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Synthetic double-stranded RNA induces multiple genes related to inflammation through Toll-like receptor 3 depending on NF- κ B and/or IRF-3 in airway epithelial cells

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Summary

Background We hypothesized that synthetic double-stranded (ds)RNA may mimic viral infection and induce expression of genes related to inflammation in airway epithelial cells.

Objective We analysed what gene was up-regulated by synthetic dsRNA poly I : C and then focused this study on the role of Toll-like receptor 3 (TLR3), a receptor of dsRNA and its transcriptional pathway.

Methods Airway epithelial cell BEAS-2B and normal human bronchial epithelial cells were cultured *in vitro*. Expression of targets RNA and protein were analysed by PCR and ELISA.

Localization of TLR3 expression in the cells was analysed with flow cytometry. To analyse the role of TLR3 and transcription factors, knockdown of these genes was performed with short interfering RNA (siRNA).

Results Real-time PCR revealed that poly I : C significantly increased the expression of mRNAs for chemokines IP-10, RANTES, LARC, MIP-1 α , IL-8, GRO- α and ENA-78 and cytokines IL-1 β , GM-CSF, IL-6 and the cell adhesion molecule ICAM-1 in both cell types. Increases in protein levels were also observed. Expression of these genes was significantly inhibited in BEAS-2B cells in which TLR3 expression was knocked down. However, pre-treatment with anti-TLR3 mAb, which interferes with the function of TLR3 expressed on the cell surface, did not inhibit the genes expression and these data were concordant with the results that TLR3 was expressed inside airway epithelial cells. The study of siRNA for NF- κ B and IRF3 showed that they transduce the signal of poly I : C, but their roles were different in each target gene.

Conclusion TLR3 is expressed inside airway epithelial cells and transduces synthetic dsRNA signals. These signals may increase expression of inflammatory cytokines, chemokines and ICAM-1 through activation of transcription factors NF- κ B and/or IRF3 in airway epithelial cells.

Keywords dsRNA, epithelial cells, IRF3, NF- κ B, TLR3

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Introduction

Viral infections are known to cause airway inflammatory diseases, such as bronchitis and pneumonia, and can exacerbate asthma and chronic obstructive pulmonary disease (COPD) [1–3]. Among the many kinds of viruses that infect the airways, human rhinovirus (HRV), respiratory syncytial virus (RSV), influenza virus and parainfluenza virus are particularly common pathogens that exacerbate asthma. These viruses are RNA viruses and synthesize double-stranded (ds)RNA during replication in

infected cells. dsRNA stimulates production of type I interferons (IFNs) in dendritic cells (DCs) and fibroblasts and plays important roles as a trigger of the host defence against viral infection [4, 5]. Recent studies revealed that these cells express Toll-like receptor 3 (TLR3), which appears to mediate the response to dsRNA, and suggested that TLR3 participates in the signalling pathway by which dsRNA induces expression of IFNs [6–8].

Airway epithelial cells may act as a host defence against virus, but these cells also express many factors that cause airway inflammation [2, 9–14]. Investigators have

hypothesized that dsRNA can mimic viral infection and reported that dsRNA induced expression of several genes in epithelial cells [15, 16]. Recent studies showed that airway epithelial cells constitutively express TLRs including TLR3 [16, 17]. We reported that airway epithelial cells express mRNA for TLR3 and its putative ligand, poly I:C, which is a synthetic dsRNA, induced expression of the chemokine regulated on activation, normal T-cell expressed and secreted (RANTES) through activation of transcription factors nuclear factor- κ B (NF- κ B) and interferon regulatory factor (IRF) [18]. However, the functional role of TLR3 and the mechanism of regulation of gene expression by dsRNA in airway epithelial cells remain unclear.

In the present study, we examined which genes were up-regulated by synthetic dsRNA in airway epithelial cells. We next observed that TLR3 is involved in this dsRNA signalling by knockdown of the gene for TLR3 in epithelial cells. Furthermore, we investigated the transcriptional regulation of genes activated through TLR3 signalling.

Materials and methods

Cell culture and reagents

BEAS-2B is a human airway epithelial cell line transformed with adenovirus 12-SV40 virus hybrid 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. Normal human bronchial epithelial (NHBE) cells were purchased from Cambrex (Walkersville, MD, USA) and cultured in SABM medium as described previously [18]. The synthetic dsRNA poly I:C was purchased from Sigma (Tokyo, Japan) and dissolved in distilled water. Recombinant human TNF- α was purchased from R&D Systems (Tokyo, Japan).

Purification of RNA and conventional reverse transcription-polymerase chain reaction

Gene expression of IL-8, IFN- γ -inducible protein-10 (IP-10) and β -actin was analysed by conventional RT-PCR as described previously [18, 19]. Total RNA was extracted with Isogen (Nippon-Gene, Tokyo, Japan) from cells after incubation with and without indicated reagents. cDNAs were synthesized from isolated RNA templates with a High-Capacity cDNA Archive Kit (Applied Biosystems, Tokyo, Japan). After cDNAs were denatured at 94 °C for 5 min, PCR was performed with Taq polymerase and PCR reaction buffer (Roche Diagnostics, Tokyo, Japan). Primers for IL-8 were 5'-ATGACTTCCAAGCTGGCCGTG-3' (forward) and 5'-TTATGAATTCTCAGCCCTCTTCAAAA-CTTCTC-3' (reverse), 5'-TGA CTCTAAGTGGCATTCAAGG

(forward) and 5'-GATTCAGACATCTCTTCTCACCC (reverse) for IP-10, and 5'-GTGGGGCGCCCCAGGCACCA-3' (forward) and 5'-CTCCTTAATGTACGCACGATTTC-3' (reverse) for β -actin. Amplification conditions consisted of 30 cycles of denaturation at 94 °C for 45 s annealing at 61 °C for 45 s, and extension at 72 °C for 1 min in a thermocycler (Perkin-Elmer Cetus, Norwalk, CT, USA). After incubation at 72 °C for 10 min, PCR products were analysed by 2% agarose gel electrophoresis and ethidium bromide staining, followed by visualization with an ultraviolet transilluminator.

Real-time PCR

Purification of RNA and synthesis of cDNA were performed as described above. Pre-designed TaqMan probe sets for the targets indicated in Table 1 were purchased from Applied Biosystems. 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 labelled 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, labelled probes and 50 ng cDNA. Amplification conditions consisted of 40 cycles of 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 the elongation step with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Data are shown as fold induction of non-stimulated control cells.

Assay of chemokine release into the culture medium

Concentrations of chemokines including IP-10, RANTES, liver and activation-regulated chemokine (LARC), macrophage inflammatory protein-1 α (MIP-1 α), IL-8 and fractalkine in collected cell culture media were determined with a commercially available ELISA kit as described previously [18]. Kits for IP-10 and IL-8 were purchased from BD Biosciences (Tokyo, Japan) and kits for RANTES, LARC, MIP-1 α and fractalkine were purchased from R&D systems. The standards and samples were added to a 96-well microtitre plate coated with an anti-chemokine antibody. After incubation at room temperature for 2 h, each well was washed five times with wash buffer. Biotinylated antibody against chemokine and avidin-horseradish peroxidase conjugate were added to the well. After incubation at room temperature for 1 or 2 h, each well was washed seven times with wash buffer. Substrate solution (stabilized hydrogen peroxide and tetramethylbenzidine) was added to each well and the plate was incubated at room temperature for 20 or 30 min. Sulphuric acid was then added to each well, and the absorbance was measured at 450 nm.

