10,[11] IFN-β,[20] interleukin-6,[21] CD161 ligand lectin-like transcript 1,[22] mucin 5AC,[23] and Tenascin-C.[24] These inducible molecules may cooperatively promote anti-viral immune and inflammatory reactions.

It has been reported that TLR3 is expressed and located in endosomes in BEAS-2B cells.[25] Herein, we report that poly IC, an authentic dsRNA widely used as a viral mimic, induces the expression of ISG56 in BEAS-2B in concentration- and time-dependent manners. Although the concentration of poly IC appears to be very high, it is similar to that in previous studies using "non-immune cells" such as brain microvascular endothelial cells [26] and bile duct epithelial cells.[27] We confirmed that TLR3 is involved in poly IC-induced ISG56 expression using RNA interference against TLR3. TLR3 signaling induces the expression of various genes in IFN-dependent or -independent manners. IFN-β is a major type I IFN produced by bronchial epithelial cells,[20] and knockdown of IFN-β almost completely inhibited poly IC- induced ISG56 expression in BEAS-2B cells. This finding suggests that ISG56 is induced by poly IC in an IFN-β-dependent manner. The expression of IFN-β mRNA was quickly increased after the treatment with poly IC and reached maximum level at 2 h. The increase of ISG56 mRNA was slower than that of IFN-β and reached a maximum level 16 h after poly IC treatment. This time course is consistent with the results of IFN-β knockdown. In addition, treatment of cells with r(h)IFN-β induced the mRNA and protein expression of ISG56. Taken together, poly IC induces the expression of IFN-β, and in turn, newly synthesized IFN-β is involved in ISG56 expression in BEAS-2B cells.

ISG56 functions as an anti-viral molecule through multiple mechanisms.[8] For example, it inhibits replication of human papillomavirus by binding to viral E1 helicase,[28] blocks replication of hepatitis C virus by interacting with the translation initiation factor eIF3,[29] alters the patterns and levels of parainfluenza virus type 5 mRNA and protein synthesis,[30] and inhibits viral mRNA translation.[31] In the present study, we found that ISG56 knockdown by RNA interference resulted in a decreased in the expression of CXCL10 induced by poly IC, while the expression of another CXC

chemokine CXCL1 was not affected. This suggests that ISG56 is selectively involved in the expression of CXCL10. CXCL10 is a member of CXC chemokine family that plays an essential role in host defence against viral infections by stimulating lymphocyte chemotaxis to the infected sites. Therefore, ISG56 may exert anti-viral immune and inflammatory reactions in bronchial epithelial cells, at least partly, by upregulating the expression of CXCL10. CXCL10 also plays diverse roles in the pathogenesis of infectious and inflammatory diseases,[32] and serum CXCL10 concentration is correlated with severity of acute respiratory viral infection.[33] Dysregulated expression of ISG56 may be associated with inflammatory lung diseases, and this should be investigated in future studies.

The results of the present study agree with our previous observation in U373 astrocytoma cells,[15] but may contradict a previous report which identified ISG56 as a mediator of negative-feedback regulation of virus-triggered signaling in Hela cells, A549 cells and macrophages.[34] Although the reason of this inconsistency was not clarified in the present study, it is possible that ISG56 functions in a complex manner under different conditions or in different cell types. The precise molecular mechanisms through which ISG56 regulates CXCL10 expression were not cleared in the present study and require further studies.

The present study was conducted in BEAS-2B cells. Although BEAS-2B cells are cell line derived from human bronchial epithelial cells, these cells have a different phenotype from primary human bronchial epithelial cells. The results of the present study should be also confirmed in primary human bronchial epithelial cells in future studies.

Conclusions

We report that TLR3/IFN-β signaling axis induces the expression of ISG56 in BEAS-2B human bronchial epithelial cells. ISG56 increases the expression of CXCL10 induced by poly IC. These findings indicate that ISG56, which is expressed in bronchial epithelial cells, may play an important role in anti-viral immune reactions and in inflammatory diseases.

Acknowledgement

We thank Kumiko Munakata and Michiko Nakata for their helps.

