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ORIGINAL

Modest antiviral activity of Toll-like receptor 3 (TLR3) against respiratory syncytial virus infection in TLR3-overexpressed human lung epithelial cells

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ABSTRACT

Human respiratory syncytial virus (RSV) is the leading cause of acute lower respiratory infections in infants and Toll-like receptors (TLRs) are the first line of host defense against such infections. In this study, we aimed to elucidate the antiviral effect of TLR3 against RSV infection. The human TLR3 gene was either transiently or stably overexpressed in A549 cells and they were infected with the Long strain of RSV. In both cases, RSV production determined by plaque assay was modestly but significantly decreased in the TLR3-overexpressed cells compared with control cells. Less interferon (IFN)-β, measured by ELISA, was produced in the supernatant of the TLR3-overexpressed cells. Neutralization of IFN-β in the supernatant of the TLR3-overexpressed cells failed to increase RSV production to the same level as controls. These results indicate that TLR3 has modest anti-RSV activity and IFN-β seems not to be a significant mediator of this activity.

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Key words: innate immunity, respiratory syncytial virus, toll-like receptor 3

INTRODUCTION

Human respiratory syncytial virus (RSV) is a single-stranded, negative-sense RNA virus belonging to the family *Paramyxoviridae* and the genus *Pneumovirus*. RSV is a major cause of acute respiratory infections in all ages of humans, but most importantly RSV causes severe lower respiratory infections in infants and young children, or immune compromised hosts and elderly persons [1-4]. RSV infection becomes epidemic in every winter season in temperate climates or in the rainy seasons in tropical areas [5, 6]. It is very contagious and almost all children are thought to be infected at least once by 2 years of age. RSV bronchiolitis in infants may be a risk factor for developing future recurrent wheezing or asthma [7, 8, 9]. RSV infection may shift the profile of cytokine production by helper T cells from T-helper type1 (Th1) to T-helper type2 (Th2), which makes the infected infants more prone to allergic states [10-12]. Thus, developing effective vaccines and therapies for RSV infection are extremely important not only for current RSV infections but to reduce future burden of asthma.

The host defense system against RSV infections must be fully understood if effective treatment strategies are to be developed. The human immune system against microbial infections consists of two major parts, i.e. innate immunity and adaptive immunity. The former is our first line of defense against microbial infections. Recent discoveries have established that the innate immunity system recognizes so called pathogen-associated molecular patterns (PAMPs) of invading pathogens and immediately reacts with them to isolate and exclude them. One of the important pattern recognition receptors is the Toll-like receptors (TLRs) which sense most microbes on the cell surface or in the intracellular compartment [13, 14]. At least 10 TLR family members (TLR1 to TLR10) have been identified in humans. Certain TLRs including TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 sense various PAMPs of viruses and activate nuclear factor-κB (NF-κB) and interferon (IFN) regulatory factors, inducing proinflammatory cytokines and type I IFNs [15-17].

RSV has been reported to interact with some TLRs and thereby activates innate immunity. TLR4 which recognizes the lipopolysaccharide of bacteria also senses

the fusion protein of RSV together with CD14 and produces interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α, and IL-1β [18]. TLR4-deficent mice delay RSV clearance in the lungs, suggesting anti-RSV response of TLR4 [18, 19]. More recently, it has been reported that TLR3, the sensor of double-stranded RNA, mediates inflammatory cytokine and chemokine production in RSV-infected epithelial cells [20]. TLR3 regulates the pulmonary immune environment in mice infected with RSV but viral clearance by TLR3 has not been identified [21].

In this study, we focused on the antiviral role of TLR3 against RSV infection and investigated whether RSV production was affected in TLR3-overexpressed human lung epithelial cells. The results indicate that TLR3 has modest anti-RSV activity in TLR3-overexpressed human lung epithelial cells.