Table 1. Quantification of expression of mRNAs for chemokines (A), cytokines (B) and factors related to host defence, ICAM-1, and NOS2 (C) in normal human bronchial epithelial (NHBE) cells and the BEAS-2B airway epithelial cell line

	NHBE cells (fold induction)	BEAS-2B (fold induction)
(A) Levels of mRNAs for chemokines in NHBE and BEAS-2B cells		
MIP-1 α	20 970.2 $\pm$ 6837.5*	1370.3 $\pm$ 304.9*
RANTES	11 061.9 $\pm$ 2132.4*	10 346.8 $\pm$ 1708.9*
IP-10	9012.2 $\pm$ 1208.2*	13 475.9 $\pm$ 3846.5*
ENA-78	654.2 $\pm$ 74*	35.4 $\pm$ 11*
IL-8	614.8 $\pm$ 128.7*	1072.3 $\pm$ 282.8*
Fractalkine	38 $\pm$ 4.2*	2.3 $\pm$ 0.8
LARC	35.7 $\pm$ 5.2*	13 148 $\pm$ 1516.1*
GRO- α	26.7 $\pm$ 3.6*	106.6 $\pm$ 20.9*
TARC	23.1 $\pm$ 5.9*	15.1 $\pm$ 5.7
MCP-1	13.4 $\pm$ 2.7*	2 $\pm$ 0.8
SR-PSOX	6.9 $\pm$ 1.5*	1.2 $\pm$ 0.6
ELC	2.4 $\pm$ 1.2	12.1 $\pm$ 6.4
SDF-1 α	ND	ND
I-309	ND	12.8 $\pm$ 2.8*
MEC	1.2 $\pm$ 0.2	0.9 $\pm$ 0.2
Eotaxin	ND	2 $\pm$ 0.4
Eotaxin-2	0.1 $\pm$ 0	ND
Eotaxin-3	0.2 $\pm$ 0.1	1.8 $\pm$ 0.5
MCP-4	ND	6.7 $\pm$ 2
(B) Levels of mRNAs for cytokines in NHBE and BEAS-2B cells		
GM-CSF	186.7 $\pm$ 36.1*	278.5 $\pm$ 64.2*
IL-6	162.8 $\pm$ 15.6*	94.5 $\pm$ 32.6*
TNF- α	76.2 $\pm$ 6.9*	11.7 $\pm$ 4.1
IL-1 β	19.4 $\pm$ 1.4*	11.2 $\pm$ 2.8*
IL-10	ND	2 $\pm$ 0.3
IL-11	1.3 $\pm$ 0.1	7.6 $\pm$ 1.3
IL-12 (p35)	3.7 $\pm$ 0.3	4.4 $\pm$ 0.7
IL-12 (p40)	ND	ND
IL-16	0.2 $\pm$ 0	0.9 $\pm$ 0.3
IL-17A	ND	ND
IL-18	0.3 $\pm$ 0	1 $\pm$ 0.3
IFN- β	2.3 $\pm$ 1.1	36.1 $\pm$ 11.1*
IFN- γ	ND	ND
TGF- β	1 $\pm$ 0.2	1.7 $\pm$ 0.3
(C) Levels of mRNAs for host defence factors, ICAM-1 and NOS in NHBE and BEAS-2B cells		
Host Defence		
α -defensin	ND	ND
β -defensin	21.4 $\pm$ 9.1*	0.9 $\pm$ 0.7
IL-1R antagonist	12.5 $\pm$ 2.1*	10.8 $\pm$ 4.4*
SLPI	1.4 $\pm$ 0.3	2.8 $\pm$ 0.6
CC10	ND	ND
SP-C	0.3 $\pm$ 0.1	0.7 $\pm$ 0.2
SP-D	0.6 $\pm$ 0.1	0.5 $\pm$ 0.1
Others		
ICAM-1	146.6 $\pm$ 23.8*	9.7 $\pm$ 1.4*
NOS 2	128.7 $\pm$ 1.8*	0.4 $\pm$ 0.3

RNA was extracted from cells stimulated with 50 μ g/mL poly I : C (synthetic dsRNA) for 24 h and subjected to real-time PCR. Levels of mRNA were calculated as fold induction compared with non-stimulated control. The data are presented as the mean $\pm$ SEM of three independent experiments.

* P < 0.05 as compared with non-stimulated cells.

ND, not detected.

Transient transfections and luciferase assay

To investigate the transcription pathway of TLR3, we used firefly luciferase reporter vectors that contain multiple binding sites for NF- κ B (pNF- κ B-Luc), IRF3 (pISRE-Luc), cAMP responsive element binding protein (CREB) (pCRE-Luc), activator protein 1 (AP1) (pAP1-Luc) and serum response factor (SRF) (pSRE-Luc). These plasmids were purchased from BD Biosciences and were transformed into DH5 α -competent cells (Invitrogen) and then purified with the Qiagen Plasmid Purification Kit (Qiagen, Tokyo, Japan). BEAS-2B cells were seeded into six-well plates and allowed to grow to 50–70% confluence. Cells were transfected with 100 ng reporter plasmid and 10 ng control renilla-luciferase vector, pRL-TK (Promega, Tokyo, Japan) with 3 μ L Eugene 6 Transfection Reagent (Roche Diagnostics) and incubated for 24 h in 2 mL medium. Poly I:C (50 μ g/mL) was then added, and 24 h later cells were washed twice with Ca²⁺- and Mg²⁺-free PBS, solubilized by incubation in 500 μ L lysis buffer for 20 min, transferred to microtubes and centrifuged to pellet cellular debris. The supernatants were stored at -80°C until luciferase activity was measured with the Dual-Luciferase Assay System (Promega) and a luminometer (Gene-Light 55, Microtech Nichion, Chiba, Japan). The firefly-luciferase activity of the reporter plasmid was normalized to renilla-luciferase activity and expressed as fold induction compared with control.

Knockdown of gene expression with short interfering RNA

Pre-designed short interfering RNA (siRNAs) for TLR3 (catalogue number #107056), NF- κ B (catalogue number #109424) and IRF3 (catalogue number #115222) were purchased from Ambion (Tokyo, Japan). BEAS-2B cells were seeded into six-well plates and allowed to grow to 50% confluence. Cells were transfected with 50 nm of each siRNA with 5 μ L Lipofectamine 2000 (Promega) and grown in DMEM/F12 with 10% FBS without antibiotics for 24 h. Then, the medium of the cells was changed to DMEM/F12 with 10% FBS, 100 U/mL penicillin and 100 ng/mL streptomycin. After 24 h, cells were stimulated with 50 μ g/mL poly I:C (dsRNA). Cells were harvested, and the supernatant was collected 24 h after stimulation. Scrambled siRNA was used as non-specific negative control of siRNA (Ambion).

Flow cytometry

Cells were detached from the plate with a non-enzymatic cell dissociation solution (Sigma). Detached cells were incubated with 5 μ g/mL of PE-labelled anti-TLR3 mAb

(eBioscience, San Diego, CA, USA), PE-labelled anti-intercellular adhesion molecule-1 (ICAM-1) Ab or PE-labelled human IgG (BD Biosciences) for 30 min at 4°C in PBS containing 1% BSA. Cells were washed with PBS twice and resuspended in PBS with 1% BSA. For intracellular staining, detached cells were resuspended in fixation and permeabilizing solution (BD Biosciences). After incubation for 20 min at 4°C , cells were washed with washing buffer (BD Biosciences) twice, resuspended in washing buffer containing Abs and incubated for 30 min at 4°C . Cells were then washed with washing buffer twice and resuspended in the buffer. Human IgG (10%) was added to both reaction mixtures. The cells were then analysed with FACSCalibur (BD Biosciences).

Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical differences were determined by ANOVA with Fisher's PLSD. Data were analysed with Stat-View IV (Abacus Concepts Inc., Berkeley, CA, USA).

Results

Time-dependent expression of chemokine mRNAs after stimulation with dsRNA

As an initial experiment, we observed the time course of gene expression in response to dsRNA by conventional RT-PCR. Peak expression of IL-8 and IP-10 mRNAs in BEAS-2B cells was observed at 24 h after stimulation with synthetic dsRNA poly I:C (50 μ g/mL) (Fig. 1). The time course data were similar to those recently reported for RANTES [18]. Therefore, our subsequent analysis of gene expression focused on the time-point 24 h after stimulation with poly I:C.

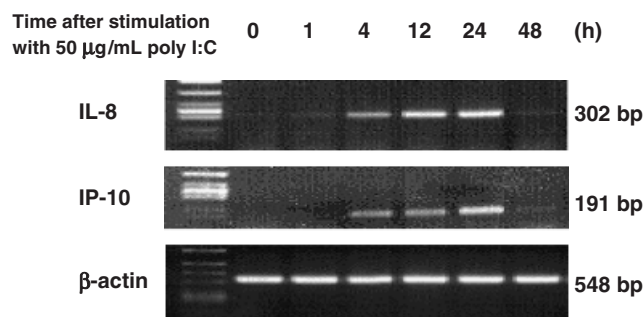


Fig. 1. Time-dependent up-regulation of expression of mRNAs for IL-8 and IP-10 in response to 50 μ g/mL poly I:C (synthetic double-stranded RNA) in the BEAS-2B airway epithelial cell line. RNA was extracted from the cells and subjected to conventional RT-PCR. Representatives of three independent experiments.

Increased expression of target gene mRNAs by dsRNA in airway epithelial cells

A variety of genes showed increased expression in NHBE cells in response to synthetic dsRNA poly I : C (50 µg/mL) for 24 h (Table 1). Inflammatory chemokines, cytokines, nitric oxide synthase 2 (NOS2) and ICAM-1 showed the greatest increases in expression after stimulation with poly I : C. In contrast, expression of chemokines such as eotaxin family members, type I IFNs, IL-12 and factors related to host defence [20] showed little change in expression. Similar results were observed in the airway epithelial cell line BEAS-2B, but expression of some genes, including scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX), fractalkine, β -defensin, was not increased significantly by poly I : C in BEAS-2B cells.

Activation of production of chemokines in the media of normal human bronchial epithelial cells

We analysed protein levels of chemokines in the culture media of NHBE cells to confirm the correlation between expression of mRNA and protein. Significant levels of IP-10, IL-8, MIP-1 α , LARC and fractalkine were detected in the supernatants of NHBE cells stimulated with synthetic dsRNA poly I : C (50 µg/mL) for 24 h (Fig. 2). Production of RANTES protein was also increased significantly by dsRNA, as reported previously [18]. Levels of chemokines of the eotaxin family, such as eotaxin, eotaxin-2 and eotaxin-3, were not increased in response to poly I : C (data not shown).

Mechanism of dsRNA-TLR3 signalling in airway epithelial cells

Activities of luciferase reporter constructs for NF- κ B and IRF were increased significantly with synthetic dsRNA poly I : C (50 µg/mL). In contrast, reporter constructs for other transcription factors, including AP-1, CREB and SRF, were not activated by poly I : C in BEAS-2B cells (Fig. 3).

Knockdown of expression of TLR3, NF- κ B and IRF3 mRNAs by siRNA

To analyse the role of TLR3 and transcription factors, NF- κ B and IRF3, in the dsRNA signalling in BEAS-2B cells, we used siRNA. Levels of these mRNAs were reduced significantly in BEAS-2B cells transfected with siRNA for each (Fig. 4).

Effect of knockdown of TLR3 expression and pre-treatment with anti-TLR3 mAb on expression of cytokines, chemokines and ICAM-1 in response to dsRNA

We next analysed the effect of siRNA for TLR3 in regulation of target genes mRNA by dsRNA. We focused on

genes that were strongly and commonly up-regulated in both NHBE and BEAS-2B cells, such as IP-10, RANTES, LARC, MIP-1 α , IL-8, growth-related oncogene (GRO- α), epithelial neutrophil-activating peptide-78 (ENA-78, IL-1 β), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and ICAM-1. As shown in Fig. 5, induction of expression of each mRNA by poly I : C (dsRNA) was remarkably inhibited in cells transfected with siRNA for TLR3. There was no significant difference in expression of mRNAs between wild-type BEAS-2B cells and cells transfected with non-specific negative control of siRNA. Pre-incubation with anti-TLR3 mAb did not inhibit the expression of these genes induced by poly I : C. We also analysed protein levels of a subset of chemokines and the data correlated with the mRNA data (Fig. 6).

Effect of knockdown of NF- κ B or IRF3 expression on expression of cytokines, chemokines and ICAM-1 in response to dsRNA

We next examined the effect of suppression of expression of specific transcription factors on the dsRNA regulation of target mRNA expression using siRNA. As shown in Fig. 5, suppression of NF- κ B expression resulted in partial inhibition of expression of genes encoding IP-10, RANTES, LARC, IL-8, GRO- α , ENA-78, IL-1 β , GM-CSF, IL-6 and ICAM-1. Up-regulation of MIP-1 α mRNA in response to poly I : C (dsRNA) was not altered in cells transfected with siRNA for NF- κ B. Suppression of IRF3 expression also resulted in partial inhibition of expression of genes such as IP-10, RANTES, LARC, MIP-1 α , IL-8, GRO- α , GM-CSF and IL-6. The up-regulation of ENA-78, IL-1 β and ICAM-1 with dsRNA was not affected by siRNA for IRF3. We also analysed levels of chemokine protein in the culture media of BEAS-2B cells by ELISA. A pattern of expression similar to that for mRNA was observed (Fig. 6).

Expression of TLR3 in airway epithelial cells

Flow cytometry revealed that BEAS-2B cells seemed to express TLR3 on the cell surface (ratio of mean fluorescence against control IgG 1.5 ± 0.2), but the level of TLR3 expression inside the cells (ratio 2.5 ± 0.4 , $*P < 0.05$ compared with control IgG) was higher than that (Fig. 7). ICAM-1 expression of the cells was confirmed as positive control (ratio 42.6 ± 6.7 , $*P < 0.05$ compared with control IgG). A significant level of TLR3 expression was also observed in NHBE cells; the level of TLR3 inside of the cells (ratio 2.8 ± 0.5 , $*P < 0.05$ compared with control IgG) seemed to be relatively higher than that on the surface (ratio 2.4 ± 0.9 , $*P < 0.05$ compared with control IgG). ICAM-1 expression of the cells was confirmed as positive control (ratio 4.0 ± 1.7 , $*P < 0.05$ compared with control IgG).

