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Original article

Toll-like receptor 3 ligand specifically induced bronchial epithelial cell death in caspase dependent manner and functionally upregulated Fas expression

Yuta Koizumi ^a, Hiroyuki Nagase ^{a,*}, Toshiharu Nakajima ^b, Masafumi Kawamura ^c, Ken Ohta ^{a,d}^a Division of Respiratory Medicine and Allergy, Department of Medicine, Teikyo University School of Medicine, Tokyo, Japan^b Department of Health Pharmacy, Yokohama University of Pharmacy, Yokohama, Japan^c Department of Surgery, Teikyo University School of Medicine, Tokyo, Japan^d National Hospital Organization, Tokyo National Hospital, Tokyo, Japan

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CXCL8, CXC motif ligand 8; CXCL10, CXC motif chemokine ligand 10; dsRNA, double-stranded RNA; FADD, Fas-associated protein with death domain; FCS, fetal calf serum; IRF-3, IFN regulatory factor-3; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; PAMPs, pathogen-associated molecular patterns; PBECs, primary cultured human bronchial epithelial cells; poly I:C, polyinosinic–polycytidylic acid; RSV, respiratory syncytial virus; ssRNA, single-stranded RNA; TLRs, Toll-like receptors

ABSTRACT

Background: Viral infections are the most common cause of asthma exacerbation. Virally infected epithelial cells undergo apoptosis. Although in healthy conditions, apoptosis may have a host-defensive role in limiting virus spread, this process may have a detrimental effect on damaged epithelium in asthma. Toll-like receptors (TLRs) are the receptors for various pathogens, and viruses possess several components that can activate TLR3, TLR4, and TLR7/8. However, as it has not been determined as to which component is responsible for virus-induced epithelial cell apoptosis, we comprehensively analyzed the effects of all TLR ligands on apoptosis.

Methods: BEAS-2B cells or primary cultured human bronchial epithelial cells (PBECs) were stimulated by TLR 2, 3, 4, 5, 7/8, and 9 ligands and cell death was analyzed by flow cytometry. Chemokine generations induced by these ligands were also analyzed.

Results: The TLR3 ligand polyinosinic–polycytidylic acid (poly I:C) specifically induced chemokine generation and apoptosis, while other TLR ligands including those for TLR5, 7/8, and 9 had no effect. The response to poly I:C had two phases, which included rapid secretion of chemokines and subsequent apoptosis in a later phase. Poly I:C induced apoptosis in a caspase-dependent manner and functionally upregulated the expression of Fas.

Conclusions: Previous findings indicating that viruses induced caspase-dependent death and upregulated Fas expression were reproduced by poly I:C, suggesting the central role of dsRNA/TLR3 in virus-induced apoptosis. Since these processes may have detrimental effects on pre-existing epithelial damage, the dsRNA/TLR3 pathway may be potential novel treatment target for virus-induced exacerbation of asthma.

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Introduction

Viral infections of the respiratory system are the most common cause of asthma exacerbation.¹ The mechanism of the worsening of allergic airway inflammation by a virus may include multiple steps. The respiratory epithelium is a frontline interface for the environment, and virally infected epithelial cells secrete various chemokines and cytokines, which lead to the accumulation of inflammatory cells.² Another important aspect of the pathology of

* Corresponding author. Division of Respiratory Medicine and Allergy, Department of Medicine, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan.

E-mail address: nagaseh@med.teikyo-u.ac.jp (H. Nagase).

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asthma worsening may include airway injury. The respiratory syncytial virus (RSV)³ or influenza virus⁴ induces apoptosis of epithelial cells. In healthy conditions, apoptosis induced in response to viral infection may work as part of the host defense response to limit virus replication and spread of the virus. However, these processes may have a detrimental effect on damaged epithelium in asthma.

As receptors for pathogen-associated molecular patterns (PAMPs), 10 human Toll-like receptors (TLRs) have been cloned, and their expression profiles have been identified in human cells.⁵ Bronchial epithelial cells express TLR1–TLR10 mRNAs and generate various cytokines or chemokines in response to TLR2, 3, 4, 5, and 7/8 ligands depending on the cell type.^{2,6} However, there has been no comprehensive study investigating the effects of TLR ligands, including TLR5, 7/8, and 9 ligands, on bronchial epithelial cell survival. Viruses possess various components that can activate different TLRs. Thus, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and the RSV fusion protein are known to activate TLR3, TLR7/8, and TLR4, respectively,⁶ but which component is responsible for epithelial cell apoptosis has not yet been determined.

Thus, we aimed to comprehensively investigate the effects of all TLR ligands in order to clarify which ligand is important for virus-induced epithelial cell apoptosis. As virus induces epithelial apoptosis in caspase-dependent manner and upregulates apoptosis-stimulating Fas expression,^{4,7} we also investigated the involvement of caspase cascade and effect on Fas expression in TLR ligand-induced apoptosis.

Methods

Reagents

TLR ligands [TLR2: N-palmitoyl-S-[2,3-bis(palmitoxyl)-(2RS)-propyl]-Cys-Ser-Lys 4 (Pam3CSK⁴); TLR3: polyinosinic–polycytidylic acid (poly I:C); TLR4: lipopolysaccharide (LPS); TLR7/8: R848; and TLR9: CpG DNA 2006] were prepared or purchased as previously described.⁸ The following reagents were purchased as indicated: TLR5 ligand flagellin (Calbiochem, San Diego, CA, USA), human recombinant tumor necrosis factor- α (TNF- α) (R&D Systems, Minneapolis, MN, USA), human recombinant interferon- γ (IFN- γ) (Shionogi Pharmaceuticals, Tokyo, Japan), anti-human TNF- α antibody (R&D Systems), anti-human IFN- γ antibody (R&D Systems), isotype control IgG (R&D Systems), caspase-8 inhibitor z-IETD-fmk (R&D Systems), pan-caspase inhibitor z-VAD-fmk (R&D Systems), anti-Fas CH-11 monoclonal antibody (mAb) (Immunotech, Marseille, France), and control mouse IgM (Organon Teknica Corp., West Chester, PA, USA).

