Retinoic acid-inducible gene-I-like receptor (RLR)-mediated antiviral innate immune responses in the lower respiratory tract: Roles of TRAF3 and TRAF5

（下気道におけるレチノイン酸誘導遺伝子-I 様受容体を介した抗ウイルス自然免疫応答：TRAF3 と TRAF5 の役割）

申 請 者　　弘前大学大学院医学研究科
成育科学領域小児病態学教育研究分野

氏　　名　　千　葉　友　揮

指導教授　　伊　藤　悦　朗

Abstract

Upon viral infection, the cytoplasmic viral sensor retinoic acid-inducible gene-I (RIG-I) recognizes viral RNA to activate antiviral signaling to induce type I interferon (IFN). RIG-I-like receptors (RLRs) activate antiviral signaling in a tissue-specific manner. The molecular mechanism underlying antiviral signaling in the respiratory system remains unclear. We studied antiviral signaling in the lower respiratory tract (LRT), which is the site of many harmful viral infections. Epithelial cells of the LRT can be roughly divided into two groups: bronchial epithelial cells (BECs) and pulmonary alveolar epithelial cells (AECs). These two cell types exhibit different phenotypes; therefore, we hypothesized that these cells may play different roles in antiviral innate immunity. We found that BECs exhibited higher antiviral activity than AECs. TNF receptor-associated factor 3 (TRAF3) has been shown to be a crucial molecule in RLR signaling. The expression levels of TRAF3 and TRAF5, which have conserved domains that are nearly identical, in the LRT were examined. We found that the bronchus exhibited the highest expression levels of TRAF3 and TRAF5 in the LRT. These findings suggest the importance of the bronchus in antiviral innate immunity in the LRT and indicate that TRAF3 and TRAF5 may contribute to RLR signaling.

Key words: RLRs, lower respiratory tract, antiviral signaling, TRAF3, TRAF5

Highlights:

- Epithelial cells in the LTR can express type I IFN in response to double-stranded RNA
- TRAF3 and TRAF5 are involved in RLR signaling in the LRT
- BECs express the highest levels of TRAF3 and TRAF5 in the LTR

Introduction

The innate immune system plays an essential role as the first line of host defense against viral infection. Pathogen-associated molecular patterns (PAMPs) are recognized by host sensors known as pattern recognition receptors (PRRs), which include retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs). The cytoplasmic viral sensors RIG-I and melanoma differentiation–associated gene 5 (MDA5), members of RLR family, recognize viral RNA in a manner that is dependent on the length of replication intermediates of genomic RNA [1]. RIG-I and MDA5 have N-terminal caspase recruitment domains (CARDs), which are essential for interactions with an adaptor molecule and activation of downstream cascades [2]. RIG-I contains two CARDs, the DExH box-helicase domain, which resides in the middle of the protein, and an RNA recognition site, the C-terminal domain (CTD) [3]. In the absence of a ligand, the CARDs are enveloped in other domains. Once a ligand is recognized by the CTD, RIG-I undergoes a conformational change and the CARDs are exposed [4], leading to an association with mitochondrial antiviral signaling (MAVS) through homotypic CARD-CARD interactions [5]. MAVS has an N-terminal CARD domain, a proline-rich region, and a C-terminal transmembrane (TM) domain [6]. Upon viral recognition by RLRs, dimerization of MAVS recruits TNF receptor-associated factors (TRAFs), leading to the activation of transcription factors such as nuclear factor-κB (NF-κB) and interferon regulatory factor (IRF)-3/7. Subsequently, homodimerized IRFs and activated NF-κB translocate from the cytoplasm to the nucleus, resulting in enhanced transcription of pro-inflammatory cytokines and type I interferons (IFNs) [7-11].

TRAFs are known to act as signal transducers of diverse receptor families, including the TNF receptor (TNF-R) superfamily. Upon activation, TRAF exhibits E3 ubiquitin ligase activity to

regulate signaling [12]. The recruitment of TRAFs also occurs in RLR signaling [6]. MAVS can interact with TRAFs via its proline-rich region. The TRAF family includes six members, TRAF1 to 6, which appear to serve non-redundant physiological roles. In RLR signaling, TRAF2, TRAF3, TRAF5, and TRAF6 have been shown to be associated with MAVS. TRAF3 is essential for IRF3 activation [9]. TRAF2 and TRAF5 have been shown to be required for the activation of IRF3 and NF-κB [8,10]. TRAF6 has been reported to be important for IRF7 and NF-κB activation [11]. However, no study has yet characterized the tissue-specific importance of the expression and function of the TRAFs in antiviral signaling.