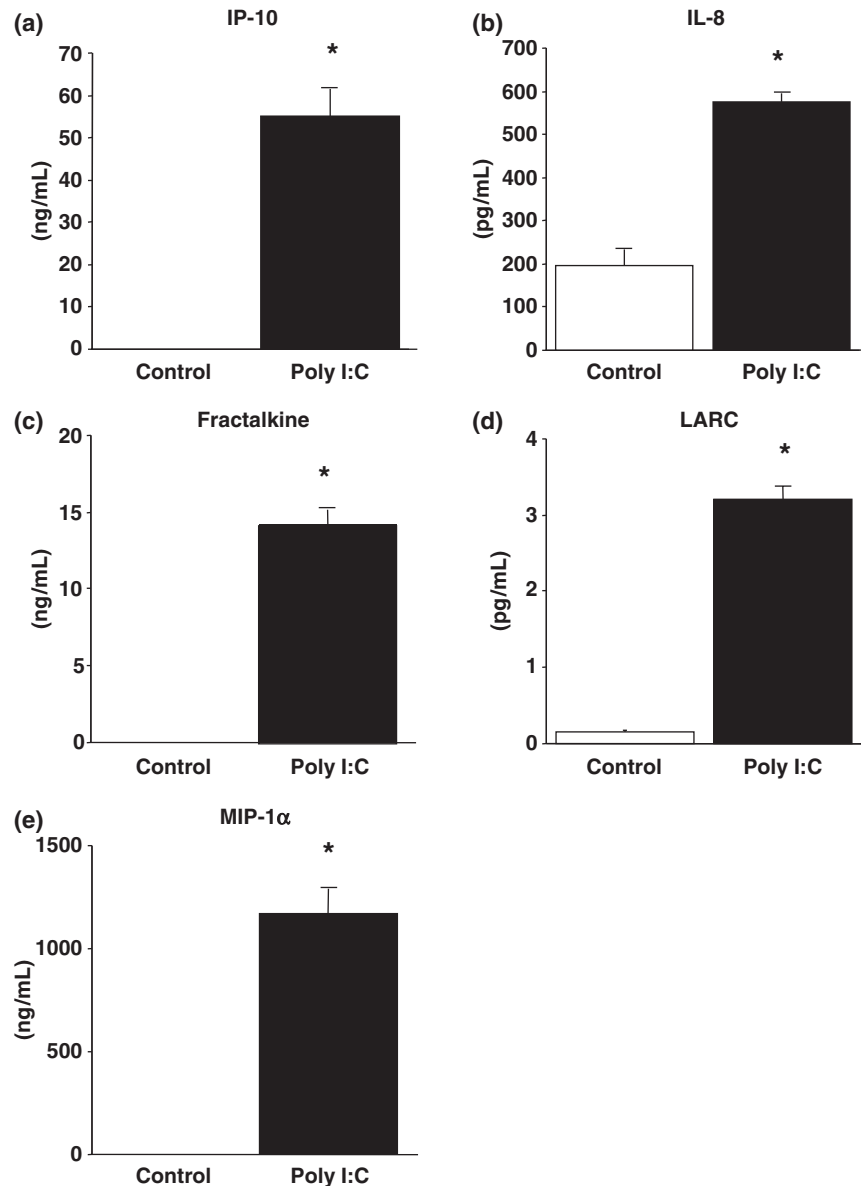


Fig. 2. Chemokine levels in the culture media of normal human bronchial epithelial (NHBE) cells. (a) IP-10, (b) IL-8, (c) fractalkine, (d) LARC and (e) MIP-1 α . Supernatants were collected from NHBE cells stimulated with 50 μ g/mL poly I:C (synthetic double-stranded RNA) for 24 h and subjected to ELISA. The data are presented as the mean $\pm$ SEM of three independent experiments (* P < 0.05 as compared with non-stimulated cells).

Discussion

We demonstrated here that airway epithelial cells, not only cell line BEAS-2B but also NHBE, express TLR3 inside and on the surface of the cells. The technique of siRNA for TLR3 and the study using anti-TLR3 mAb showed that synthetic dsRNA induce multiple inflammatory genes through TLR3 expressed inside BEAS-2B cells. In this signalling, two transcription factors NF- κ B and IRF3 play pivotal roles, but their roles were different in each target gene. It is also a new finding that dsRNA up-regulated the expression of genes for SR-PSOX, I-309

and IL-1 receptor (IL-1R) antagonist. To highlight airway epithelial cells, it might also be a novel result that dsRNA induced the expression of fractalkine, ENA-78, MIP-1 α and NOS2.

Synthetic dsRNA poly I:C increased expression of a variety of gene mRNAs in NHBE cells. Among these, the genes with the largest relative increase in expression were those encoding inflammatory chemokines such as MIP-1 α , RANTES, IP-10, ENA-78, IL-8, fractalkine, LARC, GRO- α , TARC and MCP-1, and cytokines such as GM-CSF, IL-6, TNF- α and IL-1 β . Expression of ICAM-1 and NOS2 was also increased significantly. These genes are known to

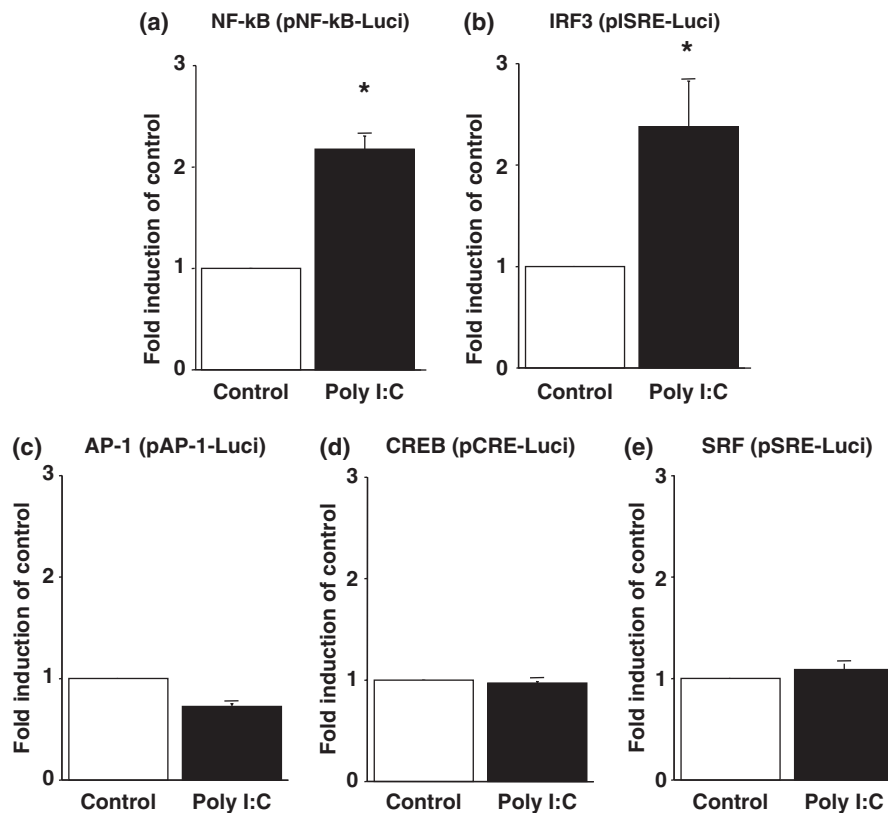


Fig. 3. Luciferase reporter assays of transcription factors. BEAS-2B cells were transfected with luciferase reporter constructs referred to as pNF- κ B-Luci that contains multiple binding sites for NF- κ B (a), pISRE-Luci for IRF3 (b), pAP-1-Luci for AP-1 (c), pCRE-Luci for CREB (d) and pSRE-Luci for SRF (e). ISRE, interferon stimulated response element. CRE, cAMP response element. SRE, serum response element. After 48 h, cells were stimulated with 50 μ g/mL poly I : C (double-stranded RNA) for 24 h and subjected to luciferase assay. The relative luciferase activity was calculated as fold induction compared with controls value. Data are presented as the mean $\pm$ SEM of three independent experiments (* P < 0.05 as compared with non-stimulated cells).

be related to airway inflammation. Respiratory viral infection can induce expression of most of these genes and cause airway inflammation.