Cell culture

BEAS-2B cells of the human bronchial epithelial cell line were cultured as described previously.⁹ BEAS-2B cells were plated on 24-well culture plates (Iwaki Glass) in Dulbecco's modified Eagle's Medium/Ham's F12K medium containing 5% fetal calf serum (FCS). Before stimulation with various TLR ligands, the culture medium in each well was replaced with an identical formulation containing

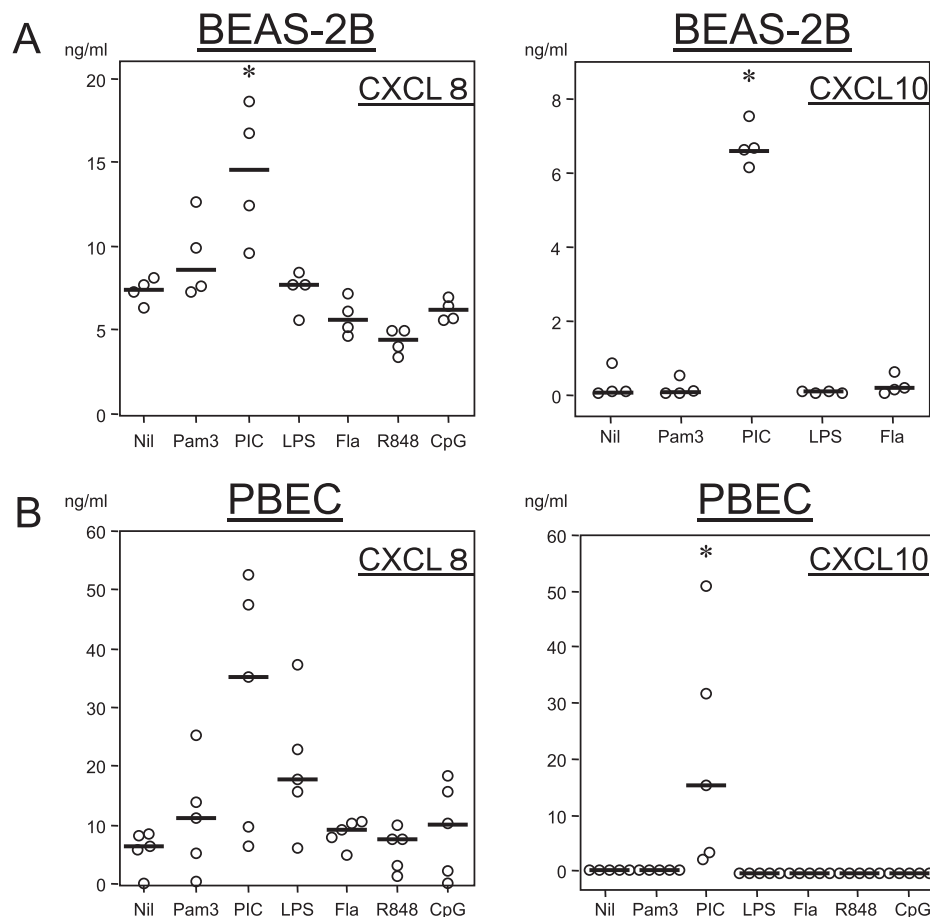


Fig. 1. Effect of TLR ligands on chemokine generation by bronchial epithelial cells. BEAS-2B cells (**A**, $n = 4$) or PBECs (**B**, $n = 5$) were incubated with the indicated TLR ligands for 72 h, and the levels of chemokines in the culture supernatants were determined by ELISA. Pam3, Pam3CSK⁴ (10 μ g/mL); PIC, poly I:C (50 μ g/mL); LPS (10 μ g/mL); Fla, flagellin (1 μ g/mL); R848 (10 μ M); CpG, CpG DNA 2006 (1 μ M). * $p < 0.05$ vs. the value of Nil.

0.1% bovine serum albumin instead of FCS as previously described.¹⁰ Primary cultured human bronchial epithelial cells (PBECs) were prepared as described previously.¹¹ The study was approved by the Ethics Committee of Teikyo University (10–088). Written informed consent was obtained from the patients before the operation.

Enzyme-linked immunosorbent assay (ELISA)

The levels of chemokines in culture supernatants and cell lysates were determined by ELISA. Cell lysates were obtained by adding 0.5% Nonidet P-40 to cultures. Concentrations of the CXC motif chemokine ligand 10 (CXCL10) were determined by ELISA using two mAbs. ELISA microtiter plates (F8 Modules, #468667; Nunc, Roskilde, Denmark) were coated with a mAb (4D5/A7/C5; BD Pharmingen, San Diego, CA, USA). Samples were added to coated plates, which were then washed and incubated with a biotinylated mAb (6D4/D6/G2, BD Pharmingen), followed by an Amdex streptavidin-horseradish peroxidase conjugate (Sigma–Aldrich, St. Louis, MO, USA). The plates were developed with the 3,3',5,5'-tetramethylbenzidine (TMB) microwell peroxidase substrate system (KPL, Gaithersburg, MD, USA), and the reactions were stopped with 3 NH₃PO₄. Absorbance was measured at 450 nm. Concentrations of the CXC motif ligand 8 (CXCL8) were determined as previously described.¹²

Analysis of cell survival

Analysis of cell survival was performed as previously described.¹³ Briefly, after removal of cells from culture plates using trypsin-EDTA, live cells were quantitatively determined by negative staining with annexin V and propidium iodide (PI) using the apoptosis detection kit MEBCYTO Apoptosis Kit (Medical and Biological Lab., Nagoya, Japan).

