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# TLR3-Mediated Synthesis and Release of *Eotaxin-1/CCL11* from Human Bronchial Smooth Muscle Cells Stimulated with Double-Stranded RNA<sup>1</sup>

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Respiratory infections with RNA viruses, such as rhinovirus or respiratory syncytial virus, are a major cause of asthma exacerbation, accompanied by enhanced neutrophilic and/or eosinophilic inflammation of the airways. We studied the effects of dsRNA synthesized during RNA virus replication, and of its receptor, TLR3, on the synthesis of eosinophilic chemokines in bronchial smooth muscle cells (BSMC). Synthetic dsRNA, polyinosinic-cystidic acid (poly(I:C)), induced the synthesis of eosinophilic chemokines, eotaxin-1/CCL11 and RANTES/CCL5, from primary cultures of human BSMC, and IL-4 increased synergistically the synthesis of poly(I:C)-induced CCL11. A robust eosinophil chemotactic activity was released from BSMC stimulated with poly(I:C) and IL-4, which was mostly inhibited by preincubation with an anti-CCL11, but not with an anti-CCL5 Ab. Although the immunoreactivity of TLR3 was detectable on the cellular surface of BSMC by flow cytometric analysis, pretreatment with an anti-TLR3-neutralizing Ab failed to block the poly(I:C)-induced synthesis of CCL11. We have determined by confocal laser-scanning microscopy that the immunoreactivity of TLR3 was aggregated intracellularly in poly(I:C)-stimulated BSMC, colocalizing with fluorescein-labeled poly(I:C). The synthesis of *CCL11* was prominently inhibited by the transfection of TLR3-specific small interfering RNA or by bafilomycin A1, an endosomal acidification inhibitor, further supporting the essential role played by intracellular TLR3 in the synthesis of poly(I:C)-induced *CCL11* in BSMC. In conclusion, these observations suggest that, by activating intracellular TLR3 in BSMC, respiratory RNA virus infections stimulate the production of *CCL11* and enhance eosinophilic inflammation of the airways in the Th2-dominant microenvironment. *The Journal of Immunology*, 2007, 178: 489–495.

**A**cute exacerbations of asthma, characterized by disease manifestations such as wheezing and dyspnea, seriously disturb the quality of life of susceptible patients. The episodes are accompanied by worsening of airflow limitations; bronchial hyperresponsiveness; and increased neutrophilic, lymphocytic, and/or eosinophilic inflammation of the airways. Respiratory RNA viral infections are a major cause of acute exacerbation of asthma, and positive-sense ssRNA viruses, such as rhinovirus and coronavirus, are often isolated in the airways of adults during exacerbations, whereas negative-sense ssRNA viruses, including respiratory syncytial virus (RSV)<sup>3</sup> and para-

influenza virus, are the main pathogens causing wheezing bronchiolitis in children (1, 2).

Eosinophils play an important role in the virus-induced deterioration of asthma. The number of eosinophils and the concentration of eosinophil cationic protein in induced sputum have been correlated with an increase in bronchial responsiveness during experimental rhinovirus infections in adult asthmatics (3). A marked eosinophilic inflammation of the airways was observed in a substantial proportion of children with RSV bronchiolitis (4, 5), and the pathologic changes in the airways of mice with RSV infection mimicked eosinophilic bronchiolitis in humans, especially in allergen-sensitized and exposed animals (6, 7).

The induction of eosinophilic chemokine synthesis is one of the putative triggering mechanisms of eosinophilia in the airways during viral infection. Infections of respiratory epithelial cells with rhinovirus, RSV, or influenza virus are associated with the production of chemokines, such as eotaxin-1/CCL11 and RANTES/CCL5 (8–11). Eosinophilic inflammation in RSV-infected mice was accompanied by the production of CCL11 and MCP-3/CCL7, and was markedly reduced by treatment with an anti-CCL11 Ab (12, 13).

It has been demonstrated that dsRNA, as a virus genome fragment or as a replicative intermediate of RNA virus (14), induces the production of chemokines, such as CCL5, MCP-1/CCL2, eotaxin-3/CCL26, IL-8/CXCL8, and IFN-inducible protein-10/CXCL10 in respiratory epithelial cells (15–20). TLR3, a receptor for dsRNA, is expressed in respiratory epithelial cells, as well as in dendritic cells, mast cells, and fibroblasts (21–23). Bronchial smooth muscle cells (BSMC) are another major source of chemokines, including CCL5 and CCL11 (24–26), although little is known regarding the expression of TLR3 and responsiveness to