Chemokines attract cells that express specific receptors to the site of inflammation. The relative position of the first two consensus cysteines provides chemokines into subclasses such as CC, CXC, CX3C and C [21]. Chemokines are named by subclass motif and gene symbol number. Name and specific receptors of analysed chemokines are shown in Table 2. Neutrophils express CXCR1 and 2. Monocytes express CCR1, 2 and 5. Th1 cells express CCR5 and CXCR3. CD8 cells express CCR5, CX3CR1 and CXCR6. Immature DCs express CCR1 and 6. It is reasonable to hypothesize that these cells migrate into the airway epithelium in response to chemokines during viral infection of the airway. These cells may act as a host defence against virus or cause airway inflammation.

We showed time-dependent up-regulation of expression of mRNAs for IL-8 and IP-10 in response to 50 μ g/mL poly I : C (synthetic dsRNA) in the BEAS-2B airway epithelial cell line. Maximum induction of mRNA was observed at 24 h after stimulation and this expression was remarkably decreased at 48 h. We have confirmed these time-depen-

dent regulation of chemokines mRNA with dsRNA in BEAS-2B cells using real-time PCR (data not shown). One of the reasons why dsRNA signals decrease rapidly after 24 h may be an inhibitor of TLR signal such as A20 [22, 23]. Synthetic dsRNA stimulated the expression of A20 in BEAS-2B at 24 h. Inhibition of the expression of A20 using siRNA caused the prolonged up-regulation of chemokines mRNA (data not shown). We are now investigating the role of A20 in the prolonged airway inflammation after viral infection *in vivo*.

Fractalkine and SR-PSOX are chemokines and are also known as scavenger receptors. Membrane bound these two chemokines can mediate adhesion and phagocytosis of bacteria in DCs [24]. There is also an interesting report that G glycoprotein of RSV is a ligand of CX3CR, a specific receptor for fractalkine [25]. The role of these chemokines in airway epithelial cells is quite unknown, but we speculate that dsRNA-induced fractalkine or SR-PSOX may act as host-defence against bacteria or virus.

IL-6, TNF- α and IL-1 β are key cytokines that regulate inflammation and host defence. GM-CSF is a potent cytokine that promotes granulocyte and macrophage maturation from haematopoietic precursors and can play

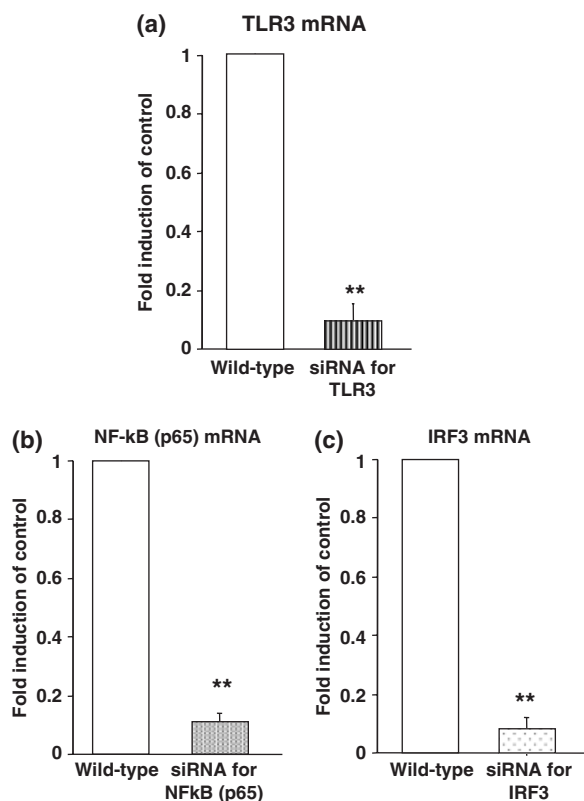


Fig. 4. Knock down of expression of genes encoding TLR3, NF- κ B and IRF3. BEAS-2B cells were transfected with or without short interfering RNA (siRNA) specific for TLR3, NF- κ B p65 or IRF3. RNA was extracted from the cells and subjected to real-time PCR for TLR3 (a), NF- κ B p65 (b) and IRF3 (c). The data are presented as the mean $\pm$ SEM of three independent experiments (** $P < 0.05$ as compared with non-transfected wild-type BEAS-2B cells).

critical roles in surfactant homeostasis and lung host defences.

ICAM-1 binds mononuclear cells and may play a role in airway inflammation. ICAM-1 is also known as a major receptor for HRV. Up-regulation of ICAM-1 in response to viral infection may contribute to activation and migration of inflammatory cells.

Levels of other mRNAs, such as those for IFN- β , IL-12 and defensin, were much lower than the inflammatory chemokines or cytokines. Type I IFNs are expressed by DCs in response to dsRNA or viral infection and appear to play pivotal roles in host defences against viruses. It would be possible that maximum induction of IFN- β mRNA by dsRNA may be observed at an earlier time point than 24 h after stimulation; however, induction of IFN- β by virus or dsRNA tends to be much lower than chemokines such as IP-10, IL-8 and RANTES in previous reports [10, 26]. Airway epithelial cells may participate in innate immunity or inflammation recruiting cells, such as Th1 lymphocytes, monocytes, CD8 cells and neutrophils by producing chemokines. IL-1R antagonist is an inhibitor of

inflammation and may play a host-protective role in HRV infection [27]. We need further studies, but it might be possible that dsRNA can induce expression of IL-1R antagonist in airway epithelial cells and also play an anti-inflammatory role at airway viral infection. In the therapy of virus-induced airway inflammation, such as exacerbation of asthma and COPD, it would be desirable to suppress inflammatory response and retain host-defensive response.

We reported previously that airway epithelial cells express TLR3 mRNA and that its putative ligand, dsRNA, activated expression of RANTES. Our present results of siRNA experiments specific for TLR3 indicated that TLR3 may be necessary for activation of expression of specific genes in response to dsRNA in airway epithelial cells. TLR3 may be present in the cytoplasm of airway epithelial cells as a functional receptor for dsRNA because the levels of TLR3 expression on the surface of epithelial cells appear lower than that of the cell inside and anti-TLR3 mAb do not inhibit dsRNA signalling in epithelial cells, although this anti-TLR3 mAb has been reported to recognize TLR3 on the cell surface of fibroblasts and to suppress dsRNA-mediated production of IFN- β [8]. In monocyte-derived immature DCs, TLR3 is localized predominantly inside the cells and not on the cell surface. Immature DCs produce IL-12 and type I IFNs in response to dsRNA. These responses were not blocked by pre-treatment of cells with anti-TLR3 mAb [28]. Guillot *et al.* [26] recently reported intracellular expression of TLR3 in airway epithelial cell line BEAS-2B. They showed that poly I:C and influenza virus activate NF- κ B through TLR3-transfecting BEAS-2B, with the plasmid encoding a dominant-negative form of TLR3. We presented the data of NHBE and clarified the role of TLR3 using a different approach using siRNA and anti-TLR3 mAb. Our data support and emphasize recent reports.

