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Original

Cross-talk between TLR3 and TNF-α or IFN-γ Signaling in Induction of CXCL8 / IL-8 and CXCL10 / IP-10 Expression in Airway Epithelial Cells

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Abstract : CXCL8 / IL-8 is a chemoattractant for neutrophils and mast cells, and regulates inflammatory cell recruitment in allergy, infection, and other neutrophil-related diseases. Interferon (IFN)-γ-inducible protein 10 (CXCL10 / IP-10) is a chemokine that attracts mononuclear cells, Th1 cells, and natural killer cells. We investigated the levels of CXCL8 / IL-8 and CXCL10 / IP-10 expression by airway epithelial cells after exposure to the inflammatory cytokines tumor necrosis factor (TNF)-α and IFN-γ, and to poly I : C, a synthetic analog of double-stranded RNA that is a ligand of Toll-like receptor 3 (TLR3). Poly I : C, TNF-α, IFN-γ, and combinations of poly I : C with TNF-α or IFN-γ were used to stimulate the airway epithelial cell line BEAS-2B. Following stimulation, we determined CXCL8 / IL-8 and CXCL10 / IP-10 mRNA levels by real-time PCR and protein levels by ELISA. Poly I : C treatment upregulated mRNA and protein expression for both CXCL8 / IL-8 and CXCL10 / IP-10. The addition of TNF-α, but not IFN-γ, to poly I : C further increased the expression of CXCL8 / IL-8 mRNA and protein. The addition of either TNF-α or IFN-γ to the poly I : C treatment further increased CXCL10 / IP-10 mRNA and protein expression. Cross-talk between TLR3 signaling and inflammatory cytokines regulates the expression of CXCL8 / IL-8 and CXCL10 / IP-10 in airway epithelial cells. From our results, TNF-α and IFN-γ produce different effects on TLR3 signaling.

Key words : bronchial asthma, COPD, TLR3, cytokine, chemokine

Introduction

A new strain of influenza virus, designated H1N1, also known as swine flu, emerged in Mexico in March 2009[1)]. Viral pathogens, including influenza, are becoming a more impor-

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tant issue in recent years and they are also important for exacerbation of bronchial asthma.

Exacerbation of bronchial asthma or chronic obstructive pulmonary disease (COPD) is usually caused by respiratory infections, commonly viruses such as influenza, respiratory syncytial virus, and human rhinovirus[2]. In recent years, other viruses have been detected in these patients, such as human metapneumovirus and human bocavirus[2-4]. Viral infection can cause direct injury to the airway epithelium, or can lead to infection of the epithelium, with subsequent production of inflammatory substances such as chemokines and cytokines[4]. Inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ, are found in bronchial lavage fluid from patients with bronchial asthma and COPD[5,6]. These cytokines induce several types of chemokines and may play an important role in exacerbation of bronchial asthma or COPD[5,6].

Toll-like receptor 3 (TLR3) is expressed in the cytoplasm of the cell and is the main receptor in the recognition of double-stranded RNA viruses by airway epithelial cells[7,8]. We have previously reported that dsRNA strongly induces the production of several types of chemokines, including CCL5 / RANTES, CXCL8 / IL-8, and CXCL10 / IP-10[9].

CXCL8 / IL-8 is an 8.5 kDa CXC chemokine that plays a major role in the initiation and maintenance of inflammatory responses in the lung via its potent chemoattractant effects on neutrophils. CXCL10 / IP-10 is a 10 kDa polypeptide which also belongs to the CXC chemokine subfamily and acts through the CXCR3 receptor. CXCL10 / IP-10, secreted by activated T lymphocytes, mononuclear cells, endothelial cells, and keratinocytes, exerts chemotactic activity toward human peripheral blood mononuclear cells and activated T lymphocytes.

In patients with severe asthma or COPD, there is a correlation between increased neutrophil numbers and CXCL8 / IL-8 levels[10]. A relationship between CXCL10 / IP-10 and severe asthma has also been reported[11,12].