Terminal deoxynucleotidyl transfer-mediated dUTP nick end-labeling staining (TUNEL)

We conducted TUNEL staining using the In Situ Cell Death Detection Kit, POD (Roche, Germany), according to the manufacturer's instructions. BEAS-2B cells were cultured on Biocoat 8-well culture slides (Corning, Tewksbury, MA, USA), and the slides were immersed in the TUNEL reaction mixture, followed by the incubation with converter-POD. The slides were analyzed by optical microscopy. The TUNEL index (%) was the ratio of the number of TUNEL-positive cells divided by the total number of cells.

Flow cytometry

Expression of Fas (CD95) and Fas ligand (FasL or CD95L) on BEAS-2B cells was analyzed by flow cytometry as previously

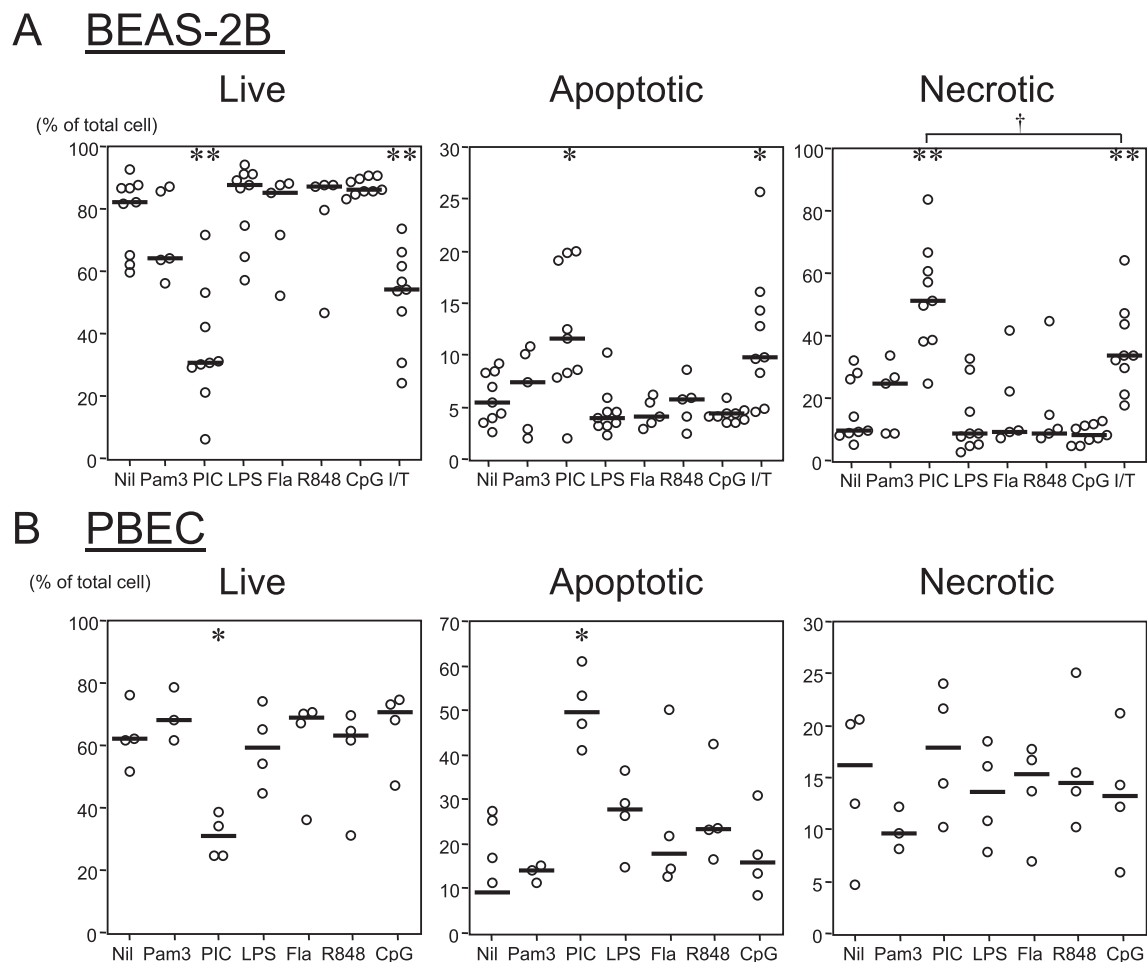


Fig. 2. Effect of TLR ligands on survival of bronchial epithelial cells. BEAS-2B cells (**A**, $n = 9$) or PBECs (**B**, $n = 4$) were incubated with the indicated TLR ligands for 72 h, and the cell status was determined by double staining with annexin V and PI. Pam3, Pam3CSK4 (10 μ g/mL); PIC, poly I:C (50 μ g/mL); LPS (10 μ g/mL); Fla, flagellin (1 μ g/mL); R848 (10 μ M); CpG, CpG DNA 2006 (1 μ M); I/T, IFN- γ (300 U/mL) + TNF- α (10 ng/mL). ** $p < 0.01$; * $p < 0.05$ vs. the value of Nil. † $p < 0.05$ between the indicated groups.

described.¹³ Cells were incubated with phycoerythrin (PE)-conjugated anti-CD95 mAb (DX2; eBioscience, San Diego, CA, USA) or PE-conjugated anti-CD95 ligand mAb (NOK-1; eBioscience). The median values of fluorescence intensity were converted into the numbers of molecules of equivalent soluble fluorochrome (MESF) units using a Quantum PE MESF Kit (QMP-2; Sigma–Aldrich). Surface expression levels were calculated using the following formula: (MESF with anti-CD95 or anti-CD95L mAb) – (MESF isotype control IgG1κ (MOPC-21/p3; eBioscience)).