Viral infections of the respiratory system occur in human populations across the globe. Newborns and infants, particularly those born prematurely, are susceptible to severe viral infections of the lower respiratory tract (LRT) because of the small size of the airways in which airway edema and sloughing of epithelial cells cause atelectasis, subsequent mismatch of ventilation, perfusion and reduced oxygenation [13]. Once a host is exposed to a virus on the surface of the respiratory tract, the antiviral innate immune response is activated, which determines the serum antibody response that prevents progression to LRT infection via virus neutralization [13-15]. Epithelial cells are among the first targets of respiratory viruses and serve both as a physical barrier and as the first line of innate host defense against viruses, such as respiratory syncytial virus (RSV), influenza virus, and human metapneumovirus [16]. Epithelial cells of the LRT can be roughly divided into two groups: bronchial epithelial cells (BECs) and pulmonary alveolar epithelial cells (AECs). Both cell types express PRRs (especially RLRs) and can express type I IFNs and pro-inflammatory cytokines upon viral infection [16]. Because these types of cells exhibit different phenotypes, we focused on the tissue-specific antiviral innate immune responses in the LRT. We also characterized the roles of TRAF3 and TRAF5 in antiviral signaling in the LRT.

Materials and Methods

Cell culture

Normal human bronchial epithelial cells (HBEpCs) and normal human pulmonary alveolar epithelial cells (HPAEpiCs) were obtained from PromoCell (Heidelberg, Germany) and ScienCell Research Laboratories (San Diego, CA, USA), respectively. HBEpCs and HPAEpiCs were grown in a 5% CO_2 atmosphere at 37°C in airway epithelial cell growth medium (PromoCell) or AEC medium (ScienCell Research Laboratories), respectively.

Human tissue extracts

Human tissue extracts (esophagus, larynx, bronchus, lung and peripheral blood leukocytes) for immunoblot analyses were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Transfection

Transient transfections of HBEpCs and HPAEpiCs were performed by plating cells at a density of 0.5×10^5 cells per well in 12-well culture plates approximately 24 h prior to transfection. RNA interference (RNAi) was induced via transfection with gene-specific siRNAs or control siRNA using Lipofectamine RNAiMAX (Life Technologies Japan, Tokyo, Japan) for 48 h, following the manufacturer's instructions. Silencer® siRNAs against TRAF3 (s14384) and TRAF5 (s14385) as well as non-silencing control siRNA were purchased from Life Technologies. To introduce a synthetic double-stranded RNA polyinosinic-polycytidylic acid (poly(I:C))-activated antiviral innate immune response, cells were transfected using TransFectin Lipid Reagent (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Cells were incubated for the indicated times, which depended on the experiment, and then were further analyzed.

Total RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using an Illustra RNAspin Mini (GE Healthcare, Buckinghamshire, England). Equal amounts of total RNA were used as a template for single-stranded cDNA synthesis in a reaction using PrimeScript RT Master Mix (Takara Bio, Otsu, Japan) under conditions indicated by the manufacturer. A CFX96 Real-Time PCR Detection System (Bio-Rad) was used for quantitative assessments of IFN-β and GAPDH mRNA transcripts. Primer sequences were as follows:

IFN-β-F (5'-ACTGCCTCAAGGACAGGATG-3'),

IFN-β-R (5'-AGCCAGGAGGTTCTCAACAA-3'),

GAPDH-F (5'-CCACCCATGGCAAATTCCATGGCA-3') and

GAPDH-R (5'-TCTAGACGGCAGGTCAGGTCCACC-3').

Thermocycling was performed using a SsoAdvanced SYBR Green Supermix (Bio-Rad) according to the manufacturer's specifications. The amplification conditions were as follows: 30 s at 95°C, followed by 95°C for 10 s and 58°C for 30 s for 39 cycles. After amplification was completed, melting curve analysis was performed by slowly heating the samples from 65°C to 95°C at 0.1°C increment per second, with continuous monitoring of fluorescence. Melting curves and quantitative analyses of data were performed using a CFX manager (Bio-Rad).

Immunoblot analyses

After a series of treatments, the cells were washed twice with 1× phosphate buffered saline (PBS) and lysed in hypotonic lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40] containing proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA).

Lysates were cleared by centrifugation at 12,000×*g* for 10 min at 4°C. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), electro-blotting, and immunodetection were performed as described previously [17]. The primary antibodies used in this study were as follows: anti-TRAF3 and anti-TRAF5 antibodies (Santa Cruz Biotechnology), anti-RIG-I and anti-MAVS antibodies (Enzo Life Sciences, Farmingdale, NY, USA), and an anti-β-actin antibody (Sigma-Aldrich).