Molecules other than TLR3, such as dsRNA-dependent protein kinase (PKR), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5), are also known as receptors for dsRNA in DCs [29]. We have examined the effect of knockdown of these receptors on the dsRNA signalling in epithelial cells using siRNA; however, these receptors were not involved in the expression of chemokines induced by poly I:C (data not shown). This discrepancy may be due to the difference in cell types. TLR3 may be an important receptor for dsRNA in airway epithelial cells.

Fire *et al.* [30] originally reported an RNA interference method to knock down gene expression. Elbashir *et al.* [31] established the gene-specific RNA interference using 21 bp of RNA (siRNA), which did not stimulate IFNs. It has been reported that siRNA can interact with TLR3 and induce signalling [32, 33]; however, siRNA did not activate TLR3 signalling or cause non-specific repression of the expression of target genes in our present experiments.

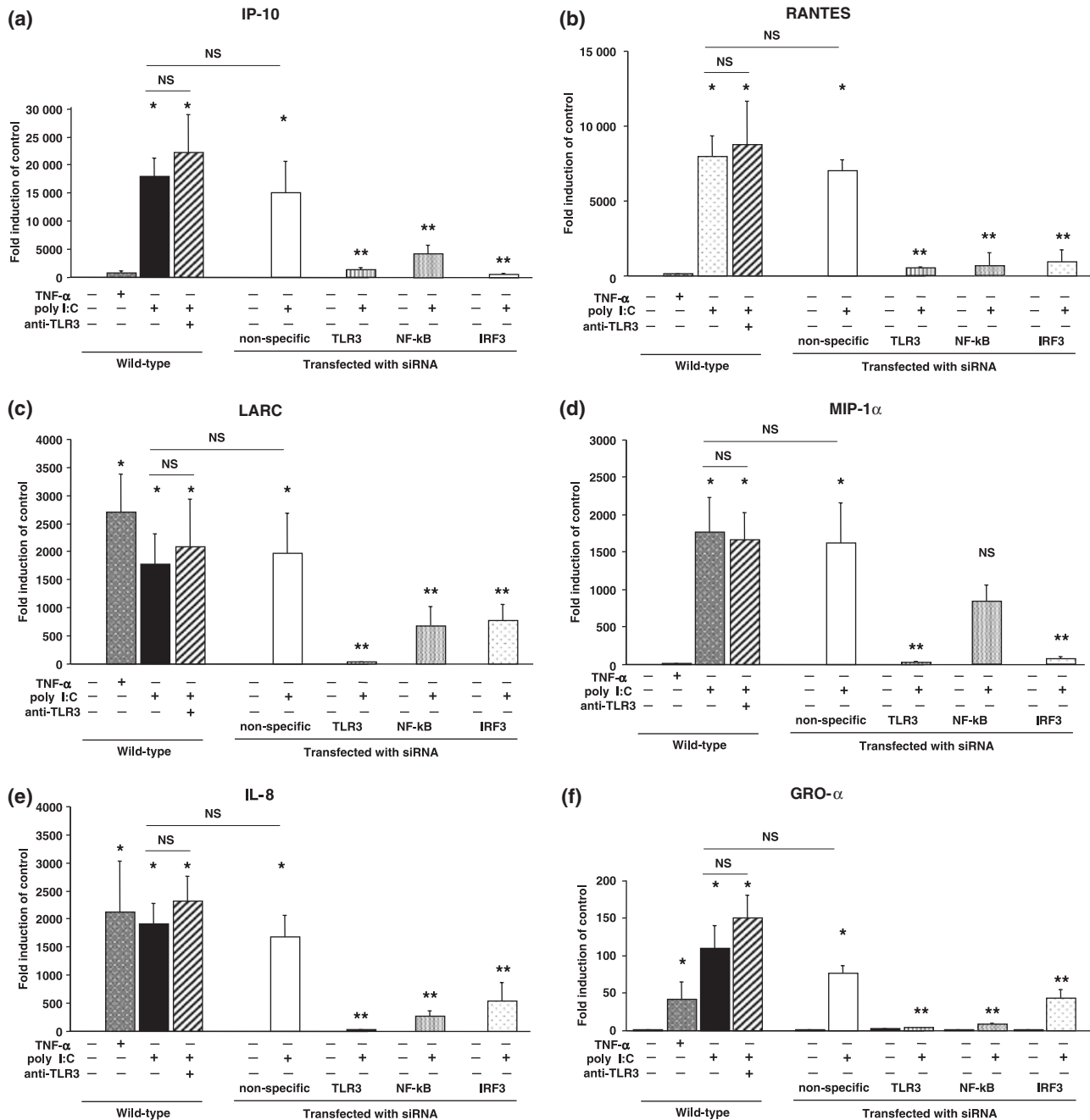
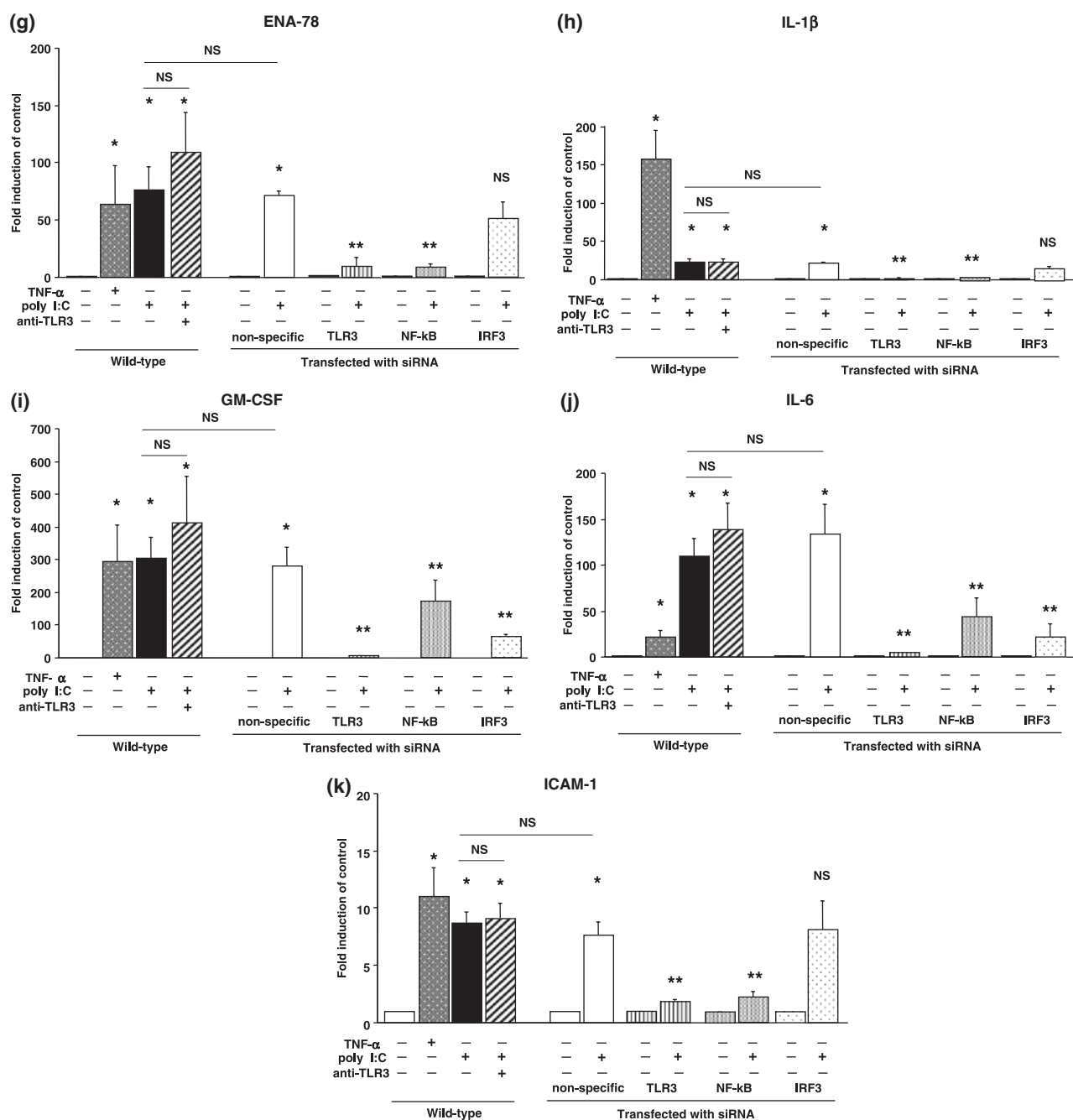