Materials and methods

Cell culture and virus infection

A549 human lung epithelial cells [22] were cultured in Dulbecco's modified Eagle medium (Invitrogen Corp., Carlsbad, CA USA) with 50μg/ml gentamicin and 10% fetal bovine serum (FBS). HEp-2 human epithelial cells were obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University and maintained in Eagle minimal essential medium (MEM) (Nissui, Tokyo, Japan) with 10% FBS. These cells were cultured in a flask at 37℃ in 5% CO_2. The Long strain of RSV [23] was propagated in HEp-2 cells and the cell culture supernatants were centrifuged, snap frozen and stored at -80℃ after considerable syncytia were observed. For RSV infection experiments, the Long strain was inoculated at a multiplicity of infection (MOI) of 3 and incubated for 2h, next the virus inoculum was removed and the cells washed once with phosphate-buffered saline (PBS). The infected cells were cultured in the medium for 12 to 24h and the supernatants were snap frozen at -80℃ for RSV titration. For IFN-β neutralization experiments, anti-IFN-β polyclonal antibody (PBL Biomedical Laboratories, Piscataway, NJ USA) was added at a concentration of 2,000U/ml after the inoculation.

Transfection and selection

For transient transfection, A549 cells were transfected with human TLR3-expression vector, pUNO-hTLR3-HA (InvivoGen, San Diego, CA USA) by using Effectene (Qiagen, Tokyo, Japan) following the instruction manual. A control vector which does not harbor TLR3, designated pUNO-HA, was made by digesting pUNO-hTLR3-HA with *EcoR* V (Takara, Otsu, Japan) which removes the majority (59.5%) of the hTLR3 sequence and ligating the rest of the vector with T4 DNA ligase (New England Biolabs, Ipswich, MA USA). At 24h post-transfection, the cells were infected with the Long strain as mentioned above. To establish the stably TLR3-overexpressed cells, A549 cells were selected in a medium with Blasticidin S (InvivoGen) at 10μg/ml at 48h post-transfection. Each Blasticidin S-resistant cell colony was picked up and propagated, and thereafter stored at -80℃ with Cell Banker 1 (Mitsubishi Chemical Medience, Tokyo, Japan) until use.

Flow cytometry analysis

To detect TLR3 overexpression on the cell surface, cells were detached from a dish with 0.5mM EDTA (ethylene diamine tetraacetic acid) and resuspended in FACS (fluorescence activated cell sorting) buffer (3% FBS, 0.02% sodium azide in PBS). After centrifuge, the cells were stained with either PE (Phycoerythrin)-labeled anti-human TLR3 (eBioscience, San Diego, CA USA) diluted at 40μg/ml with FACS buffer or PE-labeled mouse IgG1 (Beckman Coulter, Fullerton, CA USA) diluted at 100μg/ml with FACS buffer. Cytometric analysis was performed with a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA USA). For detection of intracellular TLR3 overexpression, cells were treated with BD Cytofix/Cytoperm solution (BD Biosciences) before staining with PE-labeled antibodies.

Plaque assay

Serial 10-times dilutions of the cell culture supernatants from RSV infection experiments were prepared for the plaque assay. Confluent HEp-2 cells on a well of 24-well plate were incubated with 150μl of the dilutant for 2h, then 1ml of 1% FBS, 0.75% methylcellulose-MEM (m-MEM) was overlaid. Another 1ml of m-MEM was added to the well 2 days later. Five days after the infection the cells were fixed with 10% formaldehyde for overnight and stained with 0.5% crystal violet for 1h. The plaques were counted on microscopy. The plaque number was represented as an average number of 3 wells at the same dilution.

IFN-α/β ELISA

Human IFN-α and IFN-β concentrations in the cell culture supernatants were measured with Human IFN-α

ELISA kit (PBL Biomedical Laboratories) and Human IFN-β ELISA kit (PBL Biomedical Laboratories), respectively, following the instruction manuals. The absorbance at 450nm was read by Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA USA).

