Cooperative Activation of CCL5 Expression by TLR3 and Tumor Necrosis Factor- α or Interferon- γ through Nuclear Factor- κ B or STAT-1 in Airway Epithelial Cells

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Key Words

Airway epithelial cells · CCL5/RANTES · Nuclear factor- κ B · STAT-1 · TLR3

Abstract

Background: CCL5/RANTES contributes to prolonged eosinophilic inflammation and asthma exacerbation after a viral infection. We studied the mechanism of CCL5 expression using viral product double-stranded RNA (dsRNA), a ligand of Toll-like receptor 3 (TLR3), and inflammatory cytokines in airway epithelial cells. **Methods:** The airway epithelial cell line BEAS-2B was used in our in vitro study, and the levels of CCL5 mRNA and CCL5 protein expression were determined using real-time PCR and ELISA. The activity of the CCL5 promoter region and nuclear factor (NF)- κ B was assessed by dual luciferase assay using specific luciferase reporter plasmids. We used actinomycin D to assess the stability of mRNA. Phosphorylation of signal transducer and activator of transcription 1 (STAT-1) was analyzed by Western blot. **Results:** Synthetic dsRNA up-regulated the expression of CCL5 mRNA and CCL5 protein. Adding TNF- α or IFN- γ to dsRNA further

increased the expression of CCL5. The combination of TNF- α and dsRNA cooperatively activated the CCL5 promoter region and the NF- κ B-specific reporter. IFN- γ did not activate these reporters. However, it increased the stability of CCL5 mRNA induced by dsRNA. IFN- γ phosphorylated STAT-1, but dsRNA did not. The effects of IFN- γ were not evident in the cells transfected with short interfering RNA for STAT-1. **Conclusions:** Cross-talk between TLR3 signaling and inflammatory cytokines regulates the expression of CCL5 in airway epithelial cells. In this mechanism, TNF- α may activate NF- κ B, in cooperation with TLR3 signaling. IFN- γ may stabilize CCL5 mRNA up-regulated by TLR3. This mechanism may depend on STAT-1.

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Introduction

CCL5/RANTES is a chemokine that attracts inflammatory cells, including eosinophils, into the airways. Its up-regulation in airway epithelial cells may contribute to prolonged eosinophilic inflammation and asthma exac-

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1018–2438/10/1525–0009\$26.00/0

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erbatation after an infection by viruses, such as the human rhinovirus, respiratory syncytial virus and influenza viruses [1–3].

TNF- α and IFN- γ are cytokines produced by macrophages, lymphocytes and dendritic cells during a viral infection. These cytokines are important for innate immunity against viruses. On the other hand, TNF- α or IFN- γ stimulates airway inflammation and prolong asthma exacerbation after a viral infection [4, 5]. Viruses such as human rhinovirus, respiratory syncytial virus and influenza viruses up-regulate the expression of CCL5. TNF- α and IFN- γ enhance CCL5 expression [6–10]. The mechanisms of the cooperative activation of viruses and cytokines are not fully understood.

We previously reported that double-stranded RNA (dsRNA) activates CCL5 expression, which is mediated through the Toll-like receptor 3 (TLR3) in airway epithelial cells. The activation of CCL5 transcription by dsRNA depends on the presence of the transcription factor nuclear factor (NF)- κ B and interferon regulatory factor 3 (IRF-3) [11–13]. In this study, we hypothesize that inflammatory cytokines such as TNF- α or IFN- γ may stimulate TLR3 signaling and activate CCL5 expression in airway epithelial cells. We also focused on the mechanisms of the cooperative activation of CCL5 expression by TLR3 and either TNF- α or IFN- γ .

Material and Methods

Cell Culture and Reagents

BEAS-2B is a human airway epithelial cell line transformed with an adenovirus 12-SV40 virus hybrid that we purchased from American Type 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% CO₂ in humidified air and were treated as described previously [12]. We purchased synthetic dsRNA polyinosinic:polycytidylic acid (poly I:C) from Sigma (Tokyo, Japan) and recombinant human TNF- α and IFN- γ from R&D Systems (Tokyo, Japan).