Fig. 5. (Continued)

Fig. 5. Effects of anti-TLR3 mAb or siRNA for TLR3, NF- κ B, or IRF3 on double-stranded (ds)RNA-induced expression of target genes. Wild-type BEAS-2B cells were stimulated with 10 ng/mL TNF- α or 50 μ g/mL poly I : C (synthetic dsRNA) with or without pre-incubation with 20 μ g/mL anti-TLR3 mAb. BEAS-2B cells were also transfected with non-specific control RNA or siRNA for TLR3, NF- κ B or IRF3 and stimulated with 50 μ g/mL poly I : C (synthetic dsRNA) for 24 h. RNA was extracted from the cells and subjected to real-time PCR for IP-10 (a), RANTES (b), LARC (c), MIP-1 α (d), IL-8 (e), GRO- α (f), ENA-78 (g), IL-1 β (h), GM-CSF (i), IL-6 (j) and ICAM-1 (k). Levels of mRNA were calculated as fold induction compared with unstimulated control. Data are presented as the mean $\pm$ SEM of a total of three independent experiments (* P < 0.05 as compared with non-stimulated cells. ** P < 0.05 as compared with cells transfected with non-specific control siRNA and then stimulated by dsRNA).

We previously reported that dsRNA stimulates RANTES promoter activity through cooperative activation of NF- κ B and IRF in airway epithelial cells [18]. In this report, we have shown the importance of NF- κ B and IRF3 in the induction of expression of several genes, including

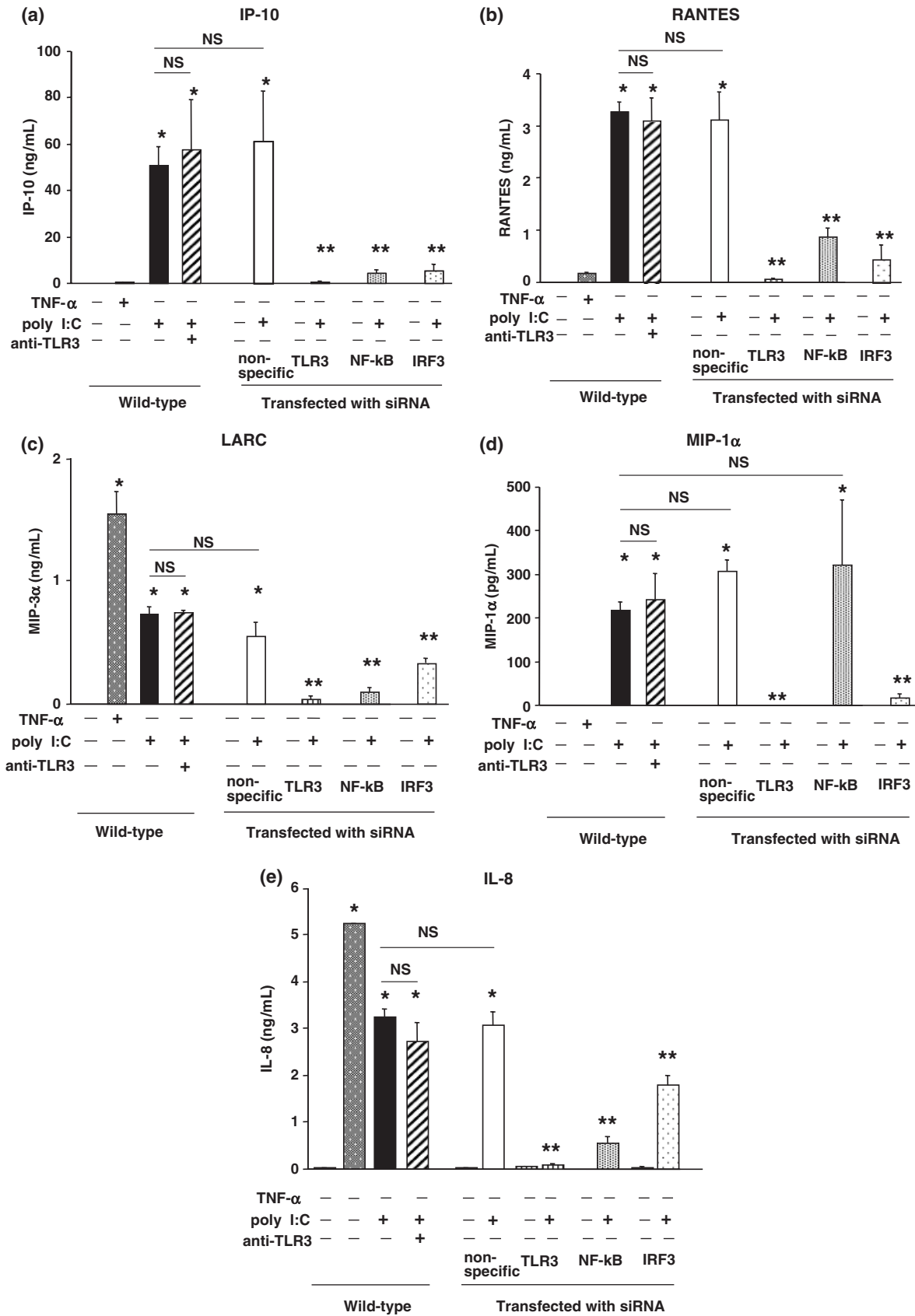
RANTES, in response to dsRNA in airway epithelial cells. Genes encoding other chemokines, such as IP-10 and IL-8, are known to contain both NF- κ B and IRF3 binding sites. Cooperative activation of target gene transcription by NF- κ B and IRF3 may yield the highest induction of gene



expression. There are several reports that indicate the activation of other transcription factors such as CREB, AP-1 and SRF (MAPK/ERK pathway) in TLRs signalling

[34–36]. In the present study, dsRNA did not activate reporter constructs for these factors, suggesting that these factors may not be involved in TLR3-mediated signalling in

Fig. 6. Effects of anti-TLR3 mAb or siRNA for TLR3, NF-κB, or IRF3 on double-stranded (ds)RNA-induced chemokine production into the culture media. Wild-type BEAS-2B cells were stimulated with 10 ng/mL TNF-α or 50 μg/mL poly I : C (synthetic dsRNA) with or without pre-incubation with 20 μg/mL anti-TLR3 mAb. BEAS-2B cells were also transfected with non-specific control RNA or siRNA for TLR3, NF-κB or IRF3 and stimulated with 50 μg/mL poly I : C (synthetic dsRNA) for 24 h. Supernatants of the cells were collected and subjected to ELISA for IP-10 (a), RANTES (b), LARC (c), MIP-1α (d), IL-8 (e), GRO-α (f), ENA-78 (g), IL-1β (h), GM-CSF (i), IL-6 (j) and ICAM-1 (k). Data are presented as the mean ± SEM of a total of three independent experiments (**P* < 0.05 as compared with non-stimulated cells. ***P* < 0.05 as compared with cells transfected with non-specific control siRNA and then stimulated by dsRNA).