Author contributions

TS and TI performed all experiments and prepared the manuscript. SK, TM and KS were involved in cell culture and treatment. KH performed quantitative real-time RT-PCR and ELISA. TI and ST contributed to the design of the study.

Data availability statement

The dataset used and analysed during the current study are available from the corresponding author on reasonable request. Before this request, users should get permission from the local ethics committee.

Disclosure of interest

The authors report no conflict of interest.

References

1. Alter KB, Fischer BM, Wright DT, Cohn LA, Becker S. Interatctions between respiratory epithelial cells and cytokines: relationships to lung inflammation. *Ann N Y Acad Sci.* 1994;725:128-145.

2. Zeytun A, Chaudhary A, Pardington P, Cary R, Guputa G. Induction of cytokines and chemokines by Toll-like receptor signaling: strategies for control of inflammation. *Crit Rev Immunol.* 2010;30:53-67.

3. 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, 280:5571-5580.

4. Hsu ACY, Parsons K, Barr I, Lowther S, Middleton D, Hansbro PM, Wark PAB. Critical role of constitutive type I interferon response in bronchial epithelial cell to influenza infection. *PLoS One*. 2012;7:e32947.

5. Frensterl V, Sen GC, The ISG56/IFIT1 gene family. *J Interferon Cytokine Res.* 2011;31:71-77.

6. 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.

7. Kan-O K, 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 glucocoticosteroids and interferon. *J Infect Dis*. 2017; 215:1536-1545.

8. Feng B, Zhang Q, Wang J, Dong H, Mu Xiang, 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;41:271-281.

9. Karin N, Razon H. Chemokines beyond chemo-attraction: CXCL10 and its significant role in cancer and autoimmunity. *Cytokine*. 2018;109:24-28.

10. Liu M, Guo S, Hibbert JM, Jain V, Singh N, Wilson NO, Stiles JK. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. *Cytokine Growth Factor Rev*. 2011;22:121-130.

11. Taima K, Imaizumi T, Yamashita K, Ishikawa A, Fujita T, Yoshida H, Takanashi S, Okumura K, Satoh K. Espression of IP-10/CXCL10 is upregulated by double-stranded RNA in BEAS-2B bronchial epithelial cells. *Respiration*. 2006;73:360-364.

12. Amstad P, Reddel RR, Pfeifer A, Malan-Shibley L, Mark GE 3rd, Harris CC. Neoplastic transformation of human bronchial epithelial cell line by a recombinant retrovirus encoding viral Harvey ras. *Mol Carcinog.* 1988;1:151-160.

13. Imaizumi T, Tanaka H, Matsumiya T, Yoshida H, Tanji K, Tsuruga K, Oki E, Yashiro T, Ito E, Satoh K. Retinoic acid-inducible gene-I is induced by double-stranded RNA and regulates the expression of CCL5 in human mesangial cells. *Nephrol Dial Transplant*. 2010;25:3534-3539.

14. Nakamoto K, Watanabe M, Sada M, Unui T, Nakamura M, Honda K, Wada H, Ishii H, Takizawa H. *Psudomonas 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;45:255-266.

15. Imaizumi T, Numata A, Yano C, Yoshida H, Meng P, Hayakari R, Xing F, Wang L, Matsumiya T, Tanji K, Tatsuta T, Murakami M, Tanaka H. ISG54 and ISG56 induced by TLR3 signaling in U373MG human astrocytoma cells: Possible involvement in CXCL10 expression. *Neuroscience Res*. 2014;84:34-42.

16. Ieki K, Matsukura S, Kokubo F, Kimura T, Kuga H, Kawaguchi M, Odaka M, Suzuki S, Watanabe S, Takeuchi H, Schleimer RP, Adachi M. Double-stranded RNA

activates RANTRES gene transcription through cooperation of nuclear factor-κB and interferon regulatory factors in human airway epithelial cells. *Clin Exp Allergy*. 2004;34:745-752.

17. Taima K, Takanashi S, Okumura K, Imaizumi T, Kumagai M, Ishikawa A, Yoshida H, Satoh K, Fijuta T. Double-stranded RNA stimulates the expression of monocyte chemoattractant protein-1 in BEAS-2B bronchial epithelial cells. *Exp Lung Res*. 2005;31:361-375.