Cross-talk between TLR and cytokine signaling needs further study, especially in chronic inflammatory airway diseases, which are commonly exacerbated by viral infections. The importance of viral infections in chronic respiratory disease is increasingly being recognized. In this study, we investigated the role of inflammatory cytokines and viral infections in bronchial epithelial cells. By determining titer and time course of inflammatory chemokines, we aimed to clarify the collaborating effects of poly I:C synthetic dsRNA and two inflammatory cytokines, TNF-α and IFN-γ, in airway epithelial cells.

Materials and Methods

Cell culture and reagents

BEAS-2B, a human airway epithelial cell line transformed with an adenovirus 12-SV40 virus hybrid, was purchased from the American Type 100 Culture Collection. BEAS-2B cells were cultured in DMEM / F12 with 10% FBS, 100 U / ml penicillin, and 100 ng / ml streptomycin (Invitrogen, Tokyo, Japan) at 37°C with 5% CO_2 in humidified air and were

treated as described previously[9]. We purchased poly I:C synthetic dsRNA from Sigma (Tokyo, Japan) and recombinant human TNF-α and IFN-γ from R&D Systems (Tokyo, Japan).

The BEAS-2B cells were seeded with 2 ml (concentration of 1.0×10^5 / ml) on 6-well plates until semi-confluent. Then the cells were exposed to either poly I : C (10 μg / ml), TNF-α (10 ng / ml), IFN-γ (50 ng / ml) or poly I : C with TNF-α or IFN-γ. Exposure times were between 4 to 48 hours. CXCL8 / IL-8 or CXCL10 / IP-10 mRNA expression and protein release to the media were assessed. The control subject was only exposed to medium.

Real-time PCR

We purified RNA and synthesized cDNA as described previously[9]. We purchased pre-designed TaqMan probe sets for CXCL8 / IL-8 mRNA and CXCL10 / IP-10 mRNA from Applied Biosystems (Tokyo, Japan). Each probe has a fluorescent reporter dye (FAM) linked to its 5' end and a downstream quencher dye (TAMRA) linked to its 3' end. We used a TaqMan ribosomal RNA probe, which is labeled with a fluorescent reporter dye (VIC), as an internal control. Each 25 μl reaction contained 2X Universal Master Mix (Applied Biosystems, Tokyo, Japan), primers, labeled probes and 50 ng cDNA. Amplification conditions consisted of 40 cycles at 95℃ for 15 seconds for denaturing and at 60℃ for 1 minute for annealing and extension after an initial incubation at 95℃ for 10 minutes. Amplification was performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), with fluorescence measurements at the end of each elongation step. The data are shown as fold induction over non-stimulated control cells.

Assay of CXCL8 / IL-8 and CXCL10 / IP-10 release into the culture medium

We collected cell culture media and determined CXCL8 / IL-8 and CXCL10 / IP-10 protein concentrations using a commercially-available enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, Tokyo, Japan), as described previously[9, 13]. The standards and samples were added to a 96-well microtiter plate coated with an anti-chemokine antibody. After incubation at room temperature for 2 hours, each well was washed five times with wash buffer. Biotinylated anti-chemokine antibodies and avidin-horseradish peroxidase conjugate were added to each well. After incubation at room temperature for 1 to 2 hours, each well was washed seven times with wash buffer of 0.05% Tween 20 (MP Biomedicals, Solon, OH). Substrate solution (stabilized hydrogen peroxide and tetramethylbenzidine) was added to each well and the plate was incubated at room temperature for 20 to 30 min. Sulfuric acid was then added to each well, and the absorbance was measured at 450 nm.

Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM). Statistical dif-

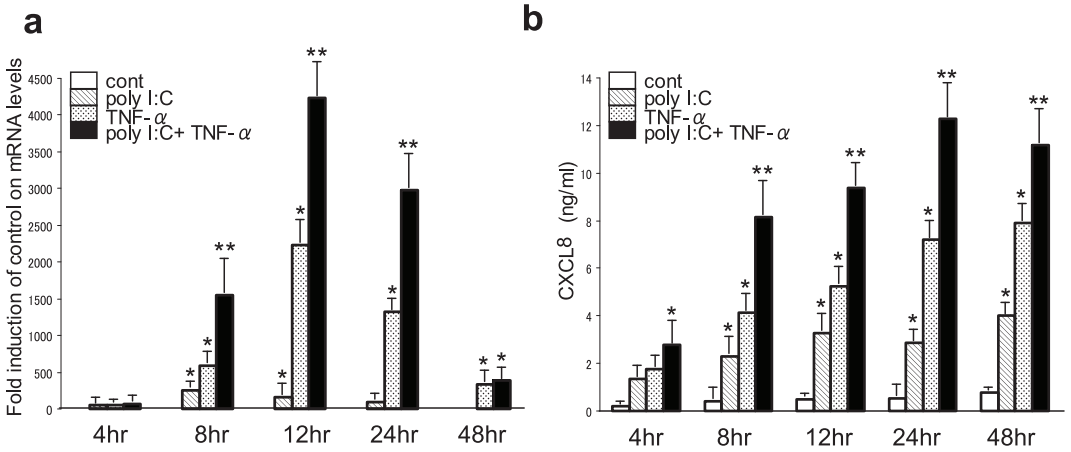

Fig. 1. The effect of treatment with poly I : C, TNF-α, or poly I : C with TNF-α on CXCL8 / IL-8 (a) mRNA expression and (b) protein release to the media. BEAS-2B cells were incubated with poly I : C (10 μg / ml) and / or TNF-α (10 ng / ml) for 4 to 48 hours, followed by real-time PCR to determine mRNA levels, and ELISA for protein levels. The data are presented as the mean ± SEM of three independent experiments. Open bar indicates control, bar with diagonal lines indicates stimulation with poly I : C, bar with dots indicates stimulation with TNF-α, and closed bar indicates stimulation with poly I : C and TNF-α.
* $P < 0.05$ (compared with non-stimulated control cells at the same time point).
** $P < 0.05$ (compared with cells stimulated with poly I : C at the same time point).

ferences were determined by analysis of variance with Fisher's protected least significant difference (PLSD) test. Stat-View IV software (Abacus Concepts, Inc., Berkeley, CA) was used to analyze the data.

Results

Synergistic or additive effects of TNF-α with poly I : C on CXCL8 / IL-8 expression

CXCL8 / IL-8 mRNA and protein expression were significantly upregulated following treatment with TNF-α or poly I : C alone (Figs. 1a and 1b). Combined stimulation using both poly I : C and TNF-α further induced CXCL8 / IL-8 mRNA and protein expression. Cells treated with TNF-α expressed more CXCL8 / IL-8 than cells treated with poly I : C. The peak of mRNA expression occurred around 12 hours after stimulation.

No additive effects of IFN-γ with poly I : C on CXCL8 / IL-8 expression

Compared with stimulation using poly I : C alone, stimulation using a combination of poly I : C and IFN-γ did not further induce CXCL8 / IL-8 mRNA (Fig. 2a) or protein (Fig. 2b) expression. IFN-γ alone did not induce any CXCL8 / IL-8mRNA or protein.