Statistical analysis

The distributions and variances across samples were checked using the Shapiro–Wilk and Bartlett's test, respectively. If majority of data were normally distributed and variances were equal, differences between two groups were analyzed using the Student's *t*-test (Fig. 7A), and the differences between more than two groups were analyzed using the two-way analysis of variance (ANOVA) test (Fig. 3A, B, 4, 5, 6, 7B). When the latter test indicated significant differences, the Fisher's protected least significant difference test was used to compare individual groups. Data were presented as the means $\pm$ standard error of the mean (SEM). If majority of data were not normally distributed or the variances were not equal, differences between two groups were analyzed using the Mann–Whitney *U* test (Fig. 3C), and the differences between more than two groups were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney *U* test and data were presented as medians

(Fig. 1, 2). The Benjamini and Hochberg false discovery rate (FDR)-controlling procedure was employed for multiple comparisons after the Kruskal–Wallis test under FDR at 0.25. The significant data shown in Figure. 1, 2 all passed the Benjamini–Hochberg procedure. The analyses were performed using the StatView software.

Results

Chemokine generation and cell death induced by TLR ligands

First, we investigated effects of various TLR ligands on chemokine generation by BEAS-2B cells. Among TLR ligands, only the TLR3 ligand poly I:C upregulated the baseline generation of CXCL8 (interleukin-8) and induced CXCL10 (IFN- γ -induced protein 10) generation by BEAS-2B cells (Fig. 1A). The other TLR ligands, including TLR2, 4, 5, 7/8, and 9 ligands, did not have significant effects on the chemokine generation by BEAS-2B cells. In the case of PBEC cells, poly I:C induced a significant generation of CXCL10 and trended toward an up-regulation of CXCL8 generation, similar to that observed in BEAS-2B cells (Fig. 1B).

We next investigated the effects of TLR ligands on bronchial epithelial cell survival. As for chemokine generation, only poly I:C significantly decreased the number of live cells and increased the number of apoptotic and necrotic cells in a BEAS-2B cell culture (Fig. 2A). The effect was significantly more prominent than that shown by IFN- γ + TNF- α as known positive controls.¹⁴ Moreover, in case of PBECs, only poly I:C significantly decreased the number of