18. Yamashita K, Imaizumi T, Taima K, Fujita T, Ishikawa A, Yoshida H, Oyama C, Satoh K. Polyinosinic-polycytidylic acid induces the expression of GRO-α in BEAS-2B cells. *Inflammation*. 2005;29:17-21.

19. Sha Q, Trouong-Tran AQ, Plitt JR, Beck LA, Schleimer RP. Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Mol Biol*. 2004;31:358-364.

20. 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;280:5571-5580.

21. Mlkamu T, Kita H, O'Grady SM. TLR3 activation evokes IL-6 secretion, autocrine regulation of Stat3 signaling and TLR2 expression in human bronchial epithelial cells. *J Cell Commun Signal*. 2013;7:109-118.

22. Satkunanathan S, Kumar N, Bajorek M, Purbhoo MA, Culley FJ. Respiratory syncytidal viras infection, TLR3 ligands, and proinflammatory cytokines induce CD161 ligand LLT1 expression on respiratory epithelium. *J Virol*. 2014;88:2366-2373.

23. Jiang J, Wang Y, Tang X, Yao Y, Zhou J. Regulation of viral infection-induced airway remodeling cytokine production by the TLR3-EGFR signaling pathway in human bronchial epithelial cells. *COPD*. 2016;13:750-755.

24. Mills JT, Schwenzer A, Marsh EK, Edwards MR, Sabroe I, Midwood KS, Parker LC. Airway epithelial cells generate pro-inflammatory tenascin-C and small extracellular vesicles in response to TLR3 stimuli and Rhinovirus infection. *Front Immunol*. 2019;10:1987.

25. Lai Y, Yi G, Chen A, Bhardwj K, Tragesser BJ, Valverde RA, Zlotnick A, Mukhopadhyay S, Ranjith-Kumar CT, Kao CC. Viral double-stranded RNA-binding proteins can enhance innate immune signalling by Toll-like receptor 3. *PloS One*. 2011; 6:e25837.

26. Sassa N, Hirono K, Shiratori T, Kawaguchi S, Matsumiya T, Yoshida H, Seya K, Tanaka H, Imaizumi T. Induction of CCL2 via TLR3 signaling in hCMEC/D3 cells: possible regulation by NF-κB. *Clin Exp Neuroimmunol*. 2019;10:197-203.

27. Shimada T, Imaizumi T, Shirai K, Tatsuta T, Hayakari R, Yoshida H, Matsumiya T, Kimura T, Kijima K, Mizukami H, Hakamada K. CCL5 is induced by TLR3 signaling in HuCCT1 human bile duct carcinoma cells: possible involvement in the pathogenesis of biliary atresia. *Biomed Res. (Tokyo)* 2017;38: 269-276.

28. Terenzi F, Saikia P, Sen GC. Interferon-inducible protein, P56, inhibits HPV replication by binding to the viral protein E1. *EMBO J*. 2008;27:3311-3321.

29. Raychoudhuri A, Shrivastava S, Steele R, Kim H, Ray R, Ray RB. ISG56 and IFITM1 proteins inhibit hepatitis C virus replication. *J Viol*. 2011;85:12881-12889.

30. Andrejeva J, Norsted H, Habjan, Thiel V, Goodbourn S, Randall RE. ISG56/IFIT1 is primarily responsible for interferon-induced changes to patterns to patterns of parainfluenza virus type 5 transcription and protein synthesis. *J Gen Virol*. 2013;94:59-68.

31. Kumar P, Sweeney TR, Skabkin MA, Skabkina OV, Hellen CU, Pestova TV. Inhibition of translation by IFIT family members is determined by their ability to

interact selectively with 5'-terminal regions of cap0, cap1-and 5'ppp- mRNAs. *Nucleic Acids Res*. 2014;42:3228-3245.

32. Liu M, Guo S, Hibbert JM, Jain V, Singh N, Wilson NO, Stiles JK. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. *Cytokine Growth Factor Rev*. 2011;22:121-130.