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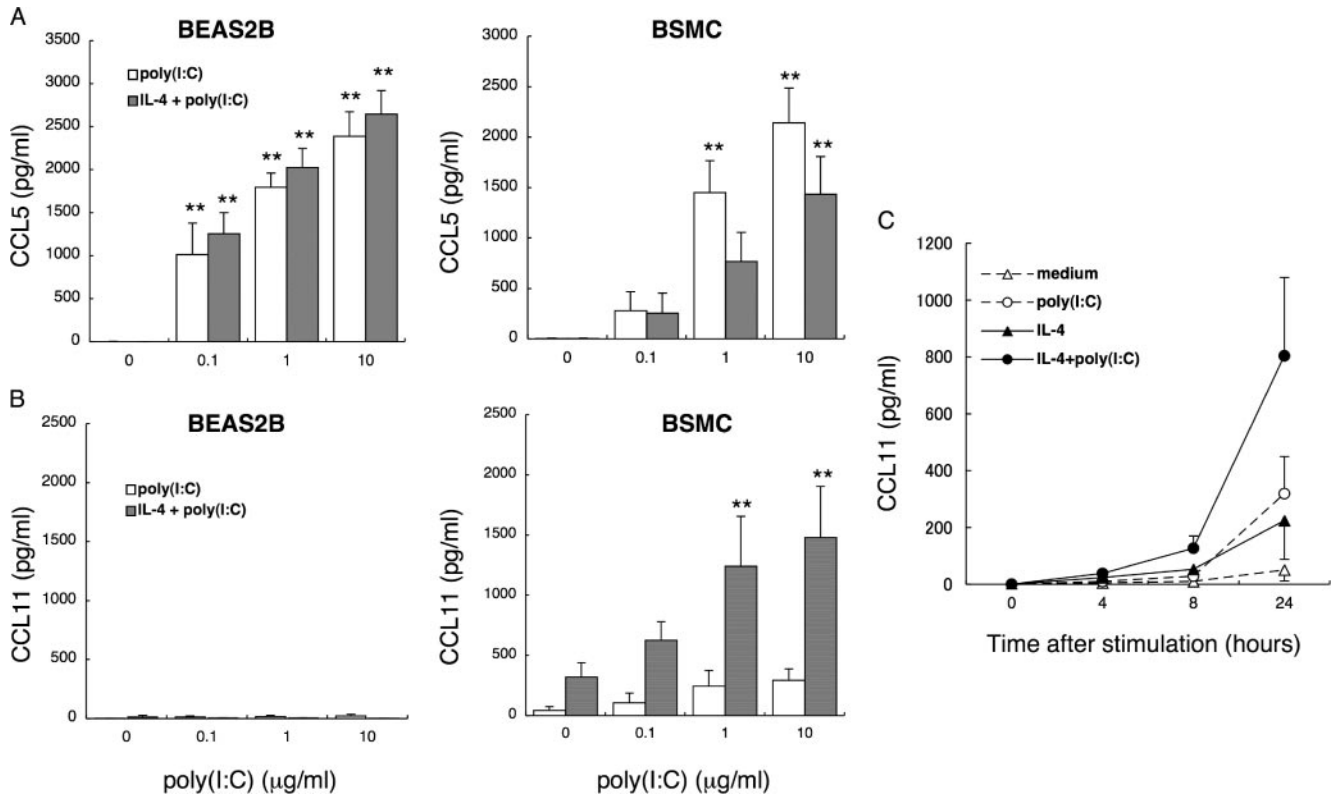
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<sup>3</sup> Abbreviations used in this paper: RSV, respiratory syncytial virus; BSMC, bronchial smooth muscle cell; EEA-1, early endosome Ag 1; poly(I:C), polyinosinic-cystidic acid; siRNA, small interfering RNA.

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**FIGURE 1.** RANTES/CCL5 (A) and eotaxin-1/CCL11 (B) concentrations in the culture supernatant of poly(I:C)-stimulated BEAS2B cells and BSMC. Cells were stimulated with poly(I:C) (0.1–10  $\mu\text{g/ml}$ ) for 24 h in presence or absence of 10 ng/ml IL-4. Mean  $\pm$  SEM from three duplicate experiments (BEAS2B), or four duplicate experiments using BSMC from different donors. \*\*,  $p < 0.005$  compared with the cells not stimulated with poly(I:C). C, Kinetics of CCL11 release from BSMC stimulated with 10  $\mu\text{g/ml}$  poly(I:C), or 10 ng/ml IL-4, or both. Mean  $\pm$  SEM from four duplicate experiments using BSMC from different donors.

dsRNA in this cell type. Therefore, we have examined, using primary cultures of human BSMC, the following: 1) whether dsRNA induces the production of eosinophilic chemokines and eosinophil chemotactic activity; 2) whether TLR3 is expressed in BSMC and its subcellular localization; and 3) whether the dsRNA-induced chemokine synthesis in BSMC is mediated by TLR3.

## Materials and Methods

### Cell culture

Primary cultures of normal human BSMC were obtained from Cambrex, and cultured in medium containing 5% FBS; 1 ng/ml human recombinant epidermal growth factor; 2 ng/ml fibroblast growth factor; and 10  $\mu\text{g/ml}$  insulin, gentamicin, and amphotericin B. BSMC from three to five different donors, at the fourth to sixth passage, were used for the experiments. A BEAS2B airway epithelial cell line was obtained from American Type Culture Collection, and maintained in DMEM/F12K medium containing 10% FBS.

### Measurements of CC chemokine release

Confluent BSMC or BEAS2B cells in 24-well plates were starved in absence of FBS and growth factors for 24 h, then stimulated with polyinosinic-cytidic acid (poly(I:C); Amersham Biosciences) for a designated period of time, in presence or absence of 10 ng/ml IL-4 (PeproTech) or 10 ng/ml IL-13 (R&D Systems). The concentration of CCL5, CCL11, CXCL8, CXCL10, and IL-6 in the culture supernatant was measured by ELISA (R&D Systems), according to the manufacturer's instructions.

