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TLR3 Activation Augments Matrix Metalloproteinase Production through Reactive Nitrogen Species Generation in Human Lung Fibroblasts

Tomohiro Ichikawa,* Hisatoshi Sugiura, † Akira Koarai, † Yoshiaki Minakata,* Takashi Kikuchi,* Yukiko Morishita,* Asako Oka,* Kuninobu Kanai,* Hiroki Kawabata,* Masataka Hiramatsu,* Keichiro Akamatsu,* Tsunahiko Hirano,* Masanori Nakanishi,* Kazuto Matsunaga,* Nobuyuki Yamamoto,* and Masakazu Ichinose ‡

Viral infection often triggers asthma exacerbation and contributes to airway remodeling. Cell signaling in viral infection is mainly mediated through TLR3. Many mediators are involved in airway remodeling, but matrix metalloproteinases (MMPs) are key players in this process in asthma. However, the role of TLR3 activation in production of MMPs is unknown. In this study, we examined the effects of polyinosinic-polycytidylic acid [poly(I:C)], a ligand for TLR3, on production of MMPs in human lung fibroblasts, with a focus on nitrosative stress in TLR3 modulation of MMP production. After lung fibroblasts were treated with poly(I:C), production of MMP-1, -2, and -9 and inducible NO synthase (iNOS) was assessed. The roles of NF-κB and IFN regulatory factor-3 (IRF-3) in the poly(I:C)-mediated production of MMPs and the responsiveness to poly(I:C) of normal lung fibroblasts and asthmatic lung fibroblasts were also investigated. Poly(I:C) augmented production of MMPs and iNOS in fibroblasts, and an iNOS inhibitor diminished this production of MMPs. Poly(I:C) stimulated translocation of NF-κB and IRF-3 into the nucleus in fibroblasts and inhibition of NF-κB or IRF-3 abrogated the poly(I:C)-induced increase in both iNOS expression and release of MMPs. Poly(I:C)-induced production of iNOS and MMPs was greater in asthmatic fibroblasts than in normal fibroblasts. We conclude that viral infection may induce nitrosative stress and subsequent MMP production via NF-κB– and IRF-3–dependent pathways, thus potentiating viral-induced airway remodeling in asthmatic airways. The Journal of Immunology, 2014, 192: 4977–4988.

*Third Department of Internal Medicine, Wakayama Medical University, School of Medicine, Wakayama 641-8509, Japan; and † Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan

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Address correspondence and reprint requests to Tomohiro Ichikawa, Third Department of Internal Medicine, Wakayama Medical University, School of Medicine, 811-1 Kimiidera, Wakayama 641-8509, Japan. E-mail address: ikawa@wakayama-med.ac.jp

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Abbreviations used in this article: CAPE, caffeic acid phenethyl ester; ECM, extracellular matrix; iNOS, inducible NO synthase; HFL-1, human fetal lung fibroblast-1; IRF-3, IFN regulatory factor-3; MMP, matrix metalloproteinase; MnTBAP, manganese (III)tetrakis(4-benzoic acid)porphyrin chloride; NHLF, normal adult human lung fibroblast; poly(I:C), polyinosinic-polycytidylic acid; RNS, reactive nitrogen species; SF-DMEEM, serum-free DMEEM; siRNA, small interfering RNA; TIMP, tissue inhibitor of metalloproteinases; 1400W, N-[3-(3-aminoethyl)benzyl] acetamide.

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The parenchymal cells of the airway, including epithelial cells, airway smooth muscle cells, and lung fibroblasts, are responsible for maintenance of the airway structure. Viral infection of airway epithelial cells is an important trigger of exacerbations (7), and viruses gain access to underlying lung fibroblasts through the epithelial disruption that occurs in asthmatic airways (8). Viral infection of these cells promotes airway remodeling characterized by airway smooth muscle hypertrophy, goblet cell hyperplasia, and subepithelial fibrosis (9–11), which are the pathological features of asthmatic airways with chronic airflow limitation (12). Differentiation of lung fibroblasts into myofibroblasts is a particularly important process in airway remodeling (13–15), and the differentiated myofibroblasts play a pivotal role in excess deposition of extracellular matrix (ECM) proteins, including collagen I, III, and V, fibronectin, and tenasin in the lamina reticularis of the basement membrane (16, 17). Recently, we showed that activation of TLR3 stimulates the transformation of lung fibroblasts into myofibroblasts, resulting in hyperproduction of ECM proteins through the NF-κB–TGF-β axis (18). These findings suggest that dsRNA could be involved in fibroblast-mediated airway remodeling of asthma during viral infection.