33. Hayney MS, Henriquez KM, Barnet JH, Ewers T, Champion HM, Flannery S, Barett B. Serum IFN-γ-induced protein 10 (IP-10) as a biomarker for severity of acute respiratory infection in healthy adults. *J Clin Virol*. 2017;90:32-37.

34. Li Y, Li C, Xue P, Zhong B, Mao AI, Ran Y, Chen H, Wang YY, Yang F, Shu HB. ISG56 is a negative-feedback regulator of virus-triggered signalling and cellular antiviral response. *Proc Natl Acad Sci USA*. 2009;106:7945-7950.

Figure captions

Figure 1. Treatment of BEAS-2B cells with poly IC induced the expression of ISG56 in a concentration-dependent manner. (a) BEAS-2B cells were cultured and treated with various concentration of poly IC for 16 h. RNA was extracted from the cells, and cDNA as a PCR template was synthesized from the RNA. Then, quantitative real-time RT-PCR analyses for ISG 56 and GAPDH were performed. Data are presented as means ± SD (n=3). (b) The cells were cultured and treated with various concentration of poly IC for 24 h. The cells were lysed and the lysates were subjected to western blotting for ISG56 and actin.

Figure 2. Treatment of BEAS-2B cells with poly IC induced the expression of ISG56 in a time-dependent manner. (a, b) The cells were treated with 30 μg/mL poly IC for up to 24 h. The mRNA expression of IFN-β and ISG56 was examined by real-time RT-PCR (a), and the protein expression of ISG56 was evaluated using western blotting (b) as in Figure 1. The data in (a) are presented as means ± SD (n=3).

Figure 3. TLR3 and IFN-β are involved in poly IC-induced expression of ISG56. (a, b) The cells were transfected with siRNA against TLR3, IFN-β or non-silencing negative control siRNA and were incubated for 48 h. The cells were then treated with 30 μg/mL poly IC. (a) After an additional 16 h incubation, RNA was extracted from the cells. Real-time RT-PCR analysis was performed for ISG56, CXCL10 and GAPDH were performed (n=3; *$p < 0.05$ and **$p<0.01$, by t-test). (b) The cells were lysed after an additional 24 h incubation, and the expression of ISG56 protein was examined by

western blot analysis. (c, d) The cells were treated with 1 ng/mL r(h)IFN-β for 16 h and subjected to real-time RT-PCR (c) and western blot (d) analyses.

Figure 4. ISG56 is involved in the poly IC-induced CXCL10 expression. The cells were transfected with siRNA against ISG56 and incubated for 48 h. Then the cells were treated with 30 μg/mL poly IC for an additional 2 h (for the mRNA analysis of IFN-β), 16 h (for the mRNA analysis of CXCL10 and CXCL1) or 24 h (for the protein analysis of CXCL10 and ISG56). (a) RNA was extracted from the cells, and the expression level of mRNA for CXCL10, CXCL1 and IFN-β was examined by real-time RT-PCR analysis (n=3; *$p<0.01$, by t-test). (b) Conditioned medium was collected from the cells and the protein concentration of CXCL10 in the medium was examined by ELISA (n=3; *$p<0.01$, by t-test). (c) The cells were lysed and western blotting was performed for ISG56 and actin proteins.