Real-Time PCR

Purification of RNA and synthesis of cDNA was performed as described previously [12]. A pre-designed TaqMan probe set for CCL5 mRNA was obtained 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 TaqMan ribosomal RNA probe, which is labeled with a fluorescent reporter dye (VIC), as an internal control. Each reaction consisted of 25 μ l containing 2 $\times$ Universal Master Mix (Applied Biosystems), primers, labeled probes and 50 ng cDNA. Amplification conditions consisted of 40 cycles at 95°C for 15 s and 60°C for 1 min after incubation with 95°C for 10 min. Amplification and fluorescent measurements were carried out during

Fig. 1. Effect of TNF- α plus dsRNA on CCL5 mRNA expression (a) and protein release into the media (b). The BEAS-2B cells were incubated with synthetic dsRNA poly I:C (10 μ g/ml) and/or TNF- α (10 ng/ml) for 4–48 h prior to real-time PCR or ELISA. Means $\pm$ SEM of three independent experiments. * $p < 0.05$ vs. non-stimulated control cells at the same time point; ** $p < 0.05$ vs. cells stimulated with dsRNA at the same time point.

the elongation step with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Data are shown as fold induction of non-stimulated control cells [12].

Assay of CCL5 Release into the Culture Medium

The concentration of CCL5 in collected cell culture media was assessed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Tokyo, Japan), as described previously [11, 12].

Transient Transfections and Luciferase Assay

To investigate transcriptional mechanisms, we used firefly-luciferase reporter vectors (a kind gift from Dr. Tomas J. Schall)

Fig. 2. Effect of IFN- γ plus dsRNA on CCL5 mRNA expression (a) and protein release into the media (b). BEAS-2B cells were incubated with synthetic dsRNA poly I:C (10 μ g/ml) and/or IFN- γ (50 ng/ml) for 4–48 h prior to real-time PCR or ELISA. Means $\pm$ SEM of three independent experiments. * $p < 0.05$ vs. non-stimulated control cells at the same time point; ** $p < 0.05$ vs. cells stimulated with dsRNA at the same time point.

that contained the 884-bp fragment of the promoter region of the CCL5 gene and multiple binding sites for NF- κ B. The cells were transfected with these plasmids, as described previously [11, 12], and incubated for 24 h. We then added poly I:C and cytokines, collected the cells 24 h later and stored them at -80°C until measuring the luciferase activity with the Dual-Luciferase Assay System (Promega). The firefly-luciferase activity of the reporter plasmid was normalized to Renilla-luciferase activity, expressed as fold induction, and compared with a control.

Knock Down of Gene Expression Using Short Interfering RNA

Pre-designed short interfering RNAs (siRNAs) for signal transducer and activator of transcription 1 (STAT-1) and negative

control of siRNA (scrambled siRNA) were purchased from Ambion (Tokyo, Japan). The cells were transfected with 50 nM of each siRNA with 5 μ l Lipofectamine 2000 (Promega), as described previously [11–13]. After 48 h, the cells were stimulated with poly I:C and cytokines. Twenty-four hours after stimulation, we harvested the cells and collected the supernatants.

Assay of the Stability of CCL5 mRNA

We treated the cells for 8 h with dsRNA alone or with dsRNA in combination with either TNF- α or IFN- γ . Afterwards, we either harvested the cells at time 0 (representing the control condition) or further treated them with the transcriptional inhibitor actinomycin D (Sigma) for 4 or 8 h and then harvested the cells. We used real-time PCR to analyze the levels of CCL5 mRNA, as described above, and expressed it as percent of the level at time 0.

Western Blotting

Western blotting was performed to investigate the activation of STAT-1, as described previously [11, 14]. We then subjected whole-cell extracts to 10% Tris-glycine gradient gel electrophoresis (Novex, San Diego, Calif., USA) and transferred the extract to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membranes were blocked with 5% non-fat milk powder in TBST (50 mM Tris, 0.15 M NaCl and 0.05% Tween 20), incubated with 1 μ g/ml rabbit anti-STAT-1 antibody (Ab) or anti-phospho-STAT-1 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) in TBST for 3 h, washed with TBST and then incubated with anti-rabbit Ig Ab (Amersham Biosciences, Tokyo, Japan) for 1 h. After extensive washing with TBST, we added a chemiluminescent substrate (ECL Western blot detection system, Amersham Biosciences) and subjected the membrane to autoradiography. Densitometry was employed to analyze the intensity of the bands, and the ratio was calculated by dividing the intensity of phosphorylated STAT-1 by the intensity of STAT-1.