Airway architectural alterations may result from tissue damage by proteases or toxic molecules released by inflammatory cells and resident lung cells. Matrix metalloproteinases (MMPs) degrade ECM proteins, participate in ECM turnover, and promote subsequent structural changes of the airways, especially in patients with refractory asthma (19). Among several MMPs detectable in asthmatic airways (20, 21), MMP-1, -2, and -9 are the key regulators of collagens and have been related to the pathophysiology of asthma in vitro and in vivo studies (22–24). Fibroblasts are...
thought to be the main producers of MMP-1 (25) and also produce MMP-2 and MMP-9 (26). However, it is unclear whether dsRNA stimulates release of MMPs in human lung fibroblasts and which signal transduction pathways are involved in fibroblast-mediated secretion of MMPs.

Excessive production of NO by inflammatory and immune responses leads to the formation of reactive nitrogen species (RNS). In inflammatory states that generate superoxide anions, NO derived from inducible NO synthase (iNOS) is rapidly consumed by reaction with superoxide to produce peroxynitrite, a potent RNS (27). Peroxynitrite causes tissue injury, lipid peroxidation, and nitration of tyrosine residues (28), as well as induction of proliferative changes in human lung fibroblasts (29). We have shown that overproduction of 3-nitrotyrosine, a footprint of RNS production, occurs in airways of patients with refractory asthma (30). Exposure to RNS also activates MMPs in human neutrophils (31) and lung fibroblasts (32), but the role of endogenous RNS production in dsRNA-stimulated secretion of MMPs in human lung fibroblasts is unknown.

The present study was designed to: determine if dsRNA enhances release of MMPs, including MMP-1, -2, and -9; examine the effects of dsRNA on expression of iNOS and RNS release; identify the signal transduction pathways that modulate release of MMPs through activation of TLR3; and determine if the response to dsRNA-induced secretion of MMPs differs between normal and asthmatic fibroblasts.

Materials and Methods

Materials

The following reagents were used in the study: manganese(III)tetraakis (4-benzoic acid)porphyrin chloride (MnTBAP), a specific peroxynitrite scavenger; caffeic acid phenethyl ester (CAPE), a specific NF-κB inhibitor; and polynicotinoyl-polyolycylic acid [poly(I)C] (all from Calbiochem, La Jolla, CA); mouse monoclonal anti-β-actin Ab, N-[3-aminomethyl]benzyl) acetamidine (1400W), recombinant active MMP-1, β-casein, anti-mouse IgG Ab, and anti-rabbit IgG Ab (all from Sigma-Aldrich, St. Louis, MO); mouse monoclonal anti-TLR3 Ab (Imgenex, San Diego, CA); FITC-conjugated rabbit polyclonal anti-p–IRF-3 Ab (Bioss, Woburn, MA); rabbit monoclonal anti-p–IRF-3 Ab (Abcam, Cambridge, MA); DAPI (Southern Biotechnology Associates, Birmingham, AL); mouse monoclonal anti-NF-κB p65 Ab, rabbit polyclonal anti-IRF-3 Ab (Abcam, Cambridge, MA); DAPI (Southern Biotechnology Associates, Birmingham, AL); mouse monoclonal anti-NF-κB p65 Ab, rabbit polyclonal anti-iNOS Ab, mouse monoclonal anti-nitrotyrosine Ab, and mouse monoclonal anti-lamin A/C Ab (all from Santa Cruz Biotechnology, Santa Cruz, CA); gelatin (Nacala Tasque, Kyoto, Japan); DMEM, FCS, and antibiotic-antimycotic (all from Invitrogen Life Technologies, Grand Island, NY); and recombinant latent MMP-1 and neutralizing Abs to IFN-β and IL-6 (all from R&D Systems, Minneapolis, MN).