Immunohistochemistry

The examined specimens were obtained from archives of pathology files maintained at the Department of Pathology and Molecular Medicine, Hirosaki University. From 2014 to 2015, lung tissues, including both AECs and BECs, were procured from six patients. Two specimens were from patients undergoing lobectomy for the treatment of lung cancer. Four were obtained from autopsy cases. A clinical summary of the specimens is briefly described in Supplementary table 1. All specimens were fixed with 10% formalin and embedded in paraffin. The study conformed to the provisions of the Declaration of Helsinki and the Ethical Declaration of the Japanese Society of Pathology. Expression of TRAF3 and TRAF5 in AECs and BECs was evaluated by immunohistochemistry. Briefly, 3.5 μm-thick paraffin embedded sections of each specimen were deparaffinized, immersed in citrate buffer (pH 6.0) at 123°C for 3 minutes for antigen retrieval, and treated with Background Sniper (Biocare Medical, Concord, CA, USA) for 10 minutes. Then, the sections were incubated with primary antibodies against TRAF3 (1:150) or TRAF5 (1:150, Genetex, Hsinchu, Taiwan) at 4°C overnight, which was followed by the quenching of endogenous peroxidase activity in 3% H_2O_2 in distilled water for 10 minutes. Finally, the sections were incubated with Dako EnVision + system-HRP Labeled polymer anti-rabbit, (Dako, Glostrup, Denmark), and the reaction products were visualized

using a Metal Enhanced DAB Substrate Kit (Thermo Scientific, MA, USA), followed by light counterstaining with hematoxylin. Immunohistostaining was evaluated by T.S. and H.M in a blinded manner.

Results

dsRNA stimulates RLR signaling in both BECs and pulmonary AECs in a concentration-dependent manner.

Type I IFN is a cytokine that is produced after the activation of RLR-mediated antiviral signaling [18]. When HBEpCs were transfected with a synthetic dsRNA, poly(I:C), the expression of a type I IFN, IFN-β, was upregulated in a concentration-dependent manner (Figure 1A). HPAEpiCs transfected with poly(I:C) exhibited a similar expression pattern to that of HBEpCs (Figure 1B). In both HBEpCs and HPAEpiCs, 50 ng/well poly(I:C) transfection resulted in significantly elevated levels of IFN-β mRNA transcripts. However, expression levels of IFN-β mRNA transcripts were much higher in HBEpCs than in HPAEpiCs when these cells were transfected with 250 ng/well poly(I:C). HBEpCs express approximately 40-fold more IFN-β mRNA transcripts than HPAEpiCs. These findings allowed us to hypothesize that BECs and AECs showed different sensitivities to foreign RNA, especially in response to RLR signaling. We therefore assessed the constitutive expression levels of the cytosolic viral sensor RIG-I and its adaptor molecule MAVS in cultured normal AECs and BECs, as shown in Figure 1C. The levels of constitutive expression of RIG-I and MAVS in those two cell types were similar, suggesting that a downstream component of RLR signaling might be associated with this difference.

TRAF3 and TRAF5 play roles in RLR signaling in epithelial cells of the LRT

Because TRAF3 is a critical molecule downstream of MAVS [19], we next examined the role of TRAF3 in HBEpCs and HPAEpiCs in inducing the expression of IFN-β in response to poly(I:C). We also investigated the effects of TRAF5, which is structurally homologous to TRAF3 [9], on IFN-β expression. As shown in Figure 2, silencing of TRAF3 significantly inhibited the expression of IFN-β, which was induced by poly(I:C) transfection in both HBEpCs and HPAEpiCs. Additionally, knockdown of TRAF5 also suppressed poly(I:C)-induced IFN-β expression in HBEpCs (Figure 2A) and HPAEpiCs (Figure 2B). No differential changes in IFN-β expression after silencing TRAF3 and TRAF5 between HBEpCs and HPAEpiCs were observed. These findings suggested the important roles of TRAF3 and TRAF5 in RLR signaling in epithelial cells of the LRT.

Robust expression of TRAF3 and TRAF5 in the bronchial epithelium

To investigate the expression levels of TRAF3 and TRAF5 in epithelial cells of the LRT *in vivo*, we performed immunoblot analyses using lysates from normal human esophagus, larynx, bronchus, and lung tissues, as well as white blood cells (WBCs). In the esophagus and larynx, both of which were used as controls of vicinal or related tissues for the LRT, low levels of TRAF3 and TRAF5 were detected (Figure 3). The bronchus tissues expressed high amounts of both of TRAF3 and TRAF5. In lung tissues, those levels of expression were reduced compared to the bronchus (Figure 3). Notably, although we applied the same amount of protein (10 μg/lane) for the protein expression level analysis, the levels of β-actin and GAPDH (data not shown) were different amongst the tissues due to tissue-specific expression. Therefore, we did not refer to these proteins as internal controls.