RNA extraction

Viral RNA was extracted from the cell culture supernatants by using MagExractor™-Viral RNA (Toyobo, Osaka, Japan). Total RNA was extracted from the RSV infected cells by using MagExractor™-RNA (Toyobo). The extracted total RNA was treated with 10U DNase I (Takara) in 40mM Tris-HCl (pH 7.5), 8mM $MgCl_2$, and 5mM DTT at 37℃ for 30min. The RNA was purified with phenol: chloroform: isoamyl alcohol (25:24:1) (Invitrogen), precipitated in isopropanol, and stored at -80℃ for further analyses.

Real-time RT-PCR

Real-time RT-PCR (reverse transcription polymerase chain reaction) was done to quantify TLR3 mRNA expression and RSV viral load (copy numbers) by using TaqMan PCR system (Applied Biosystems) with TaqMan RNA-to-Ct 1-Step Kit (Applied Biosystems). For TLR3 mRNA expression, multiplex real-time RT-PCR was employed in a 20μl mixture containing 10μl TaqMan RT-PCR mix, 1μl of each TLR3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) TaqMan Gene Expression Assay (Applied Biosystems), 0.6μg template RNA, and RNase-free water. To quantify RSV viral load, an RSV vector was prepared as follows: a part of the N protein gene (nt 1140 to 1264) of the Long strain was amplified by RT-PCR, the amplicon was cloned into pCR-4-TOPO vector with TOPO TA cloning kit (Invitrogen Corp.), and the cloned plasmid was identified by PCR (polymerase chain reaction) and the N protein gene sequence was confirmed by the DNA sequencing. For RSV quantification, the N protein gene was amplified in a 20μl mixture containing 10μl TaqMan RT-PCR mix, 10 μM forward primer
5'-CATCCAGCAAATACACCATCCA-3', 10μM reverse primer 5'-TTCTGCACATCATAATTAGGAGTATCAA-3', and 2.5μM probe 5'-FAM-CGGAGCACAGGAGAT-MGB-3', either 1μl of template RNA or the serial diluted RSV vectors, and RNase-free water [24]. RSV copy numbers were calculated using a standard curve made by the RSV vectors. PCR was done in 7500 Real-Time PCR System (Applied Biosystems) in triplicate.

Statistical analysis

Statistical analysis was performed with a two-tailed, unpaired Student's *t* test. The data were expressed as mean ± standard deviation of the mean. A value of $P<0.05$ was considered to be significant.

Results

Transiently overexpressed TLR3 suppresses RSV production

To investigate the antiviral role of TLR3 on RSV infection, human TLR3 gene was transiently overexpressed in A549 cells and the cells were infected with RSV. The overexpression of TLR3 was identified by TLR3 mRNA expression by real-time RT-PCR. Expression level of TLR3 was approximately 8 times higher in the pUNO-hTLR3-HA vector-transfected A549 cells compared to that in the control pUNO-HA vector-transfected cells at 24h post-transfection (Fig. 1a). These cells were infected with the Long strain of RSV at a MOI of 3 at 24h post-transfection. RSV copy numbers in the cell culture supernatants were significantly lower in the TLR3-overexpressed cells than in the control cells at 12h and 24h post-infection (Fig. 1b). At 24h post-infection, in particular, RSV copy number of the TLR3-overexpressed cells was about 30% of that of the control cells. These data suggest that TLR3 may suppress RSV production in human respiratory epithelial cells.