Cell culture

Human fetal lung fibroblast-1 (HFL-1) and U2-OS cells were obtained from the American Type Culture Collection (Rockville, MD). Normal adult human lung fibroblasts (NHLFs) and four strains of human lung fibroblasts from patients with asthma (DHHLF-As: from three females and one male age 18–45) were purchased from Takara Bio (Shiga, Japan). Detailed clinical data for these patients were not provided. HFL-1 cells were originally obtained from primary cultures of the lung of a Caucasian fetus. The NHLFs and DHHLF-As are also primary cells. These cells were nonimmortalized cells, and the effects of transfection could be ignored. Cells were cultured with DMEM supplemented with 10% FCS, 100 μg/ml penicillin, 250 μg/ml streptomycin, and 2.5 μg/ml fungazone at 37°C in a humidified atmosphere of 5% CO₂ and passaged. HFL-1 cells were used between the 14th and 18th passage. NHLFs and DHHLF-As were used between the third and seventh passages. To evaluate mediator production in a monolayer culture, cells were seeded in six-well tissue-culture plates at a cell density of 1 × 10⁵/ml. At 90% confluence, cells were treated with various concentrations of poly(I:C) in serum-free DMEM (SF-DMEM). To determine the effect of a neutralizing anti–IFN-β Ab or a neutralizing anti–IL-6 Ab on poly(I:C)-mediated mediator release, anti–IFN-β Ab (5 μg/ml) or anti–IL-6 Ab (10 μg/ml) was also added to the media 30 min before treatment with poly(I:C). The supernatants and cells were harvested after 48 h of treatment with poly(I:C) and stored at −80°C until assayed.

Immunohistochemical localization of TLR3, NF-κB p65, and p–IRF-3

HFL-1 cells were seeded in four-well chamber slides at a density of 1 × 10⁵/ml and cultured for 24 h, and then the media were replaced with SF-DMEM for 24 h. The cells were incubated with 30 μg/ml poly(I:C) for staining of NF-κB p65 and p–IRF-3 for up to 120 min. After washing, the slides were fixed with freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature and then blocked with blocking reagent (DakoCyto- mation, Kyoto, Japan) for 1 h at room temperature and rinsed. The cells were incubated with mouse monoclonal anti-TLR3 Ab (1:100 dilution; Imgenex) or mouse IgG as a negative control for detection of TLR3. The cells were also incubated with mouse monoclonal anti–NF-κB p65 Ab (1:100 dilution; Sigma-Aldrich) or FITC-conjugated rabbit polyclonal anti-p–IRF-3 Ab (1:100 dilution; Bioss) at 4°C overnight. Cells incubated with anti-TLR3 Ab or anti–NF-κB p65 Ab were further treated with the appropriate FITC-conjugated secondary Abs (1:1,000 dilution; Sigma-Aldrich) for 60 min at room temperature. Nuclei were stained with DAPI. Cells were viewed by epifluorescence microscopy (E-800; Nikon, Tokyo, Japan) and photographed with a digital camera (DMX-1200C; Nikon) at ×400 original magnification.

Western blotting

Cells were seeded in six-well dishes at a density of 1 × 10⁵/ml. At 90% confluence, the SF-DMEM was replaced with SF-DMEM for 24 h. Cells were then treated with various concentrations of poly(I:C) in the presence or absence of CAPE, MnTBAP, 1400W, neutralizing anti–IFN-β Ab, neutralizing anti–IL-6 Ab, and control IgG for 48 h. The cells were washed with ice-cold PBS and homogenized in cell lysis buffer. For evaluation of the effect of poly(I:C) on nuclear translocation of NF-κB and IRF-3, cells were treated with poly(I:C) for up to 120 min, and the nuclear and cytosolic fractions were obtained using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA). Equal amounts of protein were loaded and separated by electrophoresis on 12.5% SDS polyacrylamide gels. After electrophoresis, the separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The following Abs were used for detection of the target proteins: mouse monoclonal anti-TLR3 Ab (2 μg/ml), rabbit polyclonal anti-p–IRF-3 Ab (1:200 dilution; Sigma-Aldrich), mouse monoclonal anti-nitrotyrosine Ab (1:200 dilution; Santa Cruz Biotechnology), mouse monoclonal anti-β-actin Ab (1:10,000 dilution; Sigma-Aldrich), mouse monoclonal anti–NF-κB p65 Ab (1:200 dilution; Santa Cruz Biotechnology), rabbit polyclonal anti–IRF-3 Ab (1:200 dilution; Santa Cruz Biotechnology), rabbit monoclonal anti-p–IRF-3 Ab (1:1,000 dilution; Abcam), and mouse monoclonal anti-lamin A/C Ab (1:400 dilution; Santa Cruz Biotechnology). Bond Abs were visualized using the appropriate peroxidase-conjugated secondary Abs and ECL (Amersham Biosciences, Buckinghamshire, U.K.) with a chemiluminescence imaging system (Luminocapture AE9655; Atto, Tokyo, Japan). Band intensity was quantified by densitometry (ImageJ; National Institutes of Health, Frederick, MD). Band intensities of TLR3, iNOS, nitrotyrosine, and IRF-3 were standardized with that of β-actin. Similarly, band intensities of NF-κB p65 and p–IRF-3 were standardized with that of lamin A/C.