Expression levels of TRAF3 and TRAF5 in lung have been previously reported [20,21], and

lung tissues are thought to express low and moderate amounts of TRAF3 and TRAF5, respectively. Lung extracts contain various cell types, including macrophages, dendritic cells (DCs), lymphocytes, fibroblasts, smooth muscle cells, and epithelial cells. We also confirmed the low expression level of TRAF3 and weak expression level of TRAF5 in WBCs. These results indicated that bronchus tissues express high levels of TRAF3 and TRAF5 in the LRT.

Expression patterns of TRAF3 and TRAF5

In our evaluations of immunohistochemistry for TRAF3 and TRAF5, we found that both molecules were expressed in a similar pattern in lung tissues. The intensity of positive staining for TRAF3 was generally much stronger than that for TRAF5 (Figure 4). The frequency of positively stained cells in lung tissues was comparable for TRAF3 and TRAF5 staining. In the AECs of all specimens, alveolar type I (ATI) and ATII cells exhibited cytoplasmic positive reactivity for TRAF3 and TRAF5 in a patchy pattern (Figure 4A and 4B). By contrast, positive staining for TRAF3 and TRAF5 could be diffusely observed in the cytoplasm of many BECs (Figure 4C and 4D). The remaining types of cells in the lung tissues did not express TRAF3 or TARF5 in any of the specimens that we examined.

Discussion

In this present study, we examined the antiviral innate immune responses in the LRT. Most studies of respiratory viruses have been performed using HBEpCs, which are “professional” immune cells (e.g., alveolar macrophages, T cells, or dendritic cells), or cell lines derived from lung tumors [22]. We initially carried out an *in vitro* study with A549 cells, a lung epithelial cells line, as a model of AECs. Compared with HPAEpiCs, A549 cells exhibited different

phenotypes in response to dsRNA. For example, in A549 cells, the silencing of TRAF3 or TRAF5 enhanced IFN-β expression and IRF3 activation in response to poly(I:C) stimulation (data not shown), whereas knockdown of TRAF3 or TRAF5 in HPAEpiCs significantly suppressed this response (Figure 2). These findings allowed us to utilize HPAEpiCs to examine the role of RLR signaling in AECs in this present study. Human AECs can be divided into two populations: ATI and ATII epithelial cells [23]. ATI cells reside in the alveoli of the lung where they are separated from inspired air from the blood in the capillaries [24]. ATII cells cover approximately 5% of the alveolar surface but constitute 15% of total lung cells [25]. Recent studies have shown that ATII cells are involved in inflammatory responses, including antiviral innate immune responses [23]. HPAEpiCs have been reported to contain > 96% ATII cells [25] and are known to respond to foreign RNA stimuli (Figure 1). In this present study, we introduced poly(I:C) into cells to test for a role of RLR signaling. We previously showed that the application of dsRNA to cells is sensed by TLRs, whereas transfected dsRNA can activate RLR signaling [26]. Our data showed that both HBEpCs and HPAEpiCs have an intact RLR signaling pathway. To date, few reports have compared the activity of RLR signaling between AECs and BECs. Moreover, no report has yet shown a difference in tissue-specific viral susceptibility that was dependent upon RLR signaling in these cell types. Our present data clearly show that BECs are more sensitive to RLR-mediated antiviral innate immune responses than ATII cells.

The TRAF family of proteins is known to be involved in RLR signaling [3]. Among the TRAFs, TRAF3 has been shown to be the most important molecule in antiviral signaling pathways [19]. Krajewski et al. previously reported the expression pattern of TRAF3 in the LRT by immunohistochemistry and showed that TRAF3 is weakly expressed in BECs and absent in ATI and ATII cells [21], which is consistent with our present findings (Figure 4).

TRAF5 was initially identified as a transducer of CD40 and lymphotoxin-β receptor signaling, facilitating activation of the canonical NF-κB pathway [20,27]. Furthermore, TRAF5 has been suggested to also be able to activate the non-canonical NF-κB pathway. While TRAF5 has been thought to associate with various proteins, including MAVS, such characteristics have been identified using transient overexpression of TRAF5 in transformed non-immune cell lines. Our findings indicated that the TRAF3 and TRAF5 expression patterns and functions were similar in epithelial cells of the LRT. Apparently, for RLR signaling, the role of TRAF5 is more important in HBEpCs than in HPAEpiCs (Figure 2). The details of how TRAF5 contributes to RLR signaling will need to be clarified in a future study. Interestingly, TRAF5 has been shown to negatively regulate the induction of Th2 immune responses, which contribute to adaptive immunity [28]. Accordingly, lung inflammation, which is known to be critically controlled by OX40, includes conditions such as asthma that are more pronounced in $TRAF5^{-/-}$ mice and associated with higher levels of Th2 cytokines [28]. Our findings suggest that TRAF5, as well as TRAF3, is essential in not only adaptive immunity but also in RLR-mediated antiviral immune responses in the LRT.