Stable overexpressed TLR3 was detected in the intracellular compartment in A549 cells

To further explain the antiviral role of TLR3 against RSV, stable TLR3-overexpressed A549 cell clones were obtained by Blasticidin S selection after transfection. After the selection, 13 colonies of the pUNO-hTLR3-HA-transfected A549 cells, as well as 4 colonies of the pUNO-HA-transfected cells, were collected. Flow cytometry analysis showed the same level of TLR3 expression on the cell surface in pUNO-hTLR3-HA-transfected and pUNO-HA-transfected cells. However three clones of pUNO-hTLR3-HA-transfected cells demonstrated more TLR3 expression in the intracellular compartment compared to a clone of pUNO-HA-transfected cells (clone A549/HA3). Among these three, two clones (clones A549/TLR3-HA2 and A549/TLR3-HA8) were used for further experiments (Fig. 2). Exogenous TLR3 expression in pUNO-HA-transfected cells was not detected in flow cytometry analysis. Thus we concluded that A549/TLR3-HA2 and A549/TLR3-HA8 clones stably overexpressed TLR3 in the intracellular compartment and used them for further

RSV infection experiments.

Stably overexpressed TLR3 suppresses RSV production

A549/TLR3-HA2 and A549/TLR3-HA8 cells as well as A549/HA3 control cells were infected with the Long strain at MOI of 3. The cell supernatants were collected and snap-frozen at 12, 18, and 24h post-infection (only 24h in A549/TLR3-HA8 cells). RSV production was quantified at those designated times by plaque assay. TLR3-overexpressed cell clones significantly suppressed RSV production compared to the control cell clone at 18 and 24h post-infection (Fig. 3). At 24h post-infection, in particular, RSV production was less than 20% in both TLR3-overexpressed cell clones compared to the control cell clone. These data further confirmed that TLR3 had suppressed RSV production in A549 cells.

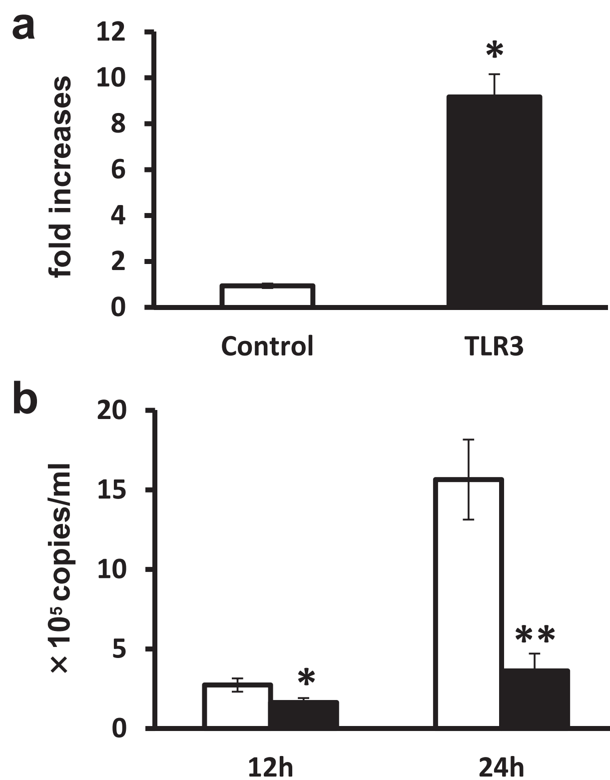

Fig.1 a TLR3 mRNA expression in transiently pUNO-TLR3-HA vector-transfected (TLR3) or pUNO-HA vector-transfected (control) A549 cells quantified by real-time RT-PCR.
b RSV copy numbers in the supernatants of the control (white bar) and the TLR3 (black bar) A549 cells at 12h and 24h after RSV infection quantified at by real-time RT-PCR. The data are representative of three experiments. *, $P < 0.05$; **, $P < 0.01$

IFN-β has a partial role as a mediator of TLR3 anti-RSV activity

The antiviral activity of TLR3 is reported to involve type I IFNs (IFN-α/β), especially IFN-β [25]. To elucidate whether IFN-α/β are involved in the anti-RSV activity of TLR3, we measured IFN-α/β production in the cell culture supernatants by ELISA. IFN-α was below minimum detection level ($<$ 12.5pg/ml) up to 24h post-RSV infection, a finding compatible with the previous report that RSV infection of human epithelial cells induced IFN-β but not IFN-α [26]. IFN-β concentration could be measured by ELISA post-18h of infection. However, in contrast to our expectation, more IFN-β was secreted in the supernatants of A549/HA3 cells than in those of A549/TLR3-HA2 and A549/TLR3-HA8 cells at 24h post-infection (Fig. 4).