Silecing of TLR3 and IRF-3

HFL-1 cells were plated in six-well dishes at an initial concentration of 5.0 × 10⁴ cells/well in 10% FCS DMEM without antibiotics. After 24 h, transfection with small interfering RNA (siRNA) was performed. In one tube, 9 μl Lipofectamine RNAiMax (Invitrogen Life Technologies) was diluted with 150 μl Opti-MEM medium (Invitrogen Life Technologies). In another tube, 240 pmol siRNA for nontargeting TLR3 or IRF-3 (siGENOME SMARTpool; Dharmacon, Lafayette, CO) was diluted with 150 μl Opti-MEM medium. Then, both solutions were mixed and incubated for 5 min at room temperature. After incubation, 250 μl combined solution (containing 200 pmol siRNA) was added to each well, and the cells were incubated at 37°C for 24 h. The infected cells were used for investigation of poly(I:C)-mediated release of MMPs, iNOS expression, and IFN-β release.

Gelatin and casein zymography

Media from monolayer cultures (500 μl per culture condition) were concentrated 10-fold by precipitation with cold ethanol and resuspended in 50 μl double-distilled H₂O. A positive control for MMP-2 and MMP-9 was provided by mixing the serum-free culture medium from HFL-1 cells with an equal volume of the serum-free culture medium from U2-OS cells. The samples were solubilized in SDS-PAGE sample buffer without 2-ME. Equal amounts of samples (20–40 μl) were separated in 10% SDS-PAGE containing gelatin (1 mg/ml) (for detection of MMP-9 and MMP-2) or casein...
(1 mg/ml) for detection of MMP-1) under nonreducing conditions. After electrophoresis, gels were soaked in zymogram renaturing buffer (Invitrogen, Carlsbad, CA) for 60 min and incubated in zymogram developing buffer (Invitrogen) for 16 h at 37˚C. The gels were stained with 0.4% Coomassie blue (Nacalai Tesque) for 15 min at room temperature and rapidly destained with destaining buffer (30% methanol and 10% acetic acid). Zones of proteolysis appeared as clear white bands against a blue background and were scanned by Light-Capture II (Atto). Band intensity was quantified using ImageJ software (National Institutes of Health).

Measurement of NO

To evaluate NO production in the monolayer culture, cells were seeded in six-well tissue-culture plates at a density of $1 \times 10^5$ cells/ml. Cells were treated with various concentrations of poly(I:C) or without poly(I:C) for 48 h, and the supernatant was harvested. The concentration of NO was determined using an NO assay kit (BioAssay Systems, Hayward, CA). NO released by NO synthase is oxidized to nitrite and nitrate in aqueous solution. This kit is designed to measure NO production following reduction of nitrate to nitrite using the Griess method.

ELISA

The concentrations of IFN-β in culture media were determined using an ELISA kit (Thermo Scientific, Rockford, IL).

Ab array

Expression levels of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 and of inflammatory cytokines such as IL-6 and IL-8 in culture...
media were determined using a Human Matrix Metalloproteinase Ab Array and Human Inflammation Ab Array 3 (Ray Biotech, Norcross, GA), respectively, and quantified by densitometry. The relative intensity was calculated by dividing each spot intensity of TIMP-1, TIMP-2, IL-6, IL-8, and RANTES by the average spot intensity of the positive controls in each membrane.

**FIGURE 2.** Poly(I:C) augments expression of iNOS, NO, and nitrotyrosine. HFL-1 cells were treated with various concentrations of poly(I:C) (filled bars) or vehicle (open bars) for 48 h. (A) Expression of iNOS in the cells was analyzed by immunoblotting \((n=4)\). (B) NO production in the media was assayed by the Griess method \((n=4)\). (C) Formation of nitrotyrosine in the cells was analyzed by immunoblotting \((n=4)\). \(*^* p < 0.01, \**^* p < 0.001\) versus controls.