Statistical Analysis

Data were expressed as means $\pm$ SEM. Statistical differences were determined by analysis of variance with Fisher's protected least significant difference test. Data were analyzed with Stat-View IV software (Abacus Concepts, Berkeley, Calif., USA).

Results

Synergistic or Additive Effects of TNF- α or IFN- γ on CCL5 Expression with dsRNA

The expression of CCL5 mRNA or CCL5 protein was up-regulated after stimulation with dsRNA. TNF- α modestly stimulated CCL5 expression, but TNF- α combined with dsRNA additively up-regulated CCL5 expression. Maximum expression was observed 12–24 h after stimulation (fig. 1). Very low levels of CCL5 mRNA or CCL5 protein were detected in cells stimulated by IFN- γ alone. However, IFN- γ significantly up-regulated dsRNA-induced CCL5 expression. Maximum expression occurred 8–24 h after stimulation (fig. 2).

Fig. 3. Effect of different doses of TNF- α or IFN- γ on CCL5 protein expression induced by dsRNA. BEAS-2B cells were incubated with synthetic dsRNA poly I:C (10 μ g/ml) and the indicated doses of TNF- α (a) or IFN- γ (b) for 24 h and analyzed by ELISA. Means $\pm$ SEM of three independent experiments. * $p < 0.05$ vs. cells stimulated with dsRNA only.

Fig. 4. Effect of preincubation with TNF- α or IFN- γ on CCL5 mRNA expression induced by dsRNA. BEAS-2B cells were preincubated with or without TNF- α (10 ng/ml) or IFN- γ (50 ng/ml) for 24 h and then incubated with synthetic dsRNA poly I:C (10 μ g/ml) after medium exchange prior to real-time PCR. Means $\pm$ SEM of three independent experiments.

Dose-Dependent Relationship of TNF- α or IFN- γ on dsRNA-Induced CCL5 Expression

The expression of CCL5 protein was up-regulated after stimulation with TNF- α (fig. 3a) or IFN- γ (fig. 3b) in a dose-dependent manner in the presence of dsRNA poly I:C (10 μ g/ml) 24 h after stimulation.

Effects of Preincubation with TNF- α or IFN- γ on the Expression of CCL5 mRNA

To analyze priming effects of TNF- α or IFN- γ , BEAS-2B cells were treated with TNF- α or IFN- γ for 24 h and then the medium was changed. Cells were stimulated with dsRNA poly I:C (10 μ g/ml), collected 24 h after stimulation and then subjected to real-time PCR. However, pre-

incubation with TNF- α or IFN- γ did not affect the expression of CCL5 mRNA in the presence of dsRNA (fig. 4).

TNF- α Activates the Promoter Region of CCL5, but IFN- γ Does Not Activate It

TNF- α alone or dsRNA alone stimulated the activity of the CCL5 promoter region (which contains binding sites for NF- κ B). The combination of TNF- α and dsRNA additively stimulated the CCL5 promoter region (fig. 5a). These data were compatible with the data of CCL5 mRNA and CCL5 protein. Similar data were observed in the NF- κ B reporter, indicating that the additive effect of TNF- α and dsRNA on CCL5 expression may depend on their cooperative activation of NF- κ B (fig. 5b). On the other

Fig. 5. Effect of TNF- α (10 ng/ml), IFN- γ (50 ng/ml) and dsRNA poly I:C (10 μ g/ml) on CCL5 promoter activity (**a**) and the NF- κ B reporter (**b**). BEAS-2B cells were transfected with the CCL5/RANTES promoter-luciferase reporter plasmids and control vector pRL-TK. Forty-eight hours later, cells were incubated for 24 h

either with or without the indicated cytokines or dsRNA. The relative luciferase activity was calculated as fold induction, compared with the control value. Means $\pm$ SEM of three independent experiments. * $p < 0.05$ vs. non-stimulated control cells; ** $p < 0.05$ vs. cells stimulated with dsRNA.