### Quantitative RT-PCR

Total RNA was extracted from BSMC with an RNeasy Mini Kit (Qiagen). The amounts of *CCL11* and *TLR3* transcripts were determined by reverse transcription using SuperScript II (Invitrogen Life Technologies), followed by quantitative PCR amplification by the TaqMan method (ABI PRISM 7000; Applied Biosystems). The comparative threshold cycle method was

validated and used to interpret the results. Premixed PCR primers and TaqMan probes for human *CCL11*, TLR3, and *GAPDH* (Assay-on-Demand) were obtained from Applied Biosystems. Conditions for PCR were as follows: 1 cycle at 95°C for 9 min, 50 cycles at 95°C for 0.5 min, 60°C for 1 min, and 1 cycle at 72°C for 5 min.

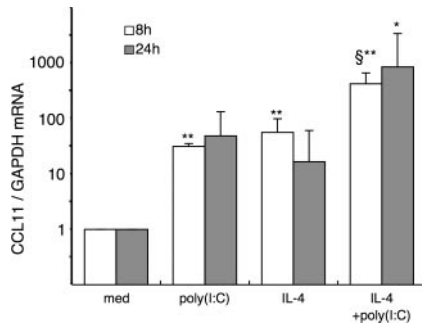
### Purification of peripheral blood eosinophils

Peripheral blood eosinophils were isolated from nonatopic healthy subjects, as described previously (27). Briefly, RBC were removed from 40 ml of heparinized peripheral blood using Dextran T-500 (Pharmacia) and mononuclear cells by centrifugation over 1.083 g/ml Histopaque (Sigma-Aldrich). After hypotonic cell lysis to remove any remaining RBC, neutrophils were removed by a CD16-negative selection method, using CD16-labeled magnetic microbeads (Miltenyi Biotec) and autoMACS (Miltenyi Biotec). Eosinophils ( $99 \pm 1\%$ , mean  $\pm$  SEM) were resuspended in RPMI 1640 supplemented with 10% FBS.

### Chemotactic activity for eosinophils

Confluent BSMC in six-well plates, growth arrested without FBS and growth factors for 24 h, were stimulated with 10  $\mu\text{g/ml}$  poly(I:C), or 10 ng/ml IL-4, or both. Culture supernatants were collected 24 h later, centrifuged for 15 min at 3000 rpm at 4°C, and stored at  $-80^\circ\text{C}$  for the subsequent experiments. In the chemokine-neutralizing experiments, the culture supernatant was incubated with 10  $\mu\text{g/ml}$  anti-CCL11-neutralizing Ab (R&D Systems), 10  $\mu\text{g/ml}$  anti-CCL5 Ab (R&D Systems), or both, at 37°C, for 1 h before the assay.

Eosinophil chemotactic activity was examined using a slightly modified method described previously (27, 28). In brief, 300  $\mu\text{l}$  of culture supernatants diluted 8-fold, or standard eosinophils ( $5 \times 10^2$ – $3 \times 10^4$  cells/well) were added to the lower compartment of a 96-well chemotaxis plate separated by a 3- $\mu\text{m}$ -pore-size filter (NeuroProbe), and eosinophils (50  $\mu\text{l}$ ,  $10^6$  cells/ml) were placed in the upper compartment. After 1 h of incubation at 37°C, the medium on the upper surface of the filter was wiped and replaced by 0.5 mM EDTA in PBS. After another hour of incubation, the plate was centrifuged at 1500 rpm at 4°C for 20 min, the filter was gently removed, and the supernatant was aspirated. The eosinophil peroxidase activity in the



**FIGURE 2.** *CCL11* mRNA levels adjusted by the amount of *GAPDH* mRNA in BSMC stimulated for 8 or 24 h with 10  $\mu\text{g/ml}$  poly(I:C), 10 ng/ml IL-4, or both combined. *CCL11/GAPDH* mRNA levels in unstimulated BSMC are defined as 1. Mean  $\pm$  SEM from four experiments using BSMC from different donors. \*,  $p < 0.05$ ; \*\*,  $p < 0.001$  compared with unstimulated cells. §,  $p < 0.05$  compared with IL-4-stimulated cells.

lower chamber was determined by incubating the plate for 10 min with substrate solution (0.5 mM *o*-phenylenediamine, 1 mM  $\text{H}_2\text{O}_2$ , and 0.1% Triton X-100 in Tris buffer (pH 8.0)), followed by the addition of 4 M  $\text{H}_2\text{SO}_4$  to stop the reaction, and the absorbance was measured at 490 nm.

#### Flow cytometric analysis of the expression of TLR3

BEAS2B cells or BSMC were resuspended in a concentration of  $10^7$  cells/ml in PBS containing 0.5% BSA and 20 mM EDTA; incubated for 1 h at 4°C with 1  $\mu\text{g}/100 \mu\text{l}$  anti-human TLR3 Ab (TLR3.7) (22) or 1  $\mu\text{g}/100 \mu\text{l}$  control mouse IgG1 (R&D Systems); washed twice in PBS; and incubated with FITC-labeled anti-mouse IgG F(ab')<sub>2</sub> (DakoCytomation) for 1 h at 4°C. A fraction of cells was prefixed with 2% paraformaldehyde in PBS (15 min, 37°C) and permeabilized with 0.1% saponin in PBS (15 min, 37°C) before the staining to examine the intracellular expression of TLR3. The labeled cells were analyzed using a FACScan flow cytometer (BD Biosciences).