BECs recognize dsRNA via TLR3 and RLRs [29,30]. Accordingly, antiviral defense in the bronchial epithelium requires the coordinated recognition of rhinovirus infection, which initially occurs via TLR3 and later involves RIG-I and MDA5 [29]. Additionally, bronchial smooth muscle cells also express RLRs and TLR3 [31]. Therefore, these types of cells are predicted to coordinately respond to viral infection in bronchi. Notably, the lung innate immune system involves a variety of effector cells—alveolar macrophages, neutrophils, dendritic cells, γδ T cells, innate lymphoid cells, and alveolar epithelial cells [32]. We showed that HPAEpiCs express lower levels of IFN-β in response to dsRNA compared with HBEpCs. This finding may indicate a lesser contribution of AECs in antiviral innate immune responses in lung because

of the abundance of professional immune cells in this tissue site.

In summary, we identified the different activities of RLR signaling between BECs and AECs. These activities appear to be associated with the expression levels of TRAF3 and TRAF5 in these cell types. Based on these results, we consider that the bronchus might be crucial in the antiviral innate immune system of the LRT to rapidly recruit professional immune cells.

Competing interests

The authors declare no competing interests.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Number 26460567 (T.M.); Takeda Science Foundation (T.M.), a Hirosaki University Institutional Research Grant (T.I.); and a Hirosaki University Institutional Research Grant for Young Scientists (T.M., T.K).

References

[1] H. Kato, O. Takeuchi, E. Mikamo-Satoh, et al., Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5, J. Exp. Med. 205 (2008) 1601-1610.

[2] M. Yoneyama, T. Fujita, Structural mechanism of RNA recognition by the RIG-I-like receptors, Immunity 29 (2008) 178-181.

[3] T. Matsumiya, T. Imaizumi, H. Yoshida, et al., Antiviral signaling through retinoic acid-inducible gene-I-like receptors, Arch. Immunol. Ther. Exp. (Warsz) 59 (2011) 41-48.

[4] S. Cui, K. Eisenacher, A. Kirchhofer, et al., The C-terminal regulatory domain is the RNA 5'-triphosphate sensor of RIG-I, Mol. Cell 29 (2008) 169-179.

[5] L.G. Xu, Y.Y. Wang, K.J. Han, et al., VISA is an adapter protein required for virus-triggered IFN-beta signaling, Mol. Cell 19 (2005) 727-740.

[6] R.B. Seth, L. Sun, C.K. Ea, et al., Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3, Cell 122 (2005) 669-682.

[7] H. Kumar, T. Kawai, S. Akira, Pathogen recognition by the innate immune system, Int. Rev. Immunol. 30 (2011) 16-34.

[8] S.S. Mikkelsen, S.B. Jensen, S. Chiliveru, et al., RIG-I-mediated activation of p38 MAPK is essential for viral induction of interferon and activation of dendritic cells: dependence on TRAF2 and TAK1, J. Biol. Chem. 284 (2009) 10774-10782.

[9] S.K. Saha, E.M. Pietras, J.Q. He, et al., Regulation of antiviral responses by a direct and specific interaction between TRAF3 and Cardif, EMBO J 25 (2006) 3257-3263.

[10] E.D. Tang, C.Y. Wang, TRAF5 is a downstream target of MAVS in antiviral innate immune signaling, PLoS One 5 (2010) e9172.

[11] R. Yoshida, G. Takaesu, H. Yoshida, et al., TRAF6 and MEKK1 play a pivotal role in the RIG-I-like helicase antiviral pathway, J. Biol. Chem. 283 (2008) 36211-36220.

[12] P. Xie, TRAF molecules in cell signaling and in human diseases, J. Mol. Signal. 8 (2013) 7.

[13] M. Lanari, S. Vandini, M.G. Capretti, et al., Respiratory syncytial virus infections in infants affected by primary immunodeficiency, J. Immunol. Res. 2014 (2014) 850831.

[14] J.B. Domachowske, H.F. Rosenberg, Respiratory syncytial virus infection: immune response, immunopathogenesis, and treatment, Clin. Microbiol. Rev. 12 (1999) 298-309.

[15] A. Mejias, S. Chavez-Bueno, A.M. Rios, et al., Comparative effects of two neutralizing anti-respiratory syncytial virus (RSV) monoclonal antibodies in the RSV murine model: time versus potency, Antimicrob. Agents Chemother. 49 (2005) 4700-4707.

[16] M. Vareille, E. Kieninger, M.R. Edwards, et al., The airway epithelium: soldier in the fight against respiratory viruses, Clin. Microbiol. Rev. 24 (2011) 210-229.