Fig. 6. Stability of CCL5 mRNA after stimulation with dsRNA poly I:C (10 μ g/ml) and/or IFN- γ (50 ng/ml) or TNF- α (10ng/ml) in BEAS-2B cells (**a**) or in cells transfected with siRNA for STAT-1 or a negative control (**b**). Cells were treated for 8 h and then ei-

ther harvested at time 0 (as a control) or further treated with actinomycin D for 4 or 8 h prior to real-time PCR. Means $\pm$ SEM of three independent experiments. * $p < 0.05$ vs. cells treated with dsRNA.

Fig. 7. Result of the Western blot for the expression of STAT-1 protein (STAT-1) or phosphorylated STAT-1 (pSTAT-1). BEAS-2B cells were stimulated either with or without dsRNA poly I:C (10 μ g/ml), IFN- γ (50 ng/ml) or dsRNA plus IFN- γ for 30 min to 4 h. **a** Whole-cell extracts were collected and then subjected to Western blotting. **b** The intensity of each band was analyzed by densitometry and the pSTAT-1/STAT-1 ratio was calculated. Means $\pm$ SEM of three independent experiments. * $p < 0.05$ vs. non-treated cells.

hand, IFN- γ did not stimulate the activity of the CCL5 promoter region. IFN- γ combined with dsRNA did not increase the activation of the CCL5 promoter region (fig. 5a). These results indicate that IFN- γ may regulate the post-transcription of the CCL5 gene.

IFN- γ Stabilizes CCL5 mRNA Expression Induced by dsRNA

After stimulation with dsRNA, the level of CCL5 mRNA decreased with actinomycin D treatment (fig. 6a). After stimulation with the combination of dsRNA and IFN- γ , the level of CCL5 mRNA was higher than its level after dsRNA stimulation alone. This effect did not occur in cells stimulated with the combination of dsRNA and TNF- α (fig. 6a) or in cells transfected with siRNA for STAT-1 and then stimulated with the combination of dsRNA and IFN- γ (fig. 6b). These data indicate that IFN- γ may stabilize dsRNA-induced CCL5 mRNA expression through activation of STAT-1.

Phosphorylation of STAT-1 by IFN- γ

IFN- γ phosphorylated STAT-1 in BEAS-2 cells from 30 min to 2 h (fig. 7). Stimulation with dsRNA did not phosphorylate STAT-1 and, therefore, did not change the level of phosphorylated STAT-1. These results indicate that activation of STAT-1 may depend on IFN- γ stimulation alone.

Effect of STAT-1 Inhibition on the Expression of CCL5 mRNA and CCL5 Protein

BEAS-2B cells transfected with siRNA for STAT-1 had significantly down-regulated the level of STAT-1 mRNA (data not shown). The negative control of siRNA did not interfere with the expression of STAT-1 mRNA. The cooperative activation of the combination of IFN- γ and dsRNA on the expression of CCL5 mRNA (fig. 8a) or CCL5 protein (fig. 8b) in BEAS-2B cells was down-regulated in the cells transfected with siRNA for STAT-1. These data indicate that STAT-1 may be important in the regulation of CCL5 expression by IFN- γ in airway epithelial cells.

Fig. 8. Effect of siRNA for STAT-1 or a negative control on CCL5 mRNA expression (**a**) and CCL5 protein expression (**b**). BEAS-2B cells were transfected with siRNA for STAT-1 or a negative control. Non-transfected cells and transfected cells were treated with

dsRNA poly I:C (10 µg/ml) or dsRNA (10 µg/ml) plus IFN-γ (50 ng/ml) for 8 h for PCR or for 24 h for ELISA. Means ± SEM of three independent experiments. * $p < 0.05$ vs. non-transfected cells.

Discussion

We demonstrate that the inflammatory cytokines TNF-α and IFN-γ, in cooperation with a ligand of TLR3 dsRNA, up-regulate the expression of the chemokine CCL5/RANTES in airway epithelial cells. In this mechanism, TNF-α may additively activate NF-κB, which is a key transcription factor regulating the CCL5 promoter region. IFN-γ may not directly regulate the transcription of CCL5, but it stabilizes CCL5 dsRNA-induced mRNA expression through activation of STAT-1.