#### Immunolocalization of TLR3 and poly(I:C) in BSMC by confocal laser-scanning microscopy

Poly(I:C) was labeled with fluorescein using a FastTag FL Kit (Vector Laboratories), according to the manufacturer's instructions. BSMC cultured on glass coverslips coated with poly(L-lysine) were incubated with poly(I:C) for 5 min at 37°C, fixed with 4% paraformaldehyde, and permeabilized with 0.1% saponin at room temperature. Fluorescein-labeled poly(I:C) was stained by serial incubation with an image-iT FX signal enhancer (Molecular Probes), 20  $\mu\text{g/ml}$  Alexa Fluor 488-conjugated rabbit anti-fluorescein IgG (Molecular Probes), and 20  $\mu\text{g/ml}$  Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) at room temperature. In another experiment, early endosome Ag 1 (EEA-1) in endosomes was stained with anti-EEA-1 Ab (10  $\mu\text{g/ml}$ ; GeneTex), followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG. After two washes with PBS, the cells were incubated for 1 h at room temperature with 20  $\mu\text{g/ml}$  anti-human

TLR3 Ab, or with control mouse IgG1 in 2% BSA/PBS, and washed with PBS. A total of 10  $\mu\text{g/ml}$  Alexa Fluor 568-conjugated rabbit anti-mouse IgG (Molecular Probes) and 10  $\mu\text{g/ml}$  Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes) was applied consecutively, followed by incubation with 1  $\mu\text{M}$  TO-PRO3 (Molecular Probes). The cells were then examined by confocal laser-scanning microscopy (LSM510; Zeiss).

#### Transfection of TLR3 small interfering RNA (siRNA) in BSMC

BSMC were cultured in 24-well plates at a density of  $2.5 \times 10^4$  cells/well overnight. TLR3 siRNA (5'-GGUUGGUAACGAUCCUUUGC-3') or randomly scrambled negative control siRNA was mixed with liposome (Lipofectamine 2000; Invitrogen Life Technologies), according to the manufacturer's instructions, and added to the culture medium containing FBS and growth factors. The cells were washed twice with PBS 72 h later, then stimulated for 3 h with FBS/growth factor-free medium containing either 10 ng/ml IL-4 only or 10  $\mu\text{g/ml}$  IL-4 plus poly(I:C). Total RNA was extracted, and quantitative RT-PCR was performed to determine the levels of *CCL11*, *TLR3*, and *TLR4* mRNA.

#### Statistical analysis

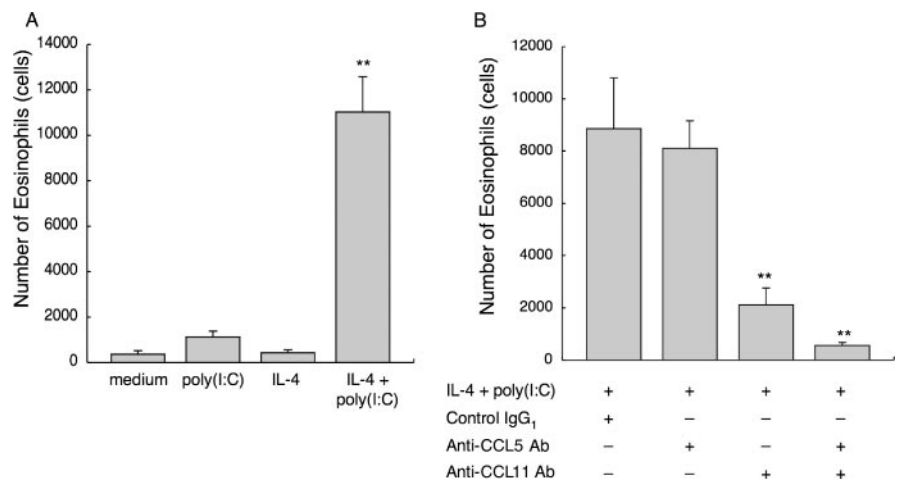
Data are presented as means  $\pm$  SEM. Chemokine concentrations in the supernatant, mRNA levels, and eosinophil chemotactic activities were tested with ANOVA, followed by Bonferroni/Dunn procedure as a post hoc test (STATVIEW 1992-98; SAS Institute). Dose-response relationships and kinetics of *CCL11* release were analyzed with repeated-measures ANOVA. A  $p$  value  $< 0.05$  was considered significant.