Synergistic or additive effects of TNF-α with poly I : C on CXCL10 / IP-10 expression

CXCL10 / IP-10 mRNA and protein expression were significantly upregulated following stimulation with TNF-α or poly I : C alone (Figs. 3a and 3b). Cells treated with TNF-α

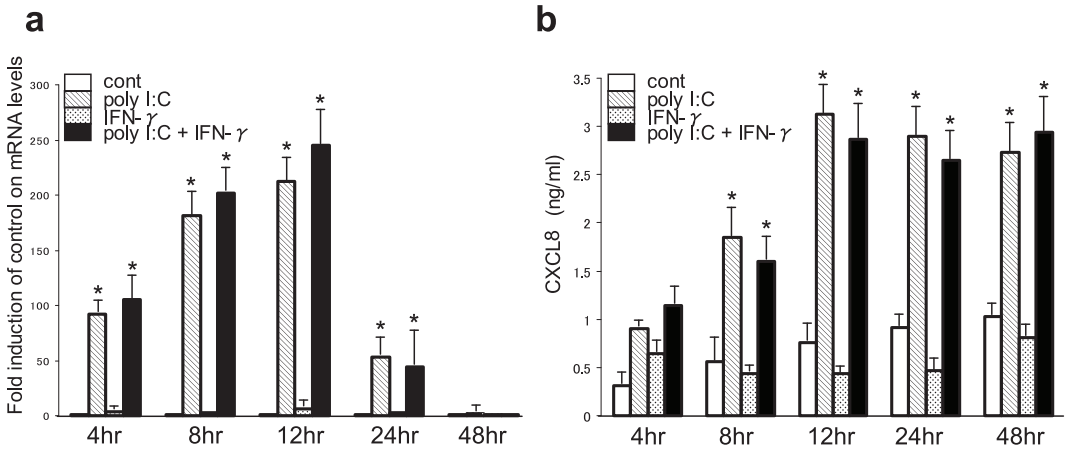

Fig. 2. The effect of treatment with poly I : C, IFN-γ, or poly I :C with IFN-γ on CXCL8 / IL-8 (a) mRNA expression and (b) protein release to the media. BEAS-2B cells were incubated with poly I : C (10 μg / ml) and / or IFN-γ (50 ng / ml) for 4 to 48 hours, followed by real-time PCR to determine mRNA levels, and ELISA for protein levels. The data are presented as the mean ± SEM of three independent experiments. Open bar indicates control, bar with diagonal lines indicates stimulation with poly I : C, bar with dots indicates stimulation with IFN-γ, and closed bar indicates stimulation with poly I : C and IFN-γ.
$^{*}P < 0.05$ (compared with non-stimulated control cells at the same time point).
$^{**}P < 0.05$ (compared with cells stimulated with poly I : C at the same time point).