To further explain whether IFN-β mediates the anti-RSV activity of TLR3, anti-IFN-β antibody which neutralizes any secreted IFN-β was added to the cell culture supernatants immediately after RSV inoculation. A concentration of 2,000U/ml of anti-IFN-β antibody was sufficient to neutralize IFN-β in the supernatants of A549 cells at 24h after RSV infection under minimum detection level ($<$ 25pg/ml) measured by ELISA (data not shown). RSV production was significantly suppressed in A549/TLR3-HA2 cells compared to A549/HA3 cells without anti-IFN-β antibody at 24h post-infection, which was compatible with the results presented in Fig. 3 (Fig. 5). After neutralizing IFN-β in the supernatants, RSV production increased significantly in both A549/HA3 and A549/TLR3-HA2 cells (Fig. 5), but the increase of RSV production in A549/TLR3-HA2 cells was much less than in A549/HA3 cells (Fig. 5). These data support the concept that secreted IFN-β in the supernatant might have a limited role as a mediator of anti-RSV activity by TLR3.

Discussion

In this study, we have shown that both transiently and stably overexpressed TLR3 inhibits RSV replication in A549 human respiratory epithelial cells. Our finding conflicts with that of Rudd et al. who reported that TLR3 had no effect on RSV replication [20]. They infected human kidney HEK293 cells with stable TLR3 expression