## Results

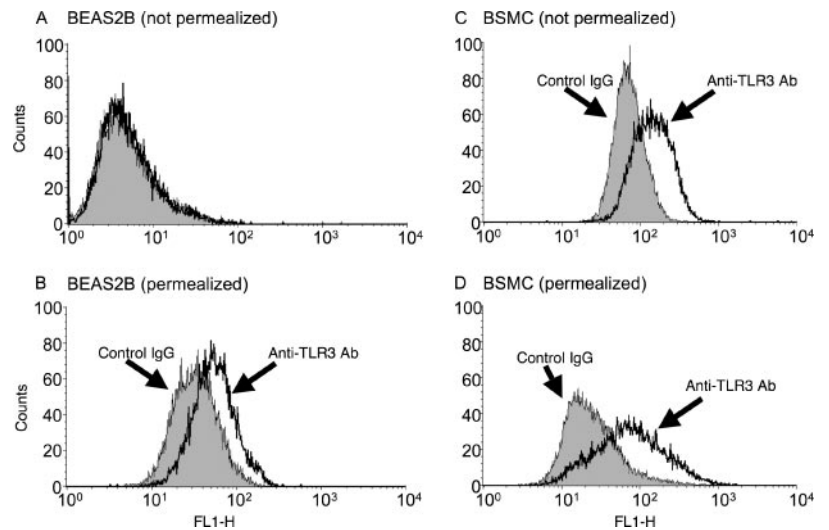
A prominent release of *CCL5* from BSMC was induced by 0.1–10  $\mu\text{g/ml}$  poly(I:C), for 24 h ( $p < 0.005$ ), similar to the release of *CCL5* from BEAS2B, a respiratory epithelial cell line (Fig. 1A). There was also a significant increase in the release of IL-6, CXCL8, and CXCL10 in BSMC stimulated with 10  $\mu\text{g/ml}$  poly(I:C), compared with those in unstimulated cells (IL-6,  $141 \pm 102$  in unstimulated cells vs  $1570 \pm 578$  pg/ml in poly(I:C)-stimulated cells,  $p < 0.01$ ,  $n = 5$ ; CXCL8,  $148 \pm 58$  vs  $1383 \pm 408$  pg/ml,  $p < 0.005$ ,  $n = 6$ ; CXCL10,  $3 \pm 2$  vs  $2598 \pm 631$  pg/ml,  $p < 0.001$ ,  $n = 6$ ). IL-4 had no synergistic effects on the production of these cytokines and chemokines (Fig. 1A and data not shown).

*CCL11* was also released from BSMC stimulated with poly(I:C) ( $p < 0.05$ ), but not from BEAS2B (Fig. 1B). In presence of 10 ng/ml IL-4, the poly(I:C)-induced release of *CCL11* from BSMC was further increased ( $p < 0.05$ ) compared with poly(I:C) alone (Fig. 1B). A total of 0.1–10  $\mu\text{g/ml}$  poly(I:C) also induced the release of *CCL11* in presence of 10 ng/ml IL-13 in a concentration-dependent fashion ( $p < 0.005$ , data not shown). The kinetics of *CCL11* release from BSMC are shown in Fig. 1C. Neither ssRNA, polycystidic acid, nor dsDNA, polydeoxyinosinic-deoxycystidic acid, up to a concentration of 100  $\mu\text{g/ml}$ , increased the IL-4-induced release of *CCL11* from BSMC (IL-4 alone,  $404 \pm 273$

**FIGURE 3.** A, Eosinophil chemotactic activity in the supernatant from BSMC stimulated with 10  $\mu\text{g/ml}$  poly(I:C), 10 ng/ml IL-4, or the combination for 24 h. Mean  $\pm$  SEM from four duplicate experiments. \*\*,  $p < 0.001$ . B, Neutralization of eosinophil chemotactic activity with anti-chemokine Abs. The supernatant from BSMC stimulated with 10  $\mu\text{g/ml}$  poly(I:C) and 10 ng/ml IL-4, for 24 h, was preincubated with an anti-CCL11 Ab, an anti-CCL5 Ab, or mouse IgG1 (control IgG1). Mean  $\pm$  SEM from four duplicate experiments. \*\*,  $p < 0.005$ .



**FIGURE 4.** Flow cytometric analysis of TLR3 expression in BEAS2B cells (A and B) and BSMC (C and D). The cells not permeabilized (A and C) or permeabilized (B and D) with 0.1% saponin were incubated with an anti-TLR3 Ab or control mouse IgG, followed by a fluorescein-labeled anti-mouse IgG Ab. Representative data from two to three separate experiments.



pg/ml; IL-4 plus polycystidic acid,  $469 \pm 380$  pg/ml; IL-4 + polydeoxyinosinic-deoxycystidic acid,  $598 \pm 522$  pg/ml,  $n = 3$ ). Preincubation of poly(I:C) for 1 h at 37°C with 10  $\mu$ g/ml polymyxin B did not modify the release of CCL11 from BSMC (data not shown), excluding an effect of LPS. Quantitative RT-PCR revealed that incubation for 8–24 h with either 10 ng/ml IL-4 or 10  $\mu$ g/ml poly(I:C) increased the concentrations of *CCL11* mRNA in BSMC significantly ( $p < 0.001$ ), and their combination synergistically enhanced the expression of *CCL11* mRNA ( $p < 0.05$ ; Fig. 2).

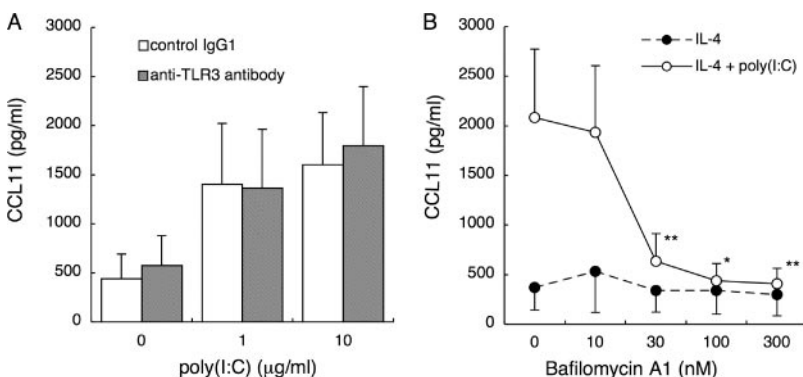
Because both CCL5 and CCL11 are potent chemoattractants of eosinophils, we examined whether the culture supernatant of stimulated BSMC exhibited a functional eosinophil chemotactic activity and, if it did, which chemokine was responsible for chemotaxis. The culture supernatant from BSMC stimulated with 10  $\mu$ g/ml poly(I:C) alone or 10 ng/ml IL-4 alone did not exhibit a significant increase in chemotactic activity for eosinophils compared with the supernatant from unstimulated BSMC (Fig. 3A). In contrast, the supernatant from BSMC costimulated with poly(I:C) and IL-4 revealed a significantly increased chemotactic activity ( $p < 0.001$ ). Preincubation of the supernatant with an anti-CCL11 Ab decreased the poly(I:C)/IL-4-induced eosinophil chemotactic activity by >85% ( $p < 0.005$ , Fig. 3B), whereas the preincubation with an anti-CCL5 Ab did not significantly inhibit eosinophil chemotaxis. This suggests that CCL11 is the major chemoattractant for eosinophils released from BSMC stimulated with poly(I:C) and IL-4.