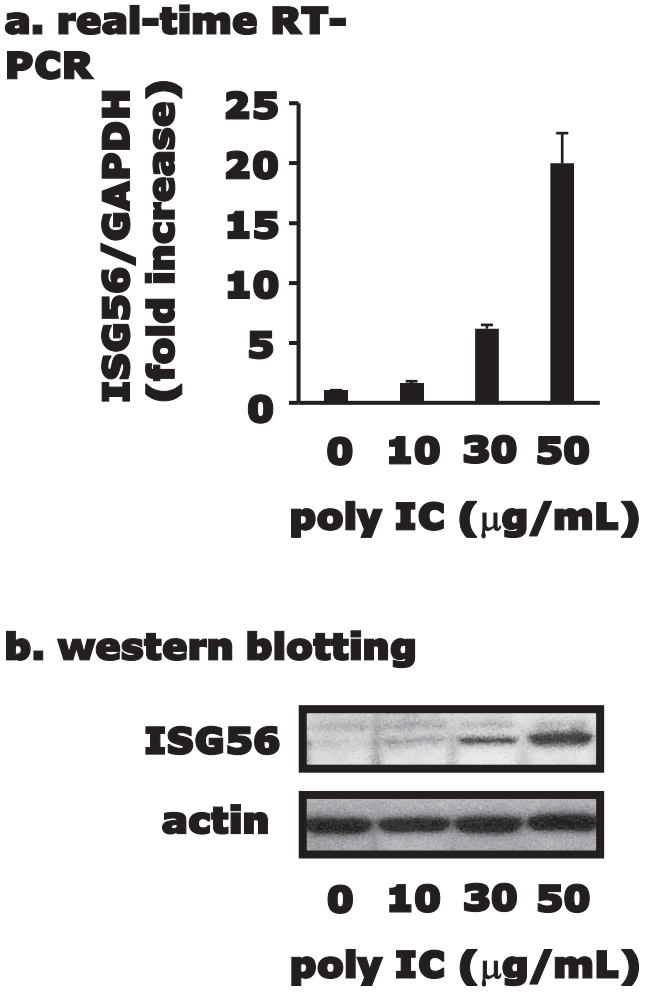
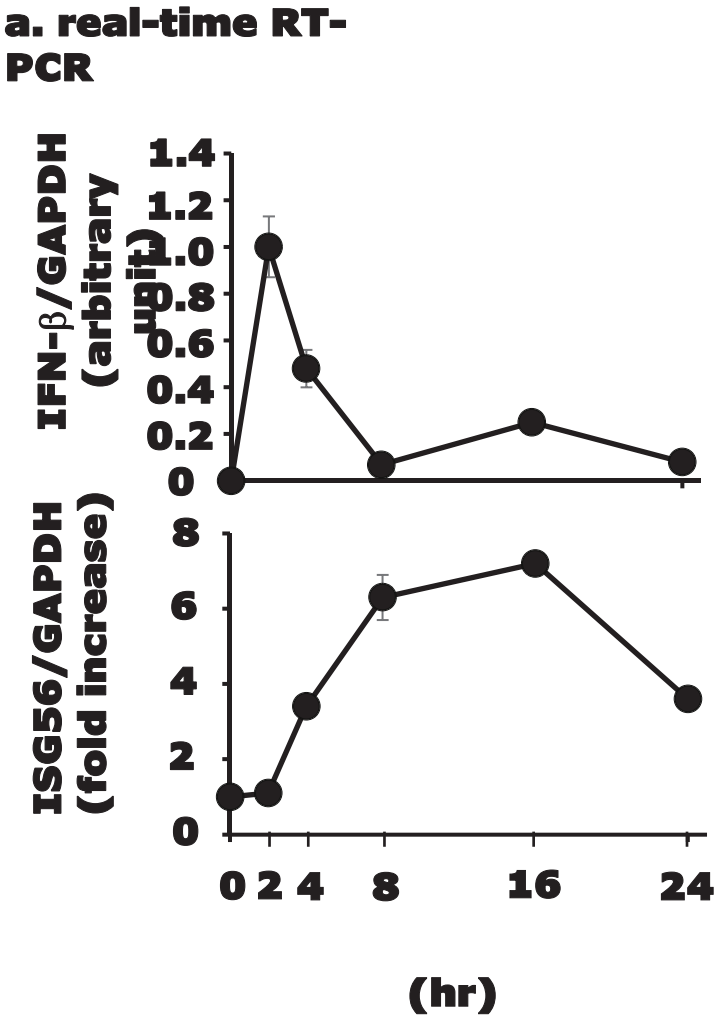


Fig. 1 Shiratori et al.



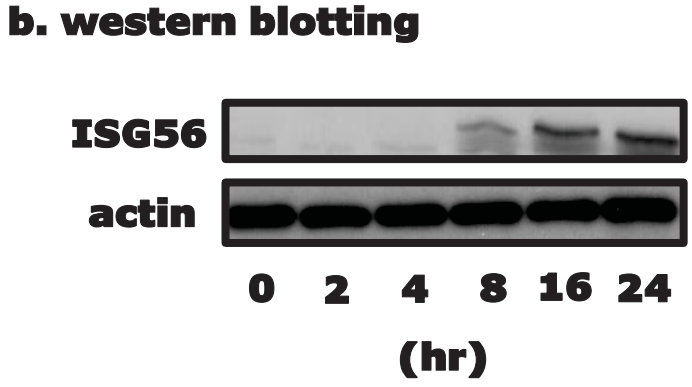


Fig.2. Shiratori et al.