[17] J. Dempoya, T. Matsumiya, T. Imaizumi, et al., Double-stranded RNA induces biphasic STAT1 phosphorylation by both type I interferon (IFN)-dependent and type I IFN-independent pathways, .J Virol. 86 (2012) 12760-12769.

[18] T. Matsumiya, D.M. Stafforini, Function and regulation of retinoic acid-inducible gene-I, Crit. Rev. Immunol. 30 (2010) 489-513.

[19] S.K. Saha, G. Cheng, TRAF3: a new regulator of type I interferons, Cell Cycle 5 (2006) 804-807.

[20] T.K. Ishida, T. Tojo, T. Aoki, et al., TRAF5, a novel tumor necrosis factor

receptor-associated factor family protein, mediates CD40 signaling, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 9437-9442.

[21] S. Krajewski, J.M. Zapata, M. Krajewska, et al., Immunohistochemical analysis of in vivo patterns of TRAF-3 expression, a member of the TNF receptor-associated factor family, J. Immunol. 159 (1997) 5841-5852.

[22] J. Wang, M.P. Nikrad, T. Phang, et al., Innate immune response to influenza A virus in differentiated human alveolar type II cells, Am. J. Respir. Cell Mol. Biol. 45 (2011) 582-591.

[23] C.J. Sanders, P.C. Doherty, P.G. Thomas, Respiratory epithelial cells in innate immunity to influenza virus infection, Cell Tissue Res. 343 (2011) 13-21.

[24] N.K. Weller, M.J. Karnovsky, Isolation of pulmonary alveolar type I cells from adult rats, Am. J. Pathol. 124 (1986) 448-456.

[25] M. Gentry, J. Taormina, R.B. Pyles, et al., Role of primary human alveolar epithelial cells in host defense against Francisella tularensis infection, Infect. Immun. 75 (2007) 3969-3978.

[26] T. Imaizumi, T. Aizawa-Yashiro, K. Tsuruga, et al., 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 (2012) 17-26.

[27] H. Nakano, H. Oshima, W. Chung, et al., TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor, J. Biol. Chem. 271 (1996) 14661-14664.

[28] T. So, S. Salek-Ardakani, H. Nakano, et al., TNF receptor-associated factor 5 limits the induction of Th2 immune responses, J. Immunol. 172 (2004) 4292-4297.

[29] L. Slater, N.W. Bartlett, J.J. Haas, et al., Co-ordinated role of TLR3, RIG-I and MDA5 in the innate response to rhinovirus in bronchial epithelium, PLoS Pathog. 6 (2010) e1001178.

[30] Q. Wang, D.R. Nagarkar, E.R. Bowman, et al., Role of double-stranded RNA pattern recognition receptors in rhinovirus-induced airway epithelial cell responses, J. Immunol. 183 (2009) 6989-6997.

[31] J. Calven, Y. Yudina, L. Uller, Rhinovirus and dsRNA induce RIG-I-like receptors and expression of interferon beta and lambda1 in human bronchial smooth muscle cells, PLoS One 8 (2013) e62718.

[32] J.L. Werner, C. Steele, Innate receptors and cellular defense against pulmonary infections, J. Immunol. 193 (2014) 3842-3850.

Figure legends

Figure 1 Concentration-dependent expression of IFN-β in BECs and AECs in response to poly(I:C). HBEpCs (A) or HPAEpiCs (B) were transfected with poly(I:C) at 0–250 ng/well for 8 h, and then cells were subjected to total RNA extraction. To measure IFN-β and GAPDH mRNA transcript abundance, qRT-PCR was performed. Data represent the average of three measurements. Means ± SD of three experiments are shown; *$p < 0.05$, **$p < 0.01$ vs "0". (C) HBEpCs or HPAEpiCs were harvested, and cell extracts were subjected to SDS-PAGE and western blotting using either anti-RIG-I, anti-MAVS, or anti-actin antibody.

Figure 2 The role of TRAF3 and TRAF5 in RLR-mediated IFN-β expression in HBEpCs and HPAEpiCs. HBEpCs (A) or HPAEpiCs (B) were transfected with TRAF3- or TRAF5-specific siRNA, or control siRNA for 48 h. Subsequently, cells were transfected with poly(I:C) at 50 ng/well for 8 h. Levels of IFN-β and GAPDH mRNA transcripts were measured using qRT-PCR. Data represent the average of three measurements. Means ± SD of three experiments are shown; *$p < 0.01$.

Figure 3 Expression levels of TRAF3 and TRAF5 protein in respiratory tract tissues. Human tissue extracts (10 μg/ lane) were subjected to SDS-PAGE and analyzed by western blotting.