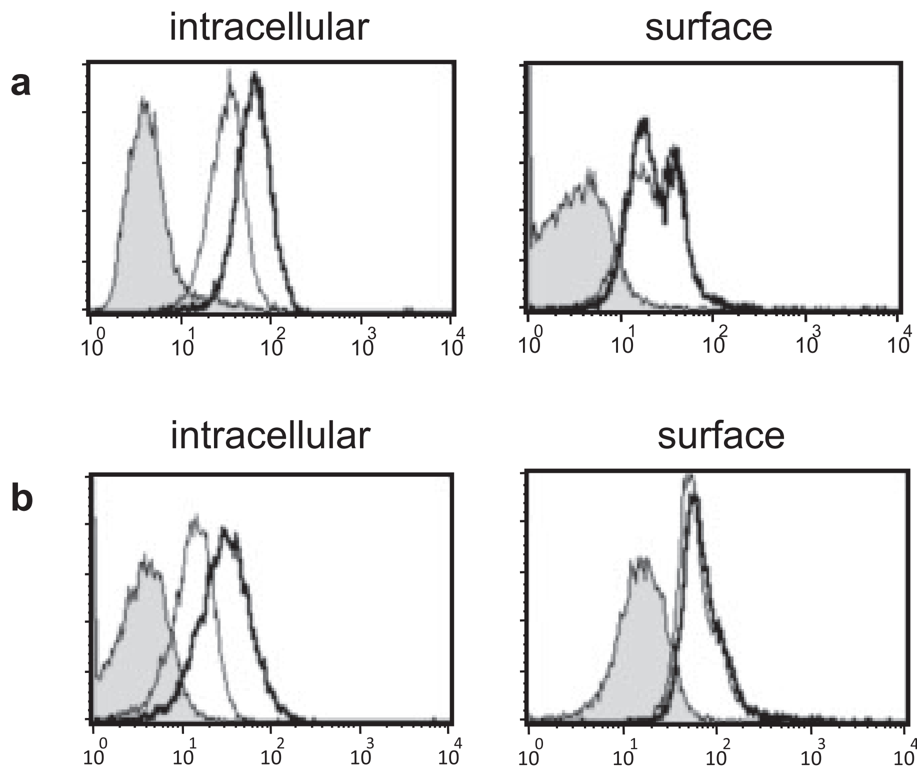

Fig.2 Flow cytometry analysis to detect TLR3 expression in the cytoplasm and on the cell surface of A549/TLR3-HA2 (a) and A549/TLR3-HA8 (b) cells. The shaded peaks indicate A549/TLR3-HA2 and A549/TLR3-HA8 cells stained with PE-labeled mouse IgG1, the thin line peaks do A549/HA3 control cells stained with PE-labeled anti-human TLR3, and the thick line peaks do A549/TLR3-HA2 and A549/TLR3-HA8 cells stained with PE-labeled anti-human TLR3

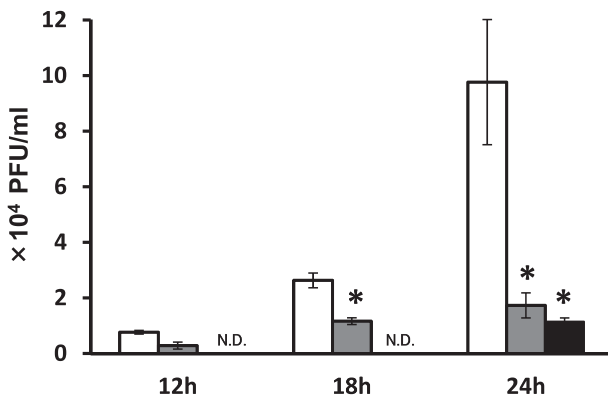

Fig.3 Plaque assay to quantify RSV production in A549/HA3 cells (white bars), A549/TLR3-HA2 cells (gray bars), and A549/TLR3-HA8 cells (a black bar) at 12h, 18h, and 24h after RSV infection. The number of plaques was designated as PFU (plaque forming units) per ml. The data are representative of three experiments. *, $P < 0.05$, *N.D.*: not determined.

and vector control cells with a clinical isolate of RSV A strain at a MOI of 0.1. They measured RSV load by plaque assay in both cell lines and the number of plaques was shown in a log scale graph. The graph shows similar peaks in both cell lines and it is difficult to see clear differences of viral replication between them. Additionally, they produced TLR3-suppressed A549 cells with small interfering RNA (siRNA) for TLR3 (TLR3i) and control cells with random siRNA and infected them with the same RSV strain at a MOI of 1. The viral loads (numbers of plaques) in TLR3i and control cells are similar on a log scale graph. In general, virus replication occurs exponentially and a log scale graph is used. In our case, the difference of viral loads between the TLR3-overexpressed cells and controls was statistically significant although it was within a single digit range. Therefore, we showed the viral loads using a linear scale graph to make the difference more evident and concluded that TLR3 had modest anti-RSV activity.

The authors' recent review concerning the roles of TLRs in regulating the immune response against RSV

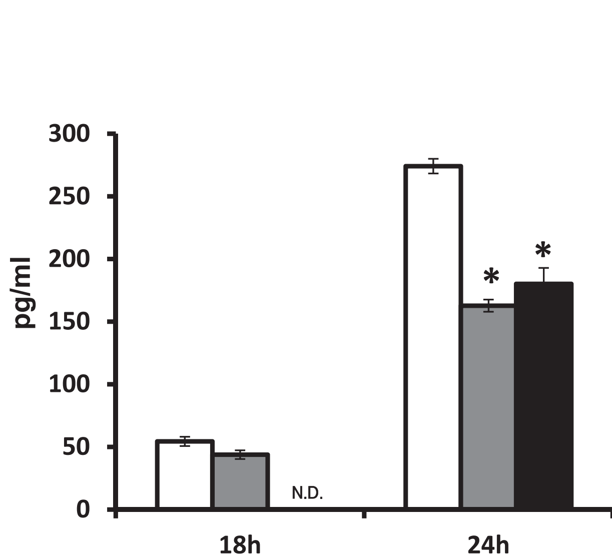

Fig.4 IFN-β ELISA of the cell culture supernatants of A549/HA3 cells (white bars), A549/TLR3-HA2 (gray bars), and A549/TLR3-HA8 (a black bar) cells at 18h and 24h after RSV infection. The data are representative of three experiments. *, $P < 0.05$, *N.D.*: not determined