**FIGURE 3.** Inhibitory effect of 1400W on poly(I:C)-augmented release of MMPs. HFL-1 cells were treated with various concentration of 1400W in the presence (filled bars) or absence (open bars) of 30 \(\mu\)g/ml poly(I:C) for 48 h. MMP-9 (A and B), -2 (A and C), and -1 (D) in the media were analyzed by zymography \((n=4)\). \(*^* p < 0.01, \**^* p < 0.001\) versus poly(I:C)-untreated controls, \(^* p < 0.05, \**^* p < 0.01, \***^* p < 0.001\) versus vehicle-pretreated poly(I:C)-treated controls. M, markers of MMPs.
Statistical analysis

Data are expressed as the mean ± SEM. Significance was tested by one-way ANOVA followed by post hoc analysis with a Tukey-Kramer test for multiple comparison or with a Dunnett test for multiple comparison versus a control, as appropriate. An unpaired two-tailed Student t test was used for single comparisons. The p values <0.05 were considered significant.

Results

Effect of poly(I:C) on release of MMPs and TIMPs in HFL-1 cells

To determine whether poly(I:C) augments production of MMPs and TIMPs in human lung fibroblasts, HFL-1 cells were incubated with various concentrations of poly(I:C) for 48 h and release of MMPs and TIMPs in the culture media was assessed by zymography and Ab array, respectively. Poly(I:C) significantly augmented release of the latent and active forms of MMP-9 (Fig. 1A, 1B) and MMP-2 (Fig. 1A, 1C) in a concentration-dependent manner compared with controls. Poly(I:C) also significantly augmented release of the latent form of MMP-1 (Fig. 1D), but the active form of MMP-1 was not detected (Fig. 1D). Poly(I:C) (30 μg/ml) had no effect on release of TIMP-1 (Fig. 1E), but significantly reduced TIMP-2 release (Fig. 1E).

Poly(I:C) induces nitrosative stress in lung fibroblasts

Because exogenously administered RNS such as peroxynitrite stimulate release of MMPs (32), we investigated the effect of poly(I:C) on iNOS expression, NO release, and nitrotyrosine formation in HFL-1 cells. Poly(I:C) significantly induced iNOS expression in a concentration-dependent manner (Fig. 2A), and also significantly augmented release of NO (Fig. 2B) and nitrotyrosine formation (Fig. 2C). To examine whether the poly(I:C)-augmented MMPs release and iNOS expression were regulated through TLR3 signaling, we investigated the effects of siRNA on these changes. HFL-1 cells expressed TLR3, as shown by immunocytochemistry (Supplemental Fig. 1A) and Western blotting (Supplemental Fig. 1B). TLR3 siRNA suppressed the expression of TLR3 compared with the nontargeting siRNA-treated group (Supplemental Fig. 1B). TLR3 siRNA significantly inhibited the poly(I:C)-augmented...
release of both MMP-9 and MMP-2 (Supplemental Fig. 1C–E) as well as MMP-1 (Supplemental Fig. 1F). Silencing of TLR3 significantly suppressed poly(I:C)-augmented iNOS expression (Supplemental Fig. 1G).

To examine whether poly(I:C) has the same effects in adult lung fibroblasts, NHLFs were incubated with various concentrations of poly(I:C) for 48 h. Poly(I:C) significantly augmented release of the latent and active forms of MMP-9 and MMP-2 (Supplemental Fig. 2A–C), and release of latent-MMP-1 (Supplemental Fig. 2D) in a concentration-dependent manner. Poly(I:C) (30 μg/ml) had no significant effect on the levels of TIMP-1 and TIMP-2 (Supplemental Fig. 2E). iNOS expression (Supplemental Fig. 3A), NO production (Supplemental Fig. 3B), and nitrotyrosine formation (Supplemental Fig. 3C) were significantly augmented by treatment with poly(I:C).

Effect of a selective iNOS inhibitor, 1400W, on poly(I:C)-augmented MMP release

Poly(I:C) clearly induced iNOS expression in human lung fibroblasts, but it was unclear whether endogenously produced NO is involved in the poly(I:C)-mediated release of MMPs. To clarify this issue, we examined the effect of 1400W, a highly selective iNOS inhibitor (33), on poly(I:C)-augmented release of MMPs in HFL-1 cells. 1400W significantly inhibited poly(I:C)-augmented release of MMP-9 (Fig. 3A, 3B), MMP-2 (Fig. 3A, 3C), and MMP-1 (Fig. 3D) in a concentration-dependent manner.