Figure 4 TRAF3 and TRAF5 are expressed in lung tissues. (A) and (B) Both TRAF3 and TRAF5 are expressed in some AECs and BECs (cases 3 and 5). The expression intensity of TRAF3 was much stronger than that of TRAF5. (C) and (D) In bronchial epithelia, TRAF3 and TRAF5 were diffusely expressed in BECs (cases 2 and 5). The expression intensity of TRAF3 was also much stronger than that of TRAF5 in both bronchial and alveolar epithelia.

Figure 1

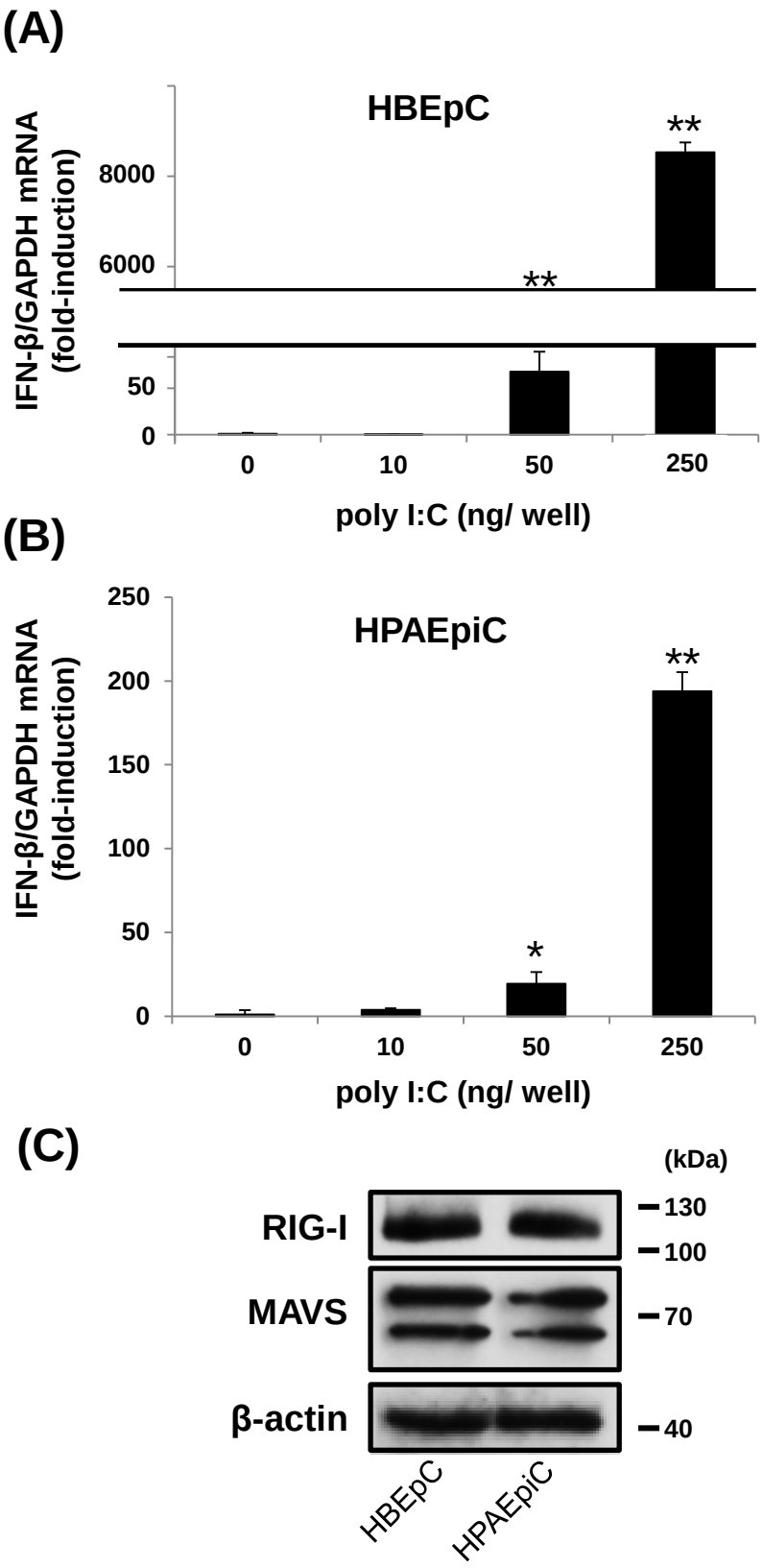
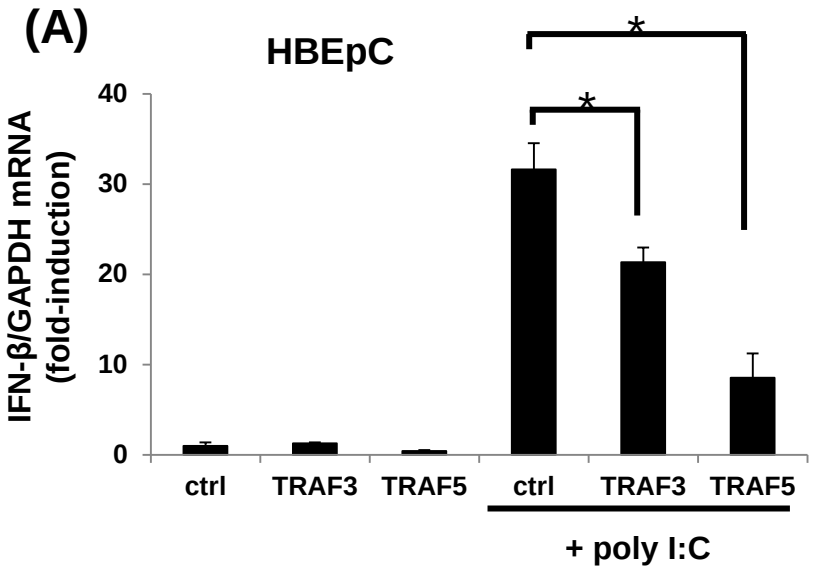