Next, we studied the expression of the dsRNA receptor, TLR3, in BSMC by two approaches. RT-PCR showed the constitutive expression of *TLR3* mRNA in the BSMC as well as BEAS2B (data

not shown). Flow cytometric analysis also showed the presence of TLR3 immunoreactivity on the cellular surface of BSMC (Fig. 4C), in sharp contrast with BEAS2B cells, in which TLR3 immunoreactivity was detectable only when they were permeabilized with saponin (Fig. 4, A and B).

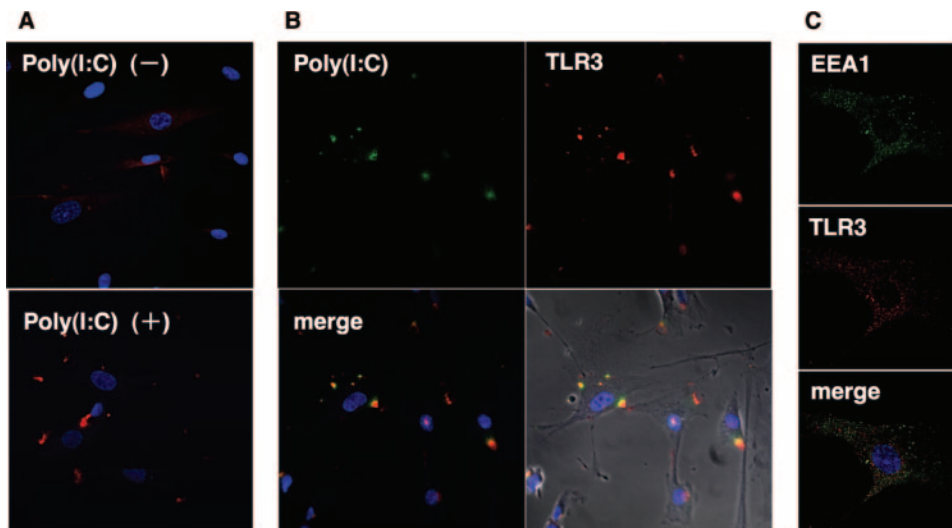
To determine the role of TLR3 in the poly(I:C)-induced synthesis of CCL11 in BSMC, 10  $\mu$ g/ml anti-TLR3-neutralizing Ab was added to the culture medium 1 h before stimulation with poly(I:C) and IL-4. We observed no effect of the anti-TLR3-neutralizing Ab on the release of CCL11 from IL-4/poly(I:C)-stimulated BSMC (Fig. 5A). In contrast, bafilomycin A1, an endosome acidification inhibitor, prominently inhibited the release of CCL11 from BSMC stimulated with IL-4 plus poly(I:C) ( $p < 0.01$ , Fig. 5B). Bafilomycin A1 had no effects on the CCL11 release induced by IL-4 alone, suggesting that the acidification of endosomes is needed for the action of poly(I:C). This suggests that the poly(I:C)-induced CCL11 release is not mediated via TLR3 on the cell surface, but via intracellular receptors.

We confirmed by confocal laser-scanning microscopy that TLR3 and exogenously administered poly(I:C) were present inside BSMC. Although, in unstimulated BSMC, the immunoreactivity of TLR3 was distributed throughout the cytoplasm, we observed a strong intracellular aggregation of TLR3 immunoreactivity 5 min after stimulation with poly(I:C) (Fig. 6A). Fluorescein-labeled poly(I:C) was also observed in the cytoplasm, and colocalized with TLR3 immunoreactivity (Fig. 6B). TLR3 immunoreactivity in poly(I:C)-stimulated BSMC was also colocalized with EEA-1, an endosomal marker (Fig. 6C).



**FIGURE 5.** A, Effects of an anti-TLR3-neutralizing Ab on poly(I:C)/IL-4-induced release of CCL11 from BSMC. Anti-TLR3-neutralizing Ab or control mouse IgG1 was added 1 h before the 24-h stimulation with 10  $\mu$ g/ml poly(I:C) and 10 ng/ml IL-4. Mean  $\pm$  SEM from three duplicate experiments, using BSMC from different donors. B, Effects of bafilomycin A1 on IL-4-induced or poly(I:C)/IL-4-induced release of CCL11 from BSMC. Bafilomycin A1 inhibited the release of CCL11 from BSMC stimulated with the combination of 10 ng/ml IL-4 and 10  $\mu$ g/ml poly(I:C), but not with IL-4 alone. Mean  $\pm$  SEM from the experiments using BSMC from four different donors. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  compared with the cells untreated with bafilomycin A1.