FIGURE 5. Poly(I:C) augments nuclear translocation of NF-κB p65, and CAPE inhibits poly(I:C)-augmented release of MMPs and iNOS expression. HFL-1 cells were treated with 30 μg/ml poly(I:C) for 0, 30, 60, 90, and 120 min, and the nuclear fraction was obtained. Nuclear translocation of NF-κB p65 was evaluated by immunoblotting (A) (n = 4) and immunocytochemistry (B). ***p < 0.001 versus controls. Cells were treated with various concentrations of CAPE in the presence (filled bars) or absence (open bars) of 30 μg/ml poly(I:C) for 48 h. MMP-9 (C and D), -2 (C and E), and -1 (F) in the media were analyzed by zymography (n = 4). (G) Expression of iNOS was analyzed by immunoblotting (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001 versus poly(I:C)-untreated controls, †p < 0.05, ††p < 0.01, †††p < 0.001 versus vehicle-pretreated poly(I:C)-treated controls. M, markers of MMPs.
Effect of a peroxynitrite scavenger, MnTBAP, on poly(I:C)-augmented MMP release

Peroxynitrite is a powerful oxidant formed from superoxide and NO and can cause nitrosative stress (34) and induce tissue remodeling (29, 35). To examine if poly(I:C)-augmented release of MMPs is mediated through a peroxynitrite-dependent pathway, we investigated the effect of MnTBAP, a specific peroxynitrite scavenger (36), on release of MMPs in HFL-1 cells. MnTBAP significantly inhibited poly(I:C)-augmented nitrotyrosine formation (Fig. 4A) and completely recovered the poly(I:C)-augmented

FIGURE 6. Poly(I:C) augments nuclear translocation of p–IRF-3, and IRF-3 silencing by siRNA inhibits poly(I:C)-augmented release of MMPs and iNOS expression. HFL-1 cells were treated with 30 μg/ml poly(I:C) for 0, 30, 60, 90, and 120 min, and the nuclear fraction was obtained. Nuclear translocation of p–IRF-3 was evaluated by immunoblotting (A) (n = 4) and immunocytochemistry (B). **p < 0.01, ***p < 0.001 versus controls. (C) Expression of IRF-3 in HFL-1 cells was measured by immunoblotting after silencing with siRNA (n = 4). ***p < 0.001 versus nontargeting siRNA. After silencing, the cells were incubated with 30 μg/ml poly(I:C) (filled bars) or vehicle (open bars) for 48 h. MMP-9 (D and E), -2 (D and F), and -1 (G) in the media were analyzed by zymography (n = 4). (H) Expression of iNOS was analyzed by immunoblotting (n = 4). *p < 0.05, **p < 0.01 versus poly(I:C)-untreated controls, †p < 0.05, ††p < 0.01 versus nontargeting siRNA-pretreated poly(I:C)-treated controls.
release of MMP-9 (Fig. 4B, 4C), MMP-2 (Fig. 4B, 4D), and the latent form of MMP-1 (Fig. 4E).

**Signal transduction in poly(I:C)-augmented MMP release and iNOS expression**

Because signal transduction of TLR3 activation is mediated through NF-κB and IRF-3, we investigated the roles of NF-κB and IRF-3 in fibroblast-mediated release of MMPs and iNOS expression in HFL-1 cells. After cells were treated with poly(I:C) for the indicated times in Fig. 5A (0–120 min), marked NF-κB p65 translocation into the nucleus was observed by Western blotting (Fig. 5A). This translocation was confirmed by immunocytochemistry (Fig. 5B). To examine whether NF-κB–related signaling is involved in the poly(I:C)-mediated responses, the effects of...
CAPE, a specific NF-κB inhibitor (37), on release of MMPs and iNOS expression were evaluated. CAPE significantly abrogated the poly(I:C)-induced increases in MMP-9 and MMP-2 (Fig. 5C–E) and MMP-1 (Fig. 5F) in a concentration-dependent manner. CAPE also significantly abrogated the poly(I:C)-induced increase in iNOS expression (Fig. 5G).

As expected, translocation of p–IRF-3 into the nucleus after treatment with poly(I:C) was observed by Western blotting (Fig. 6A) and confirmed by immunocytochemistry (Fig. 6B). To investigate whether IRF-3 is involved in the fibroblast-mediated release of MMPs and iNOS expression, the effects of IRF-3 silencing on the poly(I:C)-augmented responses were evaluated. siRNA against IRF-3 diminished expression of IRF-3 in the cells (Fig. 6C). The siRNA significantly abrogated the poly(I:C)-induced increases in MMP-9 (Fig. 6D, 6E), MMP-2 (Fig. 6D, 6F), and MMP-1 (Fig. 6G). Silencing of the IRF-3 gene also abrogated the poly(I:C)-induced increase in iNOS expression (Fig. 6H).