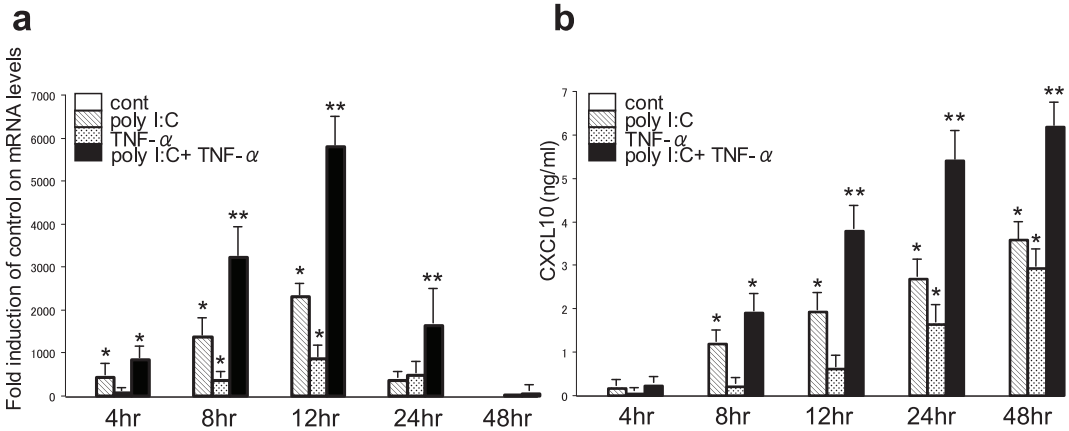

Fig. 3. The effect of treatment with poly I : C, TNF-α, or poly I :C with TNF-α on CXCL10 / IP-10 (a) mRNA expression and (b) protein release to the media. BEAS-2B cells were incubated with poly I : C (10 μg / ml) and / or TNF-α (10 ng / ml) for 4 to 48 hours, followed by real-time PCR to determine mRNA levels, and ELISA for protein levels. The data are presented as the mean ± SEM of three independent experiments. Open bar indicates control, bar with diagonal lines indicates stimulation with poly I : C, bar with dots indicates stimulation with TNF-α, and closed bar indicates stimulation with poly I : C and TNF-α.
$^{*}P < 0.05$ (compared with non-stimulated control cells at the same time point).
$^{**}P < 0.05$ (compared with cells stimulated with poly I : C at the same time point).

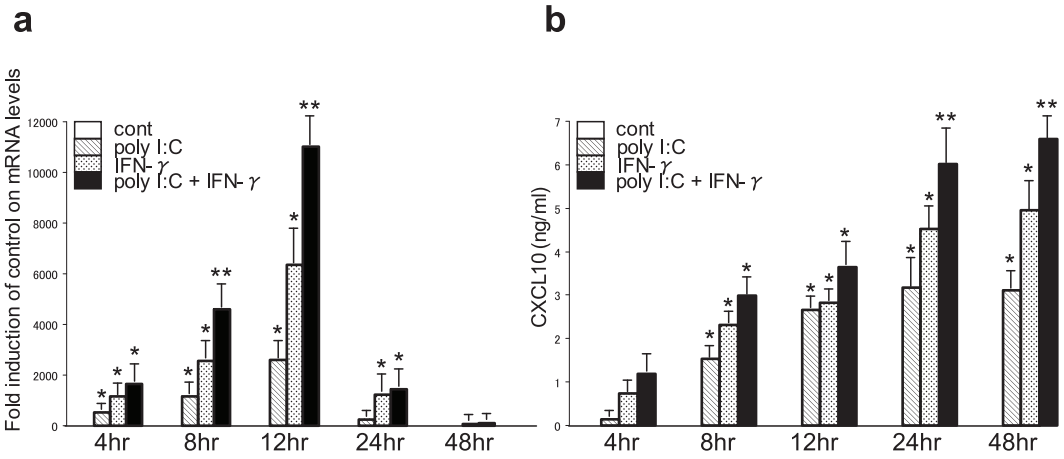

Fig. 4. The effect of poly I : C, IFN-γ, or poly I :C with IFN-γ on CXCL10 / IP-10 (a) mRNA expression and (b) protein release to the media. BEAS-2B cells were incubated with poly I : C (10 μg / ml) and / or IFN-γ (50 ng / ml) for 4 to 48 hours, followed by real-time PCR to determine mRNA levels, and ELISA for protein levels. The data are presented as the mean ± SEM of three independent experiments. Open bar indicates control, bar with diagonal lines indicates stimulation with poly I : C, bar with dots indicates stimulation with IFN-γ, and closed bar indicates stimulation with poly I : C and IFN-γ.
$^{*}P < 0.05$ (compared with non-stimulated control cells at the same time point).
$^{**}P < 0.05$ (compared with cells stimulated with poly I : C at the same time point).

expressed more CXCL10 / IP-10 than cells treated with poly I : C, at the same time point. When BEAS-2B cells were stimulated with a combination of TNF-α and poly I : C, expression of CXCL10 / IP-10 was significantly increased. Maximum mRNA expression occurred approximately 12 hours after stimulation with poly I : C or TNF-α.

Synergistic or additive effects of IFN-γ with poly I : C on CXCL10 / IP-10 expression

CXCL10 / IP-10 expression was induced by treatment with a combination of poly I : C and IFN-γ in a synergistic or additive fashion (Figs. 4a and 4b). Also IFN-γ alone induced CXCL10 / IP-10 mRNA or protein. The maximal induction of mRNA occurred approximately 12 hours after stimulation. CXCL10 / IP-10 protein levels rose significantly 8 hours after stimulation. IFN-γ treatment induced higher CXCL10 / IP-10 protein levels than did poly I : C treatment.