**FIGURE 6.** Immunolocalization of TLR3 and poly(I:C) in BSMC by confocal laser-scanning microscopy. *A*, TLR3 immunoreactivity (red) in unstimulated and poly(I:C)-stimulated BSMC (10  $\mu$ g/ml, for 5 min). *B*, Coaggregation of TLR3 (red) and poly(I:C) (green) in poly(I:C)-stimulated BSMC (10  $\mu$ g/ml, for 5 min). *C*, Coaggregation of TLR3 (red) and EEA-1 (green), an endosomal marker, in poly(I:C)-stimulated BSMC (10  $\mu$ g/ml, for 5 min). Representative data from three separate experiments.



Finally, we examined the effects of TLR3 siRNA on the poly(I:C)-induced *CCL11* mRNA expression in BSMC. Compared with liposome alone or negative control siRNA, 20 nM TLR3-specific siRNA suppressed the expression of *TLR3* mRNA by >80% ( $n = 5$ ;  $p < 0.01$ ), but did not change *TLR4* mRNA expression (Fig. 7A). A total of 10  $\mu$ g/ml poly(I:C), in presence of negative control siRNA, increased the expression of *CCL11* mRNA in IL-4-treated BSMC by  $4.7 \pm 1.1$ -fold. In contrast, the increase in the expression of *CCL11* was only  $1.7 \pm 0.2$ -fold in presence of TLR3

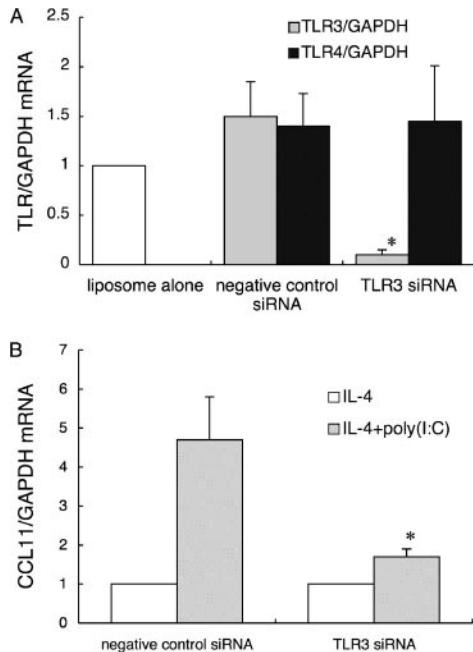
siRNA, significantly less than in presence of negative control siRNA ( $n = 5$ ;  $p < 0.05$ ; Fig. 7B).

## Discussion

We have shown that synthetic dsRNA, poly(I:C), and the Th2 cytokine, IL-4, synergistically induced the release of vigorous eosinophil chemotactic activity from primary cultures of human BSMC, an effect mostly mediated by the CC chemokine, CCL11. We have also shown that the activation of intracellular TLR3, a receptor for dsRNA, was required for the poly(I:C)-mediated induction of CCL11 synthesis in BSMC.

A synergism between a Th2-dominant microenvironment and RNA virus infections on the increase in eosinophilia in the airways has been suggested from the results of experiments in vivo (6, 29), although the exact mechanism has not been determined. Previous studies have shown that poly(I:C) or virus-derived dsRNA increases the production of cytokines and chemokines, including IL-6, CCL5, CXCL8, and CXCL10 in primary cultures or immortalized bronchial epithelial cells, such as BEAS2B (15–20). In the present study, we have confirmed that poly(I:C) is also a potent stimulant of the synthesis of CCL5 in BSMC, as well as of IL-6, CXCL8, and CXCL10. The most significant difference observed between BSMC and BEAS2B cells was the production of important amounts of CCL11 by BSMC in presence of IL-4, whereas BEAS2B cells did not produce CCL11, regardless of the presence of IL-4. Synergism between poly(I:C) and Th2 cytokines is also observed in other cell types, including bronchial epithelial cells; it has been reported that poly(I:C) induced eotaxin-3/CCL26 synthesis in BEAS2B cells in the presence of IL-4 (20). More importantly, CCL11, but not CCL5, mediated most of the eosinophil chemotactic activity in the culture supernatant of the poly(I:C)/IL-4-stimulated BSMC. These observations suggest that, in the Th2-dominant microenvironment, respiratory virus infections can exacerbate the eosinophilic inflammation of the airways via the production of CCL11, as observed in a proportion of patients with RSV infections (5).

It has been reported recently that RSV infection in mice with a TLR3 deletion results in increased eosinophil accumulation in the airway accompanied by an increase in pulmonary IL-5 and IL-13 expression (30). We have also observed that poly(I:C) administered simultaneously with aerosolized allergen attenuated airway eosinophilia in sensitized rats, accompanied by an increase in



**FIGURE 7.** Effects of TLR3 siRNA on gene expression in BSMC. *A*, Effects on the expression of *TLR3* and *TLR4* mRNA expression. TLR3 siRNA specifically suppressed the expression of *TLR3* mRNA. Mean  $\pm$  SEM from five duplicate experiments, using BSMC from different donors. \*,  $p < 0.05$  compared with negative control siRNA. *B*, Effects on the expression of poly(I:C)-induced *CCL11* mRNA expression. BSMC was stimulated with 10 ng/ml IL-4, or with the combination of 10  $\mu$ g/ml IL-4 and poly(I:C). *CCL11* mRNA levels in BSMC stimulated with IL-4 alone are presented as 1. Mean  $\pm$  SEM from five duplicate experiments, using BSMC from different donors. \*,  $p < 0.05$  compared with negative control siRNA.