Because NF-κB and IRF-3 regulate IFN-β expression, we examined whether endogenous IFN-β contributes to the poly(I:C)-mediated release of MMPs in HFL-1 cells. Poly(I:C) enhanced IFN-β production and both CAPE and siRNA against IRF-3 significantly inhibited the poly(I:C)-enhanced IFN-β release (Fig. 7A, 7B). A neutralizing anti–IFN-β Ab significantly reduced the poly(I:C)-augmented release of MMP-9 and MMP-2 (Fig. 7C–E), MMP-1 (Fig. 7F), and iNOS expression (Fig. 7G).

**FIGURE 8.** A neutralizing anti–IL-6 Ab has no significant effect on poly(I:C)-augmented release of MMPs and iNOS expression. HFL-1 cells were treated with 30 μg/ml poly(I:C) (filled bars) or vehicle (open bars) for 48 h. Levels of cytokines in the media were assayed by cytokine array (A and B) (n = 4). HFL-1 cells were treated with 30 μg/ml poly(I:C) (filled bars) or vehicle (open bars) for 48 h in the presence of a neutralizing anti–IL-6 Ab or control IgG. MMP-9 (C and D), -2 (C and E), and -1 (F) in the media were analyzed by zymography (n = 4). (G) Expression of iNOS was analyzed by immunoblotting (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001 versus poly(I:C)-untreated controls.
Poly(I:C) upregulates various proinflammatory cytokines and these mediators could cause tissue remodeling. Therefore, we further investigated the poly(I:C)-induced profiles of proinflammatory cytokines and chemokines in HFL-1 cells using cytokine and chemokine arrays. Poly(I:C) significantly augmented the levels of IL-6, IL-8, and RANTES compared with controls (Fig. 8A, 8B), whereas TIMP-2 was significantly reduced (Fig. 8A, 8B). Because expression of IL-6 was the most prominent among these cytokines, we examined the effects of IL-6 on the poly(I:C)-augmented release of MMPs and iNOS expression. A neutralizing anti–IL-6 Ab had no effects on release of MMPs or iNOS expression (Fig. 8C–G).

**Enhancement of poly(I:C)-induced responses in asthmatic human lung fibroblasts (DHLF-As)**

To determine whether poly(I:C)-mediated release of MMPs and nitrosative stress are enhanced in asthmatic airways, we compared the poly(I:C)-augmented release of MMPs and iNOS expression in primary lung fibroblasts from healthy subjects (NHLFs) with those in lung fibroblasts from asthmatic patients (DHLF-As). Poly(I:C) enhanced release of the latent and active forms of MMP-9 and MMP-2 (Fig. 9A–C) and of the latent form of MMP-1 (Fig. 9D) in both types of cells. However, the changes in the release of the latent and active forms of MMP-9 and active form of MMP-2 were significantly greater in DHLF-As than in NHLFs after stimulation with poly(I:C) (Fig. 9B, 9C). Expression of TLR3 in DHLF-As cells did not differ from that in NHLFs (Fig. 9E). The extent of iNOS induction was significantly greater in DHLF-As than in NHLFs (Fig. 9F).

**Discussion**

Our results show that TLR3 activation augments release of MMPs in lung fibroblasts through iNOS expression and subsequent RNS production. Poly(I:C) induced NF-κB and IRF-3 translocation into the nucleus, and inhibition of NF-κB or silencing of IRF-3 abrogated the poly(I:C)-induced increase in both iNOS expression and release of MMPs. In addition, poly(I:C) enhanced release of IFN-β through the NF-κB and IRF-3 pathways and endogenously produced IFN-β modulated the poly(I:C)-induced responses. Furthermore, these responses were potentiated in asthmatic lung fibroblasts compared with normal lung fibroblasts. These findings suggest that TLR3 signaling modulates production of MMPs through an IFN-β/RNS-dependent pathway in lung fibroblasts and that fibroblast-mediated release of MMPs in patients with asthma may be potentiated by excessive nitrosative stress during viral infection.