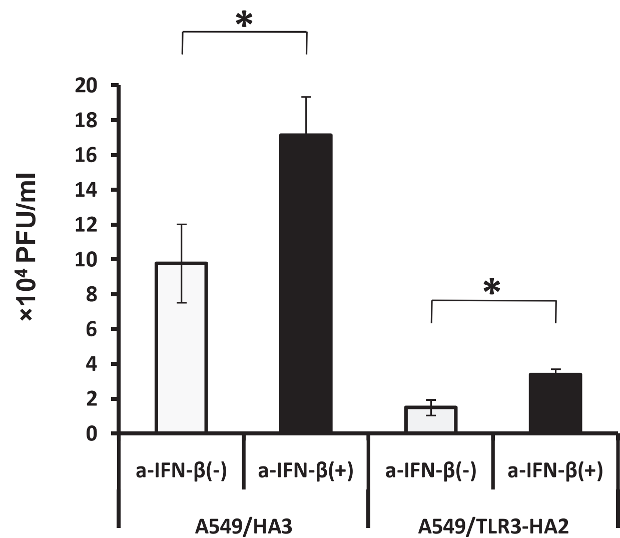

Fig.5 Plaque assay to quantify RSV production without anti-IFN-β antibody (a-IFN-β (-), white bars) or with anti-IFN-β antibody (a-IFN-β (+), black bars) in A549/HA3 and A549/TLR3-HA2 cells at 24h after RSV infection. The number of plaques was designated as PFU (plaque forming units) per ml. The data are representative of three experiments. *, $P < 0.05$

suggested that differences in infectious doses and strains of RSV and propagation cell lines employed might influence study outcome [27]. Therefore, the conflicting results by Rudd et al. and ourselves might be explained by those different factors.

An antiviral role of TLR3 for another respiratory virus was reported by Hewson et al. [28]. They infected BEAS-2B human bronchial epithelial cells with rhinovirus (RV) with or without anti-TLR3 antibody to block TLR3 which are expressed on the cell surface. Blocking TLR3 with antibody significantly increased RV release into the supernatants, suggesting an antiviral role of TLR3 against RV.

Virus infection activates a crucial transcription factor, IFN regulatory factor 3 (IRF3), and IRF3 induces IFN-α/β. The induced IFN-α/β activate the Janus kinases-signal transducers and activators of the transcription (JAK-STAT) signal cascade and induce many IFN-inducible genes involving the antiviral responses [29, 30]. Mediators of TLR3 antiviral activity were postulated by Doyle et al., after experiments in which TLR3 activated IRF3 and it induced IFN-β which inhibited murine γ herpes-virus 68 (MHV68) replication [31]. Liu et al. reported that siRNA-mediated TLR3 knockdown inhibited IFN-β expression following RSV infection in A549 cells, and suggested the IFN-β is a mediator of TLR3 bioactivity in A549 cells infected with RSV [32]. Thus, we speculated that IFN-β mediates the antiviral activity of TLR3 against RSV infection. Our IFN-β neutralization experiments, however, demonstrated that IFN-β had only a limited role as a mediator of TLR3 anti-RSV activity. This is supported by our finding that IFN-β secretion was greater in the control cells than in the TLR3-overexpressed cells. We assumed that this reflected greater production of RSV in the supernatants because RSV infection induces IFN-β not only via TLR3 but also via other sensors such as retinoic acid-inducible gene I [32]. This hypothesis is supported by the case of RV infection mentioned above [28]. In BEAS-2B cells, cell surface TLR3 mediated production of proinflammatory mediators IL-6, CXCL8, and CCL5 on stimulation with poly(IC) and this production was reduced by inhibiting TLR3 activation with anti-TLR3 antibody. In contrast, such mediators were more produced by inhibiting TLR3 activation in case of RV infection and the amount of RV was higher with TLR3 blocking. The authors explained these results that when TLR3 was blocked, the antiviral response via TLR3