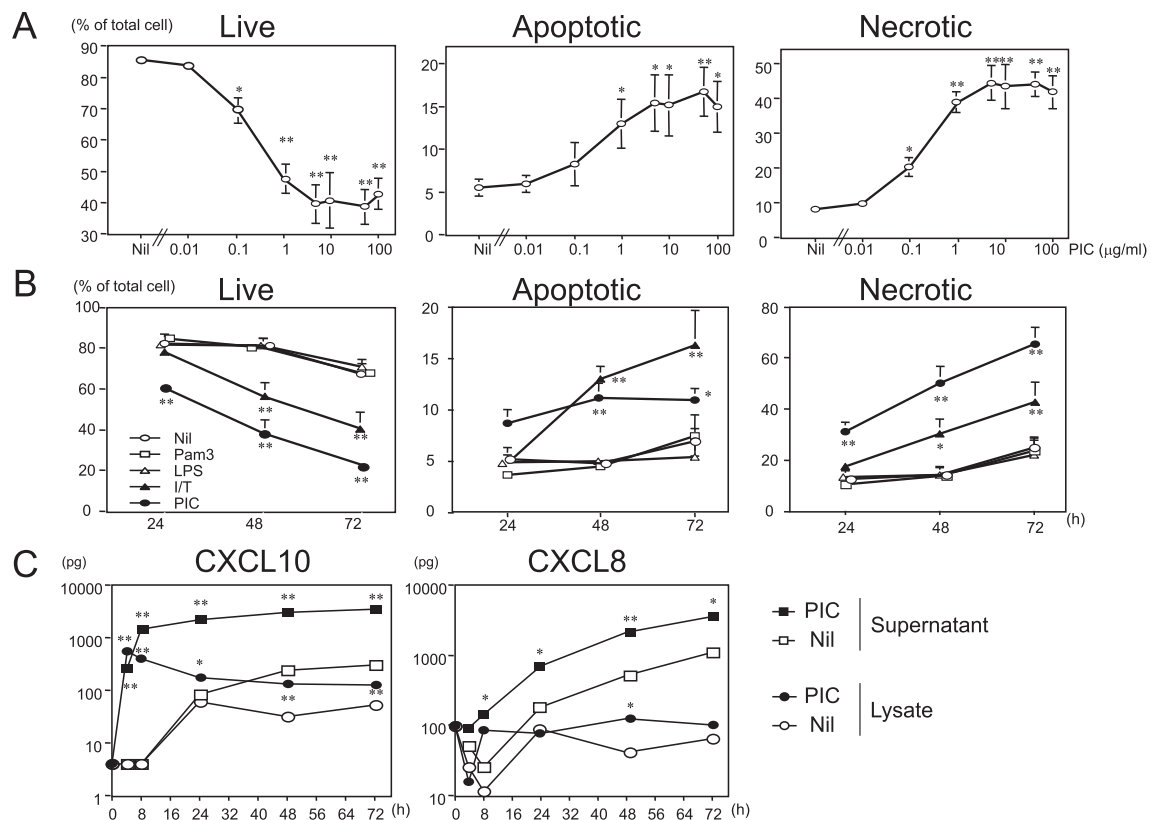


Fig. 3. Dose-dependent effects and time courses of poly I:C-induced cell death and chemokine generation in BEAS-2B cells. (A) BEAS-2B cells ($n = 4$) were incubated with the indicated concentrations of poly I:C for 72 h, and the cell status was determined by double staining with annexin V and PI. ** $p < 0.01$; * $p < 0.05$ vs. the value of Nil. (B) BEAS-2B cells were incubated with various TLR ligands for up to 72 h ($n = 4$). Pam3, Pam3CSK4 (10 μ g/mL); LPS (10 μ g/mL); I/T, IFN- γ (300 U/mL) + TNF- α (10 ng/mL); PIC, poly I:C (50 μ g/mL). ** $p < 0.01$; * $p < 0.05$ vs. the value of Nil at the same time point. (C) BEAS-2B cells ($n = 6$) were incubated with 50 μ g/mL of poly I:C for up to 72 h, and the levels of chemokines in the culture supernatants or cell lysates were determined by ELISA. The amounts of chemokines were calculated according to following formulas: total amount in the supernatant = (concentration) $\times$ (total volume of the supernatant) and total amount in the cell lysate = (concentration in the medium containing the cell lysate) $\times$ (total volume of the medium containing the cell lysate). ** $p < 0.01$; * $p < 0.05$ vs. the value of Nil at the same time point.

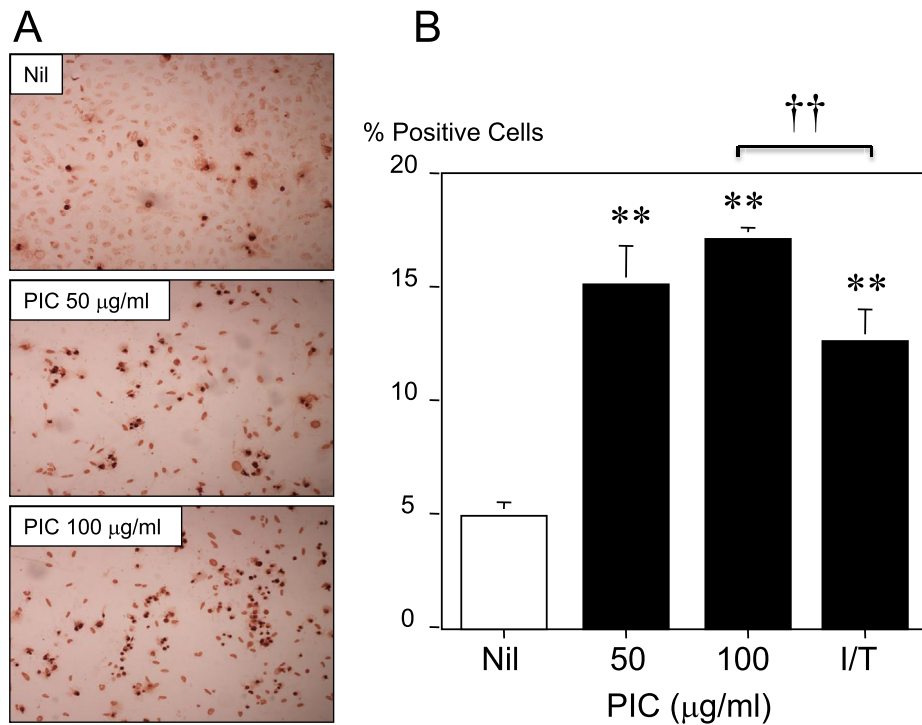


Fig. 4. Detection of poly I:C-induced DNA fragmentation by the TUNEL assay. (A) Representative photomicrograph of TUNEL staining of the BEAS-2B cells treated with poly I:C for 72 h (from four independent experiments). (B) Ratio of TUNEL staining-positive cells after 72 h of incubation ($n = 4$). PIC, poly I:C; I/T, IFN- γ (300 U/mL) + TNF- α (10 ng/mL). ** $p < 0.01$ vs. the value of Nil. †† $p < 0.01$ between the indicated groups.

live cells and increased the number of apoptotic cells (Fig. 2B). In contrast to BEAS-2B cells, the number of apoptotic cells was markedly increased, but no significant change was observed in the fraction of necrotic cells in a PBEC culture.