Viral infection, especially by rhinovirus and respiratory syncytial virus, causes exacerbation of asthma and is a major cause of prolonged worsening of disease (3, 8, 38). Rhinovirus detection in the lower airway tissue or severe exacerbation has also been associated with a decline in lung function in patients with asthma (39, 40). In an OVA-induced asthma rat model, intratracheal administration of poly(I:C) enhanced airway remodeling, including goblet hyperplasia and increased airway smooth muscle mass (41). These findings suggest that viral infection or TLR3 activation may promote architectural changes such as airway remodeling in asthmatic airways. The MMP-9/TIMP-1 ratio in the airways of patients with asthma is increased during exacerbation (42), and

![FIGURE 9](http://www.jimmunol.org/)

**FIGURE 9.** Responsiveness of NHLFs and DHLF-As to poly(I:C). NHLFs (normal) (n = 3) and DHLF-As (asthma) (n = 4) were incubated with 30 μg/ml poly(I:C) or vehicle for 48 h. MMP-9 (A and B), -2 (A and C) and -1 (D) were analyzed by zymography. Fold increase was calculated by dividing the band intensity of MMPs in the poly(I:C)-treated group by that in the vehicle-treated group. The fold increases in the MMPs from NHLFs are compared with those from DHLF-As (B–D). Expression of TLR3 (E) and iNOS (F) in the cells was analyzed by immunoblotting. *p < 0.05 versus NHLFs. M, markers of MMPs.
this is also associated with development of airway remodeling (43). Viral infection or TLR3 activation increases secretion of MMPs without elevation of TIMPs in airway epithelial cells (11, 44). Collectively, these findings and the results in the current study suggest that viral infection and TLR3 activation may cause disruption of the balance between MMPs and TIMPs and thus may promote airway remodeling.

Our study showed that RNS released by iNOS contributed to TLR3-mediated release of MMPs. Peroxynitrite, an RNS, has been shown to stimulate release of MMPs through the NF-κB–TGF-β axis in lung fibroblasts (32) and also activate pro-MMPs such as pro-MMP-8 and pro-MMP-9 by oxidizing cysteine residues in the active site of the enzymes (31, 45). Peroxynitrite also stimulates profibrotic responses, including myofibroblast differentiation and production of ECM proteins (29). These data suggest that TLR3 activation during viral infection may cause tissue remodeling through RNS production.

TLR3 signaling activates IRF3 and NF-κB via the adapter molecule Toll/IL-1R homologous domain-containing adapter protein inducing IFN-β (46). The coordinated nuclear translocation of NF-κB and IRF-3 during activation of TLR3 leads to upregulation of a set of primary response genes, including IFN-β (5, 47). In particular, IFN-β transcription begins with delivery of NF-κB to the IFN-β enhancer, followed by binding of ATF-2/c-Jun and IRF-3 in a highly cooperative manner upon viral infection (48). In addition to its antiviral effect, IFN-β induces expression of iNOS (49–51). These data suggest that IFN-β has the potential to aggravate ECM remodeling by amplifying RNS production.

In this study, lung fibroblasts from patients with asthma produced greater amounts of iNOS and MMPs than normal fibroblasts after poly(I:C) treatment. Airway fibroblasts from patients with asthma have also been shown to release more MMP-2 after IL-13 stimulation compared with fibroblasts from healthy subjects (52). Furthermore, rhinovirus infection augmented iNOS expression in a greater extent in asthmatic bronchial epithelial cells compared with normal cells (53). These findings are compatible with our results showing greater susceptibility to TLR3 in asthmatic fibroblasts. However, the mechanism of the susceptibility to poly(I:C) is unclear. To explore this mechanism, we examined the levels of TLR3 in the fibroblasts, but these levels did not differ between asthmatic and normal fibroblasts. This suggests that the mechanism of susceptibility may be independent of the amount of TLR3 expressed.

The limitations of the current study include the lack of in vivo data and gain-of-function experiments. Thus, the effect of TLR3 activation on iNOS expression and subsequent release of MMPs in human lungs remains unknown. MMP-9 secretion is increased in airways of mice infected with influenza virus through TLR3 signaling (54), and expression of MMP-9 in the lungs in an influenza pneumonia model was accompanied by nitrotyrosine formation (55). These data suggest that TLR3 activation might have modulated release of MMPs in these animal models. However, we do not have data showing that TLR3 activation is involved in RNS-mediated release of MMPs in vivo, and a further study is needed to confirm our in vitro findings.

In conclusion, this study shows that TLR3 activation causes nitrosative stress through an IFN-β– and iNOS-dependent pathway. This leads to excessive release of MMPs in lung fibroblasts, which may contribute to airway remodeling. We also found that susceptibility to dsRNA is greater in asthmatic lung fibroblasts than in normal lung fibroblasts. These findings may provide a possible mechanism for the pathogenesis of viral infection–induced airway remodeling.

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