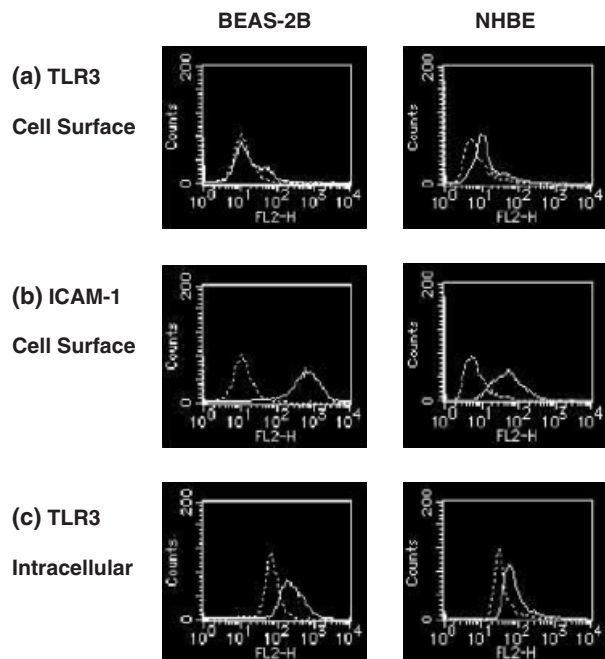


Fig. 7. Expression of TLR3 in the airway epithelial cells. BEAS-2B cells or normal human bronchial epithelial cells were incubated with human PE-labelled anti-TLR3 mAb, PE-labelled anti-ICAM-1 Ab or PE-labelled control IgG. For intracellular staining, cells were subjected to permeabilizing solution and then incubated with anti-TLR3 mAb or control IgG. Then cells were analysed by flow cytometry. Thick lines indicate as follows: (a) cell surface TLR3, (b) cell surface ICAM-1, (c) intracellular TLR3. Dot lines indicate control IgG.

response to dsRNA in BEAS-2B cells. NF- κ B and IRF3 may be major transcription factors in this signalling.

NF- κ B is a homodimer or heterodimer of Rel family proteins. The p50/p65 heterodimer may be the most important NF- κ B to activate transcription of inflammatory cytokines or chemokines. p50/p65 is sequestered in an inactive form in the cytoplasm by inhibitory molecules known as I κ B. It has been reported that NF- κ B regulates expression of a variety of genes and plays a crucial role in inflammatory diseases. Several different viruses induce activation and subsequent binding of NF- κ B to the promoters of target genes [37]. NF- κ B is believed to be a key transcription factor in the signalling of TLRs. NF- κ B has been reported to activate transcription of most of the genes examined in this study, including RANTES [38–40], IP-10 [41, 42], IL-8 [43], LARC [44], ENA-78 [45, 46], GM-CSF [47], GRO- α [48], ICAM-1 [49], IL-6 [50] and IL-1 β [51].

Transcription of MIP-1 α is also reported to be regulated by NF- κ B [52]; however, induction of MIP-1 α by dsRNA in airway epithelial cells does not depend on NF- κ B in our study. It is known that TNF- α activates NF- κ B and stimulates expression of several genes in airway epithelial cells. We have confirmed that TNF- α stimulates the expression of IP-10, RANTES, LARC, IL-8, GRO- α ,

Table 2. Chemokines are named by subclass motif and gene symbol number

Subclass motif and gene symbol number	Chemokine name	Receptors
CXCL1	GRO- α	CXCR1, 2
CXCL5	ENA-78	CXCR2
CXCL8	IL-8	CXCR1, 2
CXCL10	IP-10	CXCR3
CXCL12	SDF-1 α	CXCR4
CXCL16	SR-PSOX	CXCR6
CCL1	I-309	CCR8
CCL2	MCP-1	CCR2
CCL3	MIP-1 α	CCR1, 5
CCL5	RANTES	CCR1, 3, 5
CCL11	Eotaxin	CCR3
CCL13	MCP-4	CCR2, 3
CCL17	TARC	CCR4
CCL19	ELC/MIP-3 β	CCR7
CCL20	LARC/MIP-3 α	CCR6
CCL24	Eotaxin-2	CCR3
CCL26	Eotaxin-3	CCR3
CCL28	MEC	CCR3, 10
CX3CL1	Fractalkine	CX3CR1

Name and specific receptors of analysed chemokines are indicated.

GRO, growth-related oncogene; ENA, epithelial neutrophil-activating peptide; IL, interleukin; IP, inducible protein; SR-PSOX, scavenger receptor for phosphatidylserine and oxidized lipoprotein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T-cell expressed and secreted; LARC, liver and activation-regulated chemokine.

ENA-78, IL-1 β , GM-CSF, IL-6 and ICAM-1 in BEAS-2B cells. This expression was dependent on NF- κ B (data not shown). However, TNF- α did not significantly stimulate the expression of MIP-1 α in BEAS-2B cells. Previous data suggested that TNF- α did not stimulate the expression of MIP-1 α in airway epithelial cell line A549 [53]. These data may indicate that NF- κ B may not play a pivotal role in the regulation of MIP-1 α expression in airway epithelial cells.

IRF3 is an important transcriptional regulator of the anti-viral immune response. Viral infection causes phosphorylation and translocation of IRF3 into the nucleus [54]. IRF3 then binds to IFN-stimulated response element (ISRE) in the promoters of type I IFNs to stimulate transcription of these genes. IRF3 is also believed to be one of the key transcription factors in the signalling of TLRs. Other genes in the present study, including RANTES [18, 38, 55], IP-10 [41, 42], IL-6 [50], IL-8 [56, 57] and ICAM-1 [58], are known to have the ISRE motif in their promoters. To our knowledge, the presence of an ISRE in the promoters, including MIP-1 α , LARC, GRO- α and GM-CSF, has not been investigated. Further studies, including assays of promoter activities, are needed; however, our present results indicate that IRF3 may activate the

promoters of these chemokines and cytokines in response to dsRNA.

Taken together, our present findings indicate that synthetic dsRNA up-regulates expression of inflammatory cytokines and chemokines through the TLR3–NF- κ B and TLR3–IRF3 signalling in airway epithelial cells. These results might expand our understanding of the mechanisms of airway viral infection and subsequent airway inflammation; however, we need further studies including experiments using viruses and studies *in vivo*.

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