As shown in Figure 3A, a significant decrease in the number of live cells was observed at 0.1 µg/mL of poly I:C, and a significant increase in the number of apoptotic cells was observed at 1 µg/mL. The median effective dose value of poly I:C in cell death induction was 0.1–1 µg/mL. A time-course experiment showed that a

significant decrease in the number of live cells was observed after 24 h, and the number of live cells linearly decreased up to 72 h (Fig. 3B). In contrast, significant induction of CXCL10 in the supernatant by poly I:C was observed after 4 h, and the effect reached a plateau level at 24 h, suggesting that the peak of CXCL10 generation was approximately 8 h (Fig. 3C). Significant induction of CXCL10 in a BEAS-2B cell lysate was observed as soon as after 4 h, and the level gradually decreased up to 72 h. Although the effect of poly I:C on CXCL8 generation was delayed compared with that on CXCL10

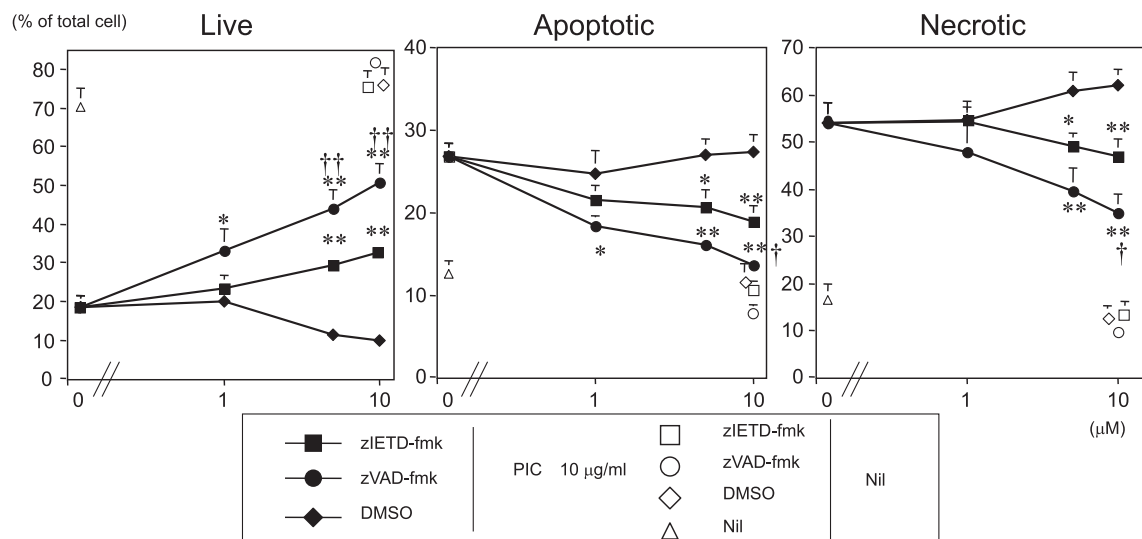


Fig. 5. Effect of caspase inhibitors on poly I:C-induced cell death. BEAS-2B cells were incubated with 10 µg/mL of poly I:C and the indicated concentrations of the caspase inhibitor z-VAD-fmk or z-IETD-fmk for 72 h, and the cell status was determined by double staining with annexin V and PI ($n = 5$). ** $p < 0.01$; * $p < 0.05$ vs. the same concentration of dimethyl sulfoxide (DMSO). †† $p < 0.01$; † $p < 0.05$ vs. the same concentration of z-IETD-fmk.

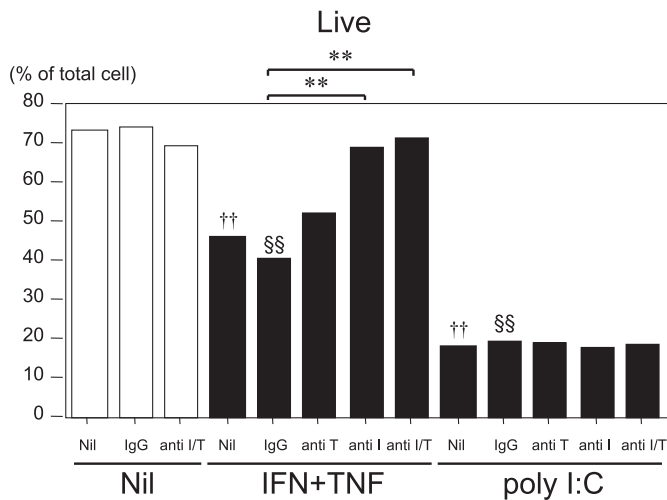


Fig. 6. Effect of anti-TNF- α Ab and anti-IFN- γ Ab on poly I:C-induced cell death. BEAS-2B cells were incubated with IFN- γ (30 U/mL) + TNF- α (10 ng/mL) (IFN + TNF) or poly I:C (10 μ g/mL) for 72 h. The effects of anti-TNF- α Ab (anti T: 5 μ g/mL), anti-IFN- γ Ab (anti I: 5 μ g/mL), or both (anti I/T) were determined by double staining with annexin V and PI ($n = 3$). ** $p < 0.01$ vs. control IgG-treated cells stimulated with IFN + TNF. †† $p < 0.01$ vs. Nil and §§ $p < 0.01$ vs. control IgG-treated cells without stimulation.

generation, the former reached a plateau after 48 h. Thus, the effect of poly I:C on chemokine generation was rapid but transient compared with the effect on cell death.

We also confirmed poly I:C-induced cell death using the TUNEL assay. As shown in Figure 4, the number of TUNEL-positive cells was significantly increased by poly I:C, suggesting that DNA fragmentation occurred due to the poly I:C treatment.

Pathway of poly I:C-induced death of bronchial epithelial cells

The signaling pathway of viral infection-induced cell death includes a caspase cascade.⁴ We explored if the caspase pathway was also involved in poly I:C-induced cell death (Fig. 5). A treatment

with the pan-caspase inhibitor z-VAD-fmk or the caspase-8 inhibitor z-IETD-fmk did not have a significant effect on spontaneous cell death (Nil). However, these inhibitors partially but significantly reversed poly I:C-induced cell death.