Discussion

The main aim of this study was to investigate chemokine expression following treatment with a combination of poly I : C, as a model for dsRNA viral infection, and inflammatory cytokines.

In the present study we examined CXCL8 / IL-8 and CXCL10 / IP-10 mRNA and protein expression following stimulation with poly I : C, TNF-α, IFN-γ, or a combination of poly I : C with TNF-α or IFN-γ. We confirmed that TLR3 and cytokine signaling collaborate to

produce greater induction of chemokines, which are important in the exacerbation of chronic inflammatory respiratory diseases like bronchial asthma and COPD.

TNF-α was a potent inducer of CXCL8 / IL-8 and CXCL10 / IP-10 mRNA and protein (Figs. 1a, 1b, 3a, and 3b). Stimulation with a combination of poly I : C and TNF-α further induced this expression. IFN-γ alone did not induce CXCL8 / IL-8 but strongly induced CXCL10 / IP-10 expression (Figs. 2a, 2b, 4a, and 4b). The combination of poly I : C with IFN-γ produced a stronger induction of CXCL10 / IP-10 mRNA and protein expression.

From our results, mRNA expression for both CXCL8 / IL-8 and CXCL10 / IP-10 peaked around 12 hours after stimulation with poly I : C, TNF-α, or a combination of poly I : C and TNF-α. CXCL8 / IL-8 protein levels peaked approximately 24 to 48 hours after stimulation. We previously reported that infection of the airway epithelium by influenza A virus induced CCL11 / eotaxin and CCL5 / RANTES proteins, which were detectable at least 24 hours after infection[14, 15]. Viral particles need time for replication, which may explain why the time to peak protein expression differs between synthetic dsRNA and actual influenza A virus.

CXCL10 / IP-10 protein expression was induced by IFN-γ, and further induced by treatment with a combination of IFN-γ and poly I : C. Stimulation with IFN-γ alone did not induce CXCL8 / IL-8 in the BEAS-2B airway cell line, which is consistent with a previous report[16].

TNF-α and poly I :C are thought to induce expression of chemokines through activation of nuclear factor-kappa B (NF-κB)[9, 17]. CXCL8 / IL-8 and CXCL10 / IP-10 promoter regions contain NF-κB binding sites[18, 19]. Our results suggest additive or synergistic activation of NF-κB following stimulation with a combination of poly I : C and TNF-α. TLR3 and TNF-α signals probably come together to activate NF-κB, which may able to induce more chemokines than a single stimulant. Our data do not suggest that IFN-γ activates NF-κB. IFN-γ is able to induce signal transducer and activator of transcription (STAT) -1[20]. As the CXCL10 / IP-10 promoter region has a STAT binding site, our results suggest that the induction of CXCL10 / IP-10 expression by IFN-γ occurs through STAT. Control of CXCL8 / IL-8 transcription does not appear to involve a STAT-1 mediated mechanism. IFN-γ also has a role in post-transcriptional mRNA stabilization[20], however from our time course of CXCL10 / IP-10 mRNA expression, there is no evidence for increased stabilization of CXCL10 / IP-10 mRNA.

Viral-induced exacerbations are the most common cause for asthmatic patients to be admitted to hospital. The results of this study suggest collaboration between viral infections and inflammatory cytokines in the airway epithelium. In combating viral infections, inflammatory cytokines appear to worsen the conditions in the airway epithelium. This study also indicates that daily control of inflammatory cytokines, both systemically and locally, in the bronchus, may diminish exacerbations during viral infections. Inhaled corticosteroids are the main agent used to control inflammatory cytokines at this time. Finally, asymptomatic

patients with bronchial asthma may still need inhaled corticosteroids to reduce inflammatory cytokines.

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