Several reports indicate the priming effects of TNF-α or IFN-γ on TLR signaling. These cytokines up-regulate the expression of receptors for dsRNA, such as TLR3, dsRNA-dependent protein kinase (PKR), melanoma differentiation-associated gene 5 (MDA-5) and retinoic acid-inducible gene 1 (RIG-1) [15, 16]. We have also confirmed that TNF-α up-regulated TLR3 mRNA expression in airway epithelial cells [unpubl. data]. We then hypothesized that up-regulation of TLR3 expression by cytokines may contribute to the hyperresponsiveness to dsRNA and subsequent cooperative activation of CCL5 expression. In this study, we examined the effects of pre-treatment with TNF-α or IFN-γ on CCL5 expression in epithelial cells stimulated with dsRNA. However, we could not detect any priming effects. Moreover, the ef-

fects of these cytokines under dsRNA stimulation were different among the various target genes. For example, IFN-γ did not stimulate CXCL8/IL-8 expression induced by dsRNA [unpubl. data]. Taken together, up-regulation of TLR3 mRNA expression may not contribute to the cooperative up-regulation of CCL5 with dsRNA and TNF-α/IFN-γ in our study.

NF-κB reportedly activates the transcription of CCL5 in response to TNF-α, viral infection and dsRNA [11, 17, 18]. Our result showing an additive activation of NF-κB by the combination of TNF-α and dsRNA and the subsequent stimulation of CCL5 transcription may be concordant with previous reports. Other transcription factors, such as AP-1, may regulate CCL5 transcription in response to TNF-α and/or dsRNA. However, AP-1 did not activate CCL5 expression in our previous studies [11, 12]. Some reports suggest constitutive activation of NF-κB in the airway epithelium of asthmatics [19]. Therefore, the excessive response to a virus and overexpression of CCL5 may easily occur in asthma exacerbation induced by viral infection.

IFN-γ activates transcription factors such as STAT-1 and IRF-1. STAT-1 homodimers bind to the γ-activated sequence element of DNA in the promoter region of a target gene. IRF-1 expression can be directly activated by IFN-γ or up-regulated by IFN-γ through STAT-1. Acti-

vated IRF-1 binds the IFN-stimulated response element of DNA in the promoter region of the target gene. Several reports indicate that STAT-1 or IRF-1 binds to the promoter region of CCL5 and stimulates its activity [20–22]. In our study, however, IFN- γ did not stimulate the CCL5 promoter either with or without dsRNA stimulation. Further studies are needed to clarify this discrepancy. One reason may be related to the differences among the various cell types.

We next focused on the post-transcriptional regulation of CCL5 by IFN- γ . Stellato et al. [3] first reported CCL5 expression in airway epithelial cells and suggested that IFN- γ in combination with TNF- α may stabilize CCL5 mRNA. IFN- γ also stabilizes other genes [23] and dsRNA-induced expression of CCL5 mRNA. To investigate this mechanism, we further observed STAT-1, which transduces the initial signal of IFN- γ [24]. IFN- γ phosphorylated STAT-1 in BEAS-2B cells and the effect of IFN- γ was not evident in the cells in which siRNA had down-regulated STAT-1 expression. Further studies are required to clarify how STAT-1 translates the IFN- γ signal and how it stabilizes CCL5 mRNA induced by dsRNA. However, we hypothesize that STAT-1 may interact with RNA-binding protein and may regulate the post-transcriptional modulation of CCL5 RNA. STAT-3 is associated with Sam68 (an RNA-binding protein) in response to leptin [25]. The up-regulation of STAT-1 in

the airway epithelium of asthmatics indicates the possible expression of CCL5 during viral infection in asthma [26].

Our results show the cross-talk between inflammatory cytokines and TLR3 in airway epithelial cells. Epithelial-derived CCL5 may play an important role in host defense against viral infections. However, CCL5 overexpression may contribute to the pathogenesis of airway inflammation, including asthma exacerbation. Further investigation of these mechanisms may contribute to the understanding of airway diseases.

Acknowledgments

The authors would like to thank Dr. Tomas J. Schall for providing CCL5/RANTES promoter plasmid and Drs. Fumio Kokubu, Tsutomu Hirano and Takeshi Kasama for their excellent assistance and helpful discussions. This work was supported by the Environmental Restoration and Conservation Agency and GlaxoSmithKline.

Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.

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