To investigate the autocrine or paracrine involvement of IFN- γ or TNF- α in poly I:C-induced cell death, BEAS-2B cells were treated with an anti-TNF- α Ab and/or an anti-IFN- γ Ab (Fig. 6). As previously reported,¹⁴ IFN- γ + TNF- α induced a significant decrease in the number of live cells. The anti-IFN- γ Ab in combination with the anti-TNF- α Ab almost completely restored the survival of the IFN- γ + TNF- α -treated BEAS-2B cells. However, the anti-IFN- γ Ab and/or TNF- α Ab had no effect on poly I:C-induced cell death, suggesting that the autocrine or paracrine pathway of these cytokines was not involved in the poly I:C-induced cell death.

Effect of poly I:C on Fas expression

Fas and FasL are functionally expressed on bronchial epithelial cells¹⁵ and the expression of Fas is upregulated by viral infection.⁷ However, it is still unclear if dsRNA alone can exert the same effect on bronchial epithelial cells. To examine if poly I:C-induced cell death is mediated by Fas/FasL expressed in neighboring epithelial cells, we investigated the effect of poly I:C on expression levels of Fas and FasL.

Although we analyzed FasL expression on BEAS-2B cells, no significant expression was detected before and after poly I:C stimulation (data not shown). In contrast, poly I:C significantly upregulated the surface expression of Fas on BEAS-2B cells (Fig. 7A). The upregulation of Fas expression seemed to have functional relevance. When BEAS-2B cells were treated without prior stimulation by poly I:C, the agonistic Fas antibody CH-11 did not have any effect on cell survival. However, when BEAS-2B cells were stimulated by CH-11 after incubation with poly I:C for 24 h, the Fas-induced decrease in the number of live cells and the increase in the number of apoptotic cells were significantly upregulated (indicated at 48 h). These findings suggested that the upregulation of Fas expression by poly I:C led to upregulation of Fas-induced cell death and had a functional significance.

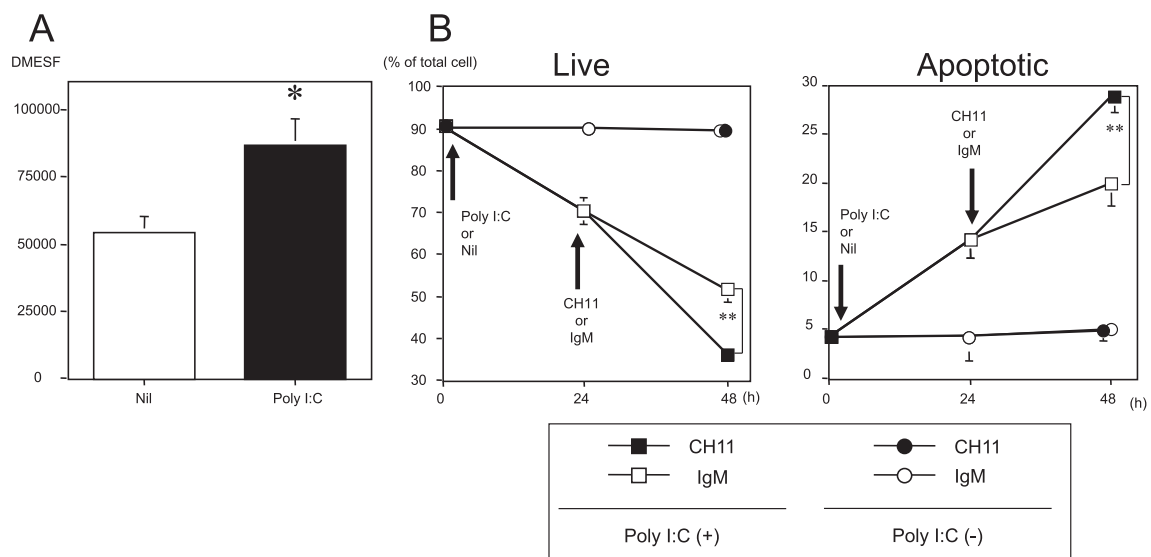


Fig. 7. Effect of poly I:C on the expression and function of Fas. (A) Fas expression on BEAS-2B cells. BEAS-2B cells were incubated with 10 μ g/mL of poly I:C for 72 h, and surface expression of Fas was determined by flow cytometry ($n = 5$). The level of surface expression was calculated using the following formula: Δ MESF = (MESF with anti-CD95 mAb) – (MESF with isotype control IgG1). * $p < 0.05$ vs. the value of Nil. (B) Effect of poly I:C on FasL-induced cell death in BEAS-2B cells. After incubation with poly I:C (10 μ g/mL) for 24 h, the BEAS-2B cells were additionally stimulated with agonistic Fas Ab CH-11 (1 μ g/mL) or control IgM for 24 h (a total of 48 h). ** $p < 0.01$ vs. control IgM ($n = 5$).

Discussion

In this study, among various TLR ligands, only the TLR3 ligand poly I:C specifically and exclusively reduced bronchial epithelial cell survival in a caspase-dependent manner (Fig. 2, 5) and also functionally upregulated the expression of Fas (Fig. 7).

Although the effect of the TLR3 ligand poly I:C on epithelial apoptosis has been reported,^{16,17} no effects of other TLR ligands, including TLR2, TLR5, TLR7/8, and TLR9, have been reported to date. As virus components, dsRNA, ssRNA, and the RSV fusion protein activate TLR3, TLR7/8, and TLR4, respectively.⁶ As for bacterial components, *Pseudomonas aeruginosa* exotoxin A induces epithelial cell death,¹⁸ but the effect of a TLR5 or TLR9 ligand has remained unclear so far. In this study, we showed that the TLR3 ligand poly I:C selectively and strongly induced bronchial epithelial cell apoptosis (Fig. 2). Although live RSV³ or influenza virus⁴ induces bronchial epithelial cell death, our study suggested that dsRNA/TLR3 exclusively mediates viral infection-induced death of bronchial epithelial cells among various TLRs.