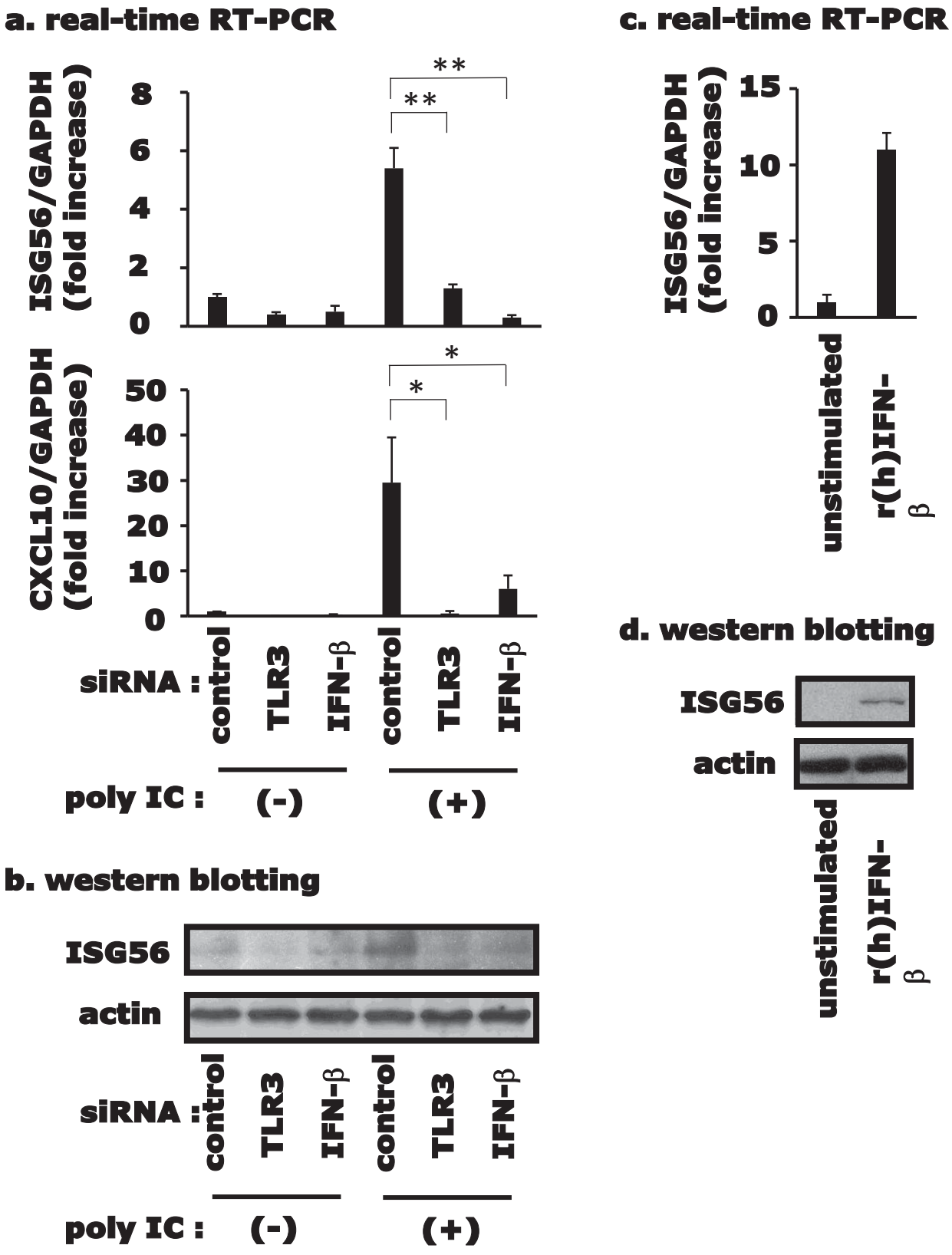


Figure 3. Shiratori et al.