(A)
HBEpC
IFN-β/GAPDH mRNA (fold-induction)
8000
6000
50
0
**
**
0
10
50
250
poly I:C (ng/ well)
(B)
HPAEpiC
IFN-β/GAPDH mRNA (fold-induction)
250
200
150
100
50
0
*
**
0
10
50
250
poly I:C (ng/ well)
(C)
(kDa)
RIG-I
MAVS
β-actin
130
100
70
40
HBEpC
HPAEpiC

Figure 2


(A)
HBEpC
IFN-β/GAPDH mRNA (fold-induction)
40
30
20
10
0
*
*
ctrl
TRAF3
TRAF5
ctrl
TRAF3
TRAF5
+ poly I:C

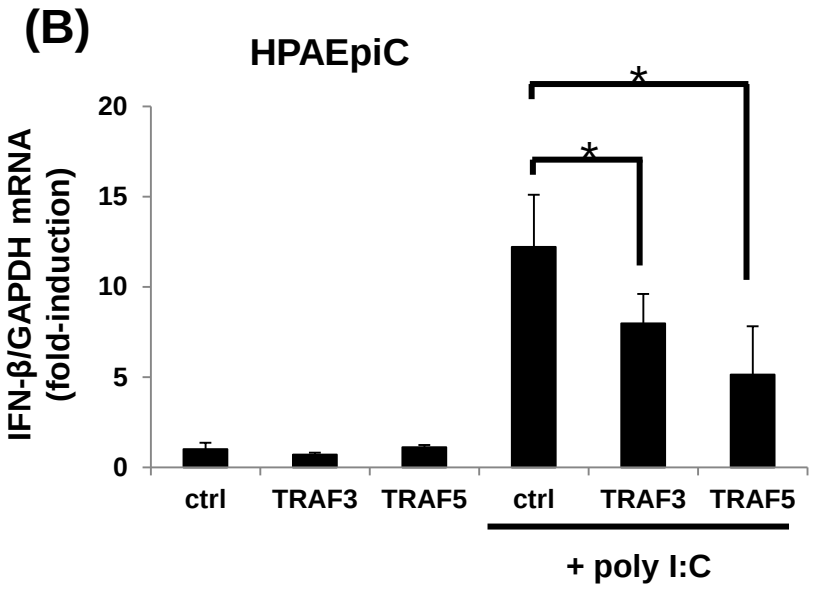

(B)
HPAEpiC
IFN-β/GAPDH mRNA (fold-induction)
20
15
10
5
0
*
*
ctrl
TRAF3
TRAF5
ctrl
TRAF3
TRAF5
+ poly I:C

Figure 3

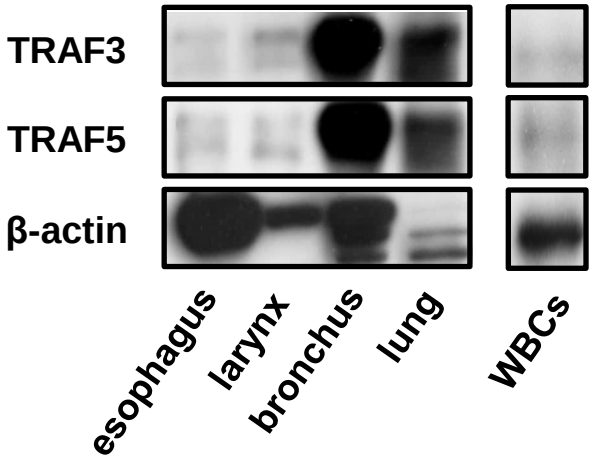


Figure 4