Importantly, the time courses of chemokine generation and cell death were different (Fig. 3). In previous studies, time course experiments were performed for up to 24–48 h to examine stimulation of cytokine production,^{19,20} but the exact time course of poly I:C-induced epithelial apoptosis has been unclear. In this study, we simultaneously analyzed both time courses and showed that the CXCL10 and CXCL8 concentrations in the supernatant reached a plateau after 24 and 48 h, respectively (Fig. 3). In line with our observations, previous studies have shown that poly I:C upregulates CXCL8 and CCL5 mRNA expression at 4 and 24 h, respectively, in BEAS-2B and that levels of both mRNAs rapidly return to baseline.^{19,21} In contrast, the time course of cell death was slower as shown in Figure 3B. Those results suggest that the increased concentrations of chemokines we observed here were primarily generated by live cells. Thus, the response of bronchial epithelial cells to poly I:C seemed to have two phases. In the early phase, cells rapidly secrete chemokines and may attract inflammatory cells to combat invading viruses, and in the later phase, infected bronchial epithelial cells undergo apoptosis and may inhibit dissemination of viruses or excess inflammation.

Individual TLRs trigger specific biological responses based on different Toll/interleukin-1 receptor (TIR) domain-containing adaptor molecules, including MyD88, TIRAP, TRAM, and TRIF.⁵ Among these adaptor molecules, TRIF is the sole adaptor of TLR3, and it is unique for TRIF signaling that it interacts with Fas-associated protein with death domain (FADD) and activates procaspase-8 to initiate cell apoptosis. TRIF-induced apoptosis pathways and the cytokine generation pathway via IFN regulatory factor (IRF) or nuclear factor kappa B (NF- κ B) activation are uncoupled,²² which might partly explain the different time courses of chemokine generation and apoptosis in the current study (Fig. 3).

There are two main pathways that lead to apoptosis: a death receptor (such as FADD)-mediated extrinsic pathway initiated by caspase-8 and a mitochondrial-dependent intrinsic pathway initiated by caspase-9; both pathways lead to activation of caspase-3, -6, and -7. In bronchial epithelial cells, poly I:C has been reported to activate only caspase-8, and not caspase-9,¹⁶ suggesting that an extrinsic pathway works in this process. Although the functional effects of caspase-8 (Z-IETD-fmk) and pan-caspase (caspases-1, -3, -6, and -7; Z-VAD-fmk) inhibitors on poly I:C-induced bronchial epithelial cell death have not been reported, these inhibitors significantly suppressed poly I:C-induced cell death in the current study (Fig. 5). This finding is in line with a previous report showing that these inhibitors inhibit influenza A virus-induced apoptosis in NCI-H292 cells⁴ and suggests that an extrinsic pathway is also involved in dsRNA-induced cell death.

Fas is a transmembrane protein, and the activation of Fas induces apoptosis. Influenza virus upregulates the expression of Fas in HeLa cells,²³ and poly I:C functionally upregulated the Fas expression in murine macrophages.²⁴ Although human bronchial epithelial cells express Fas²⁵ and RSV infection increases the expression of Fas in A549 epithelial cells,⁷ the effect of dsRNA on Fas expression in bronchial epithelial cells has not been reported previously. This study is the first to report that poly I:C could upregulate Fas expression and functionally increased the cell sensitivity to FasL. The upregulation of Fas expression might lead to increased apoptosis by FasL, which is expressed in various inflammatory cells, including activated T cells, natural killer cells, or neutrophils. This process might limit virus replication and prevent persistent infection and the spread of viruses. On the other hand, Fas-induced cell death may worsen preexisting inflammatory disorders.

In pulmonary fibrosis, the blockade of Fas seems to be protective for fibrosis, as indicated by the facts that Fas-deficient *lpr* mice are resistant to fibrosis²⁶ and inhibition of the Fas pathway by z-VAD-fmk reduced bleomycin-induced fibrosis.²⁷ These findings suggested that Fas activation might lead to the deterioration of fibrosis. In asthmatic airways, the Fas/FasL system seems to limit airway inflammation under stable conditions. Administration of agonistic anti Fas mAb to ovalbumin-immunized mice induced eosinophil apoptosis and significantly alleviated airway hyperresponsiveness.²⁸ However, the *in vivo* role of dsRNA-induced upregulation of epithelial Fas in virally infected asthma has not been investigated; therefore, further investigation is required in the future.

In conclusion, we determined that among various TLR ligands, a TLR3 ligand specifically and exclusively induced chemokine generation and cell death. The previous findings that viruses induced caspase-dependent cell death or upregulation of Fas expression were reproduced only with dsRNA stimulation, suggesting the central role for dsRNA/TLR3 interactions in virus-induced apoptosis. In healthy conditions, dsRNA/TLR3-induced apoptosis or an increase in Fas expression during viral infection may play a host-defensive role, but may also have a detrimental effect on pre-existing airway damage. In this view, the dsRNA/TLR3 pathway may be a novel treatment target of virus-induced exacerbation of airway inflammatory diseases, especially asthma.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

YK and HN designed the study, conducted experiments, interpreted the results, and wrote a manuscript. TN developed CXCL10 ELISA and interpreted the results. MK provided surgical specimens. KO designed the study and interpreted the results. All the authors approved final version of the manuscript.

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