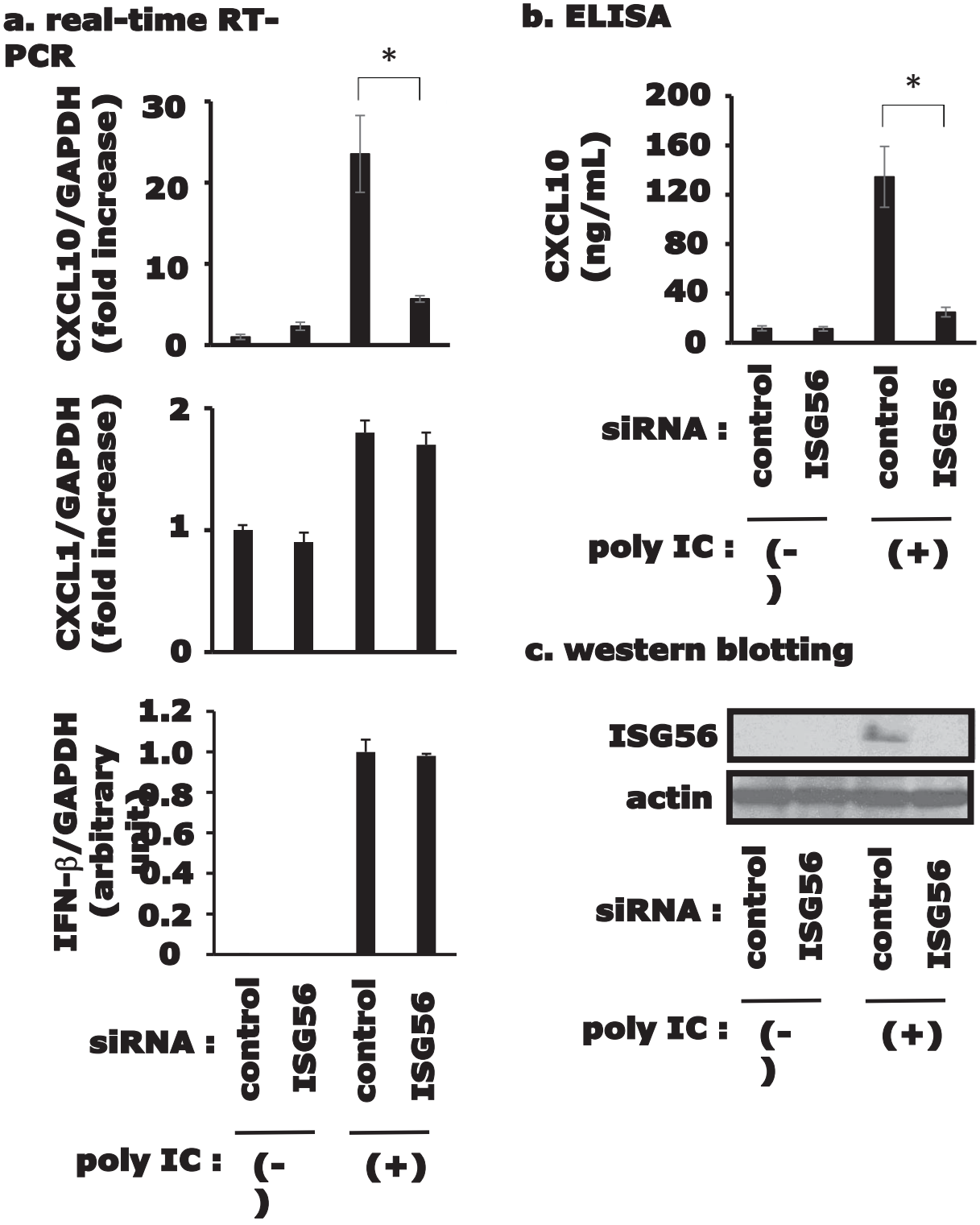


Fig.4. Shiratori et al.