IFN- $\gamma$  and IL-12 and a decrease in IL-13 in bronchoalveolar lavage fluid. In contrast, poly(I:C) administered in rats 16 h after allergen exposure, at the time when Th2 responses have already been established, significantly increased the number of eosinophils in bronchoalveolar lavage fluid (Y. Shiraishi, K. Asano, K. Niimi, K. Fukunaga, M. Wakaki, J. Kagyo, T. Takihara, T. Nakajima, T. Oguma, Y. Suzuki, T. Shiomi, K. Sayama, E. Ikeda, H. Hirai, M. Nakamura, and A. Ishizaka, manuscript in preparation). These findings as well as the results of the present study suggest that the activation of TLR3 exacerbates the pre-existed Th2-dependent eosinophilic airway inflammation, although it undermines the development of Th2 responses.

Although the major cellular target for the respiratory RNA virus are the upper and lower respiratory epithelial cells, rhinovirus, RSV, or parainfluenza virus can infect primary cultures of respiratory or gastrointestinal smooth muscle cells directly, and modulate various cellular functions (31–33). For example, the administration of poly(I:C) or RSV infection in colonic smooth muscle cells promoted the adhesion of leukocytes on these cells (33). During the revision of this manuscript, Oliver et al. (34) reported that rhinovirus can infect and replicate in nonasthmatic and asthmatic BSMCs, and induced the expression of IL-6 and CXCL8. Viral infection might also have damaged the infected epithelial cells, causing the leak of virus-derived dsRNA that stimulates surrounding cells, including smooth muscle cells. Epithelial cells in the airways of asthmatics can release even larger amounts of viral RNA than epithelial cells from normal airways, because the impaired synthesis of IFN- $\beta$  in asthmatic epithelial cells allows a greater viral replication and cellular necrosis (not apoptosis) accompanied by membrane disruption (35). It is further supported by the recent finding that the leakage of rhinovirus-derived RNA into the blood during upper respiratory rhinovirus infections is more prominent in asthmatics than nonasthmatics (36). Besides viral RNA, cellular mRNA leaked from, or associated with, necrotic cells is also capable of activating TLR3 with its secondary structures that contain double-stranded sequences (37).

The subcellular localization of TLR3 differs markedly among various cell types. Its expression is limited to the intracellular compartment in dendritic and mast cells (21, 23), whereas, in fibroblasts, it is expressed on the cell surface, and can be blocked with an anti-TLR3 Ab (22). The localization of TLR3 in respiratory epithelial cells remains controversial. By flow cytometric analysis, its immunoreactivity is limited to the intracellular space, as in dendritic and mast cells, although the anti-TLR3 Ab can partially inhibit the synthesis of poly(I:C)-induced cytokine (38, 39). Although we were able to confirm the expression of TLR3 immunoreactivity on the surface of BSMC by flow cytometric analysis, the anti-TLR3-neutralizing Ab failed to suppress the synthesis of CCL11 in poly(I:C)-stimulated BSMC in the concentration that successfully blocked the synthesis of IFN(I:C)-stimulated fibroblasts (22). This suggests that poly(I:C) is recognized in BSMC by intracellular TLR3 or other cytoplasmic dsRNA receptors, such as protein kinase R or retinoic acid-inducible gene-I (15, 40). Because bafilomycin A1, a vacuolar H<sup>+</sup>-ATPase inhibitor, inhibits the acidification of endosome and suppresses the intracellular uptake of poly(I:C) (41), the suppression of the synthesis of CCL11 in poly(I:C)-stimulated BSMC by treatment with bafilomycin A1 further confirmed the interaction between intracellular receptor(s) and poly(I:C).

We next examined whether TLR3 mediates the poly(I:C)-induced synthesis of CCL11 in BSMC. Although TLR3 mediates the cellular response to RSV and influenza virus in the respiratory epithelial cells and fibroblasts (38, 39, 42), other cytoplasmic receptors for dsRNA, such as protein kinase R and retinoic acid-

inducible gene-I, play a major role in different situations (15, 40). First, we showed that poly(I:C) was coaggregated with TLR3 intracellularly, which is possibly located in endosomes. The inhibitory effects of bafilomycin A1 suggest that the endosomes are the site of interaction between poly(I:C) and TLR3. A recent analysis using TLR3-CD32 chimeric receptors has also suggested that an acidic pH, observed in endosomes and phagolysosomes, is necessary for the multimerization and signaling of TLR3 (43). Second, we have shown that targeting the expression of TLR3 with a TLR3-specific siRNA prominently decreased the poly(I:C)-induced mRNA expression of *CCL11* in BSMC. Therefore, we have concluded that the poly(I:C)-induced synthesis of CCL11 in BSMC is mediated, at least partially, by intracellular TLR3.

In conclusion, our observations suggest that BSMC participate in the pathogenesis of exacerbation of inflammation during respiratory viral infections in asthmatic patients. The cellular tropism of the virus, extent of damage in infected epithelial cells, and the airway microenvironment are the main factors that determine the type and intensity of inflammation.

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## Disclosures

The authors have no financial conflict of interest.

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