was inhibited and RV replication increased, resulting in increase in RV infection and stimulation of non-TLR3 receptors such as protein kinase R, which overwhelmed reduced production of the mediators due to TLR3 inhibition.

Currently we do not know what factor(s) other than IFN-β mediate the antiviral activity of TLR3. Recently, new members of the IFN family, type III IFNs (IFN-λ1/IL-29, IFN-λ2/IL-28A, IFN-λ3/IL-28B), have been identified [33, 34]. These IFN-λs were induced by several viruses including RSV in different cell lines including A549 and displayed potent antiviral activity [35, 36]. Further, the TLR3 signal transduction pathway activated IFN-λ1 promoter and the IFN-λ1 gene is regulated by IRF3 and IRF7 [37]. Thus, IFN-λs could be involved in the TLR3-mediated anti-RSV activity.

Localization of TLR3 expression is reported to differ depending on cell type. In human fibroblasts, TLR3 was naturally expressed on the cell surface whereas it was expressed only in the intracellular compartment in monocyte-derived immature dendritic cells (iDCs) [38, 39]. According to previous reports, A549 cells do not express TLR3 on the cells surface at baseline, but express intracellularly at baseline and on the cell surface after RSV infection [40, 41, 42]. In our study, however, TLR3 was expressed on the cell surface at baseline in both control vector- and TLR3 gene-transfected A549 cells, and TLR3 gene-transfected A549 cells further overexpressed TLR3 in the intracellular compartment. We do not know why TLR3 was expressed on the cell surface in those control vector- and TLR3 gene-transfected A549 cells without RSV infection. Sha et al. reported that TLR3 expressed on the plasma membrane of the BEAS-2B airway epithelial cell line and primary bronchial epithelial cells and the TLR3 expression was further upregulated by dsRNA stimulation detected by flow cytometric analysis [43]. Thus, the transfected exogenous gene might stimulate TLR3 induction by dsRNA originating from the transcriptional intermediate of the exogenous gene.

In conclusion, we have shown that TLR3 expressed in human lung epithelial cells can modestly inhibit RSV replication and IFN-β may not be a major mediator of this activity. Further study is necessary to elucidate other factor(s) which mediate anti-RSV activity of TLR3 and details of their mechanisms.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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RS ウイルス感染に対する Toll-like receptor 3 の抗ウイルス作用

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ヒト RS ウイルス（RSV）は乳幼児に急性細気管支炎などの下気道感染症をしばしば引き起こす．一方，自然免疫のパターン認識受容体として，広範囲に病原体を認識する Toll-like receptors（TLRs）は，感染症に対する宿主側の最前線の防御機構である．本研究は，2 本鎖 RNA を認識する Toll-like receptor 3（TLR3）の抗 RSV 作用を検討することを目的とした．ヒト TLR3 発現プラスミドベクターを A549 細胞に導入し，TLR3 を一過性または恒常的に過剰発現させ，RSV Long 株を感染させた．いずれの TLR3 過剰発現細胞でも，コントロール細胞と比較して，わずかだが有意に RSV 産生が抑制された．TLR3 過剰発現細胞の上清では，ELISA にて測定した結果，インターフェロン（IFN）-β 産生量が減少していた．また，TLR3 過剰発現細胞の上清において IFN-β を中和しても，RSV 量はコントロールと同じ水準にまで増加しなかった．以上の結果から，TLR3 は弱いながら抗 RSV 作用を有し，IFN-β はこの作用における主要な媒介物質ではないと考えられた．