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Original article

Epithelial-mesenchymal transition promotes reactivity of human lung adenocarcinoma A549 cells to CpG ODN

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EMT	epithelial-mesenchymal transition
ODN	oligodeoxynucleotides
TLR	Toll-like receptor

ABSTRACT

Background: Epithelial-mesenchymal transition (EMT) is reported to promote airway remodeling in asthmatics, which is the main histological change that causes complex and severe symptoms in asthmatics. However, little is known about whether EMT also plays a role in acute exacerbations of asthma evoked by respiratory tract infections.

Methods: A human lung adenocarcinoma line, A549, was incubated with TGF-β1 at 10 ng/ml to induce EMT. Then the cells were stimulated with CpG ODN. Expression of surface and intracellular molecules was analyzed by flow cytometry. IL-6, IL-8 and MCP-1 in the culture supernatant were measured by Cytometric Bead Assay, and the expression of mRNA was quantitated by real-time PCR. CpG ODN uptake was analyzed by flow cytometry.

Results: The culture supernatant levels of IL-6, IL-8 and MCP-1 and the expression of mRNA for these cytokines in CpG ODN-stimulated A549 cells that had undergone EMT was significantly higher compared to those that had not. Addition of ODN H154, a TLR9-inhibiting DNA, significantly suppressed the CpG ODN-induced production of those cytokines. However, flow cytometry found the level of TLR9 expression to be slightly lower in A549 cells that had undergone EMT compared to those that had not. On the other hand, CpG ODN uptake was increased in cells that had undergone EMT.

Conclusions: EMT induction of A549 cells enhanced CpG ODN uptake and CpG ODN-induced production of IL-6, IL-8 and MCP-1. These results suggest that EMT plays an important role in exacerbation in asthmatics with airway remodeling by enhancing sensitivity to extrinsic pathogens.

Introduction

Bronchial asthma is an allergic disease of the airways that is increasing throughout the world, with significant impacts on public health. Based on WHO estimates, 334 million people suffer from asthma,[1] and in Japan the prevalence rates of asthmatic symptoms and current asthma among Japanese adults have been reported to be as high as 10.1% and 4.2%, respectively.[2] While most asthmatics' symptoms can be controlled well with inhaled corticosteroids, symptoms of severe asthmatics remain difficult to control and can even become fatal, especially when acute exacerbation is evoked. The known potential causes of acute exacerbation include infections, allergens, occupational exposure, hormones, drugs, exercise, stress and air pollutants.[3] Among these, bacterial infection is reported to be frequently involved in asthma exacerbation in adults[4,5] as well as young children.[6]

Especially in severely asthmatic bronchi, airway remodeling is often identified as one of the main histological changes, together with airway inflammation. Histopathological findings of airway remodeling include goblet cell metaplasia/hyperplasia, subepithelial fibrosis, reticular basement membrane thickening and extracellular matrix deposition, increased airway smooth muscle mass and vascular changes.[7] Airway remodeling leads to irreversible airflow limitation[8] and airway hyperresponsiveness,[9] which are the main physiological changes seen in severe asthmatics. Indeed, the degrees of subepithelial layer thickening,[10] fibroblast accumulation and airway smooth muscle hypertrophy[11] are associated with the severity of asthma. One of the underlying causes of these histological changes is thought to be epithelial-mesenchymal transition (EMT).[12,13]

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EMT is a biologic process in which epithelial cells are phenotypically transformed into mesenchymal cells.[14,15] EMT is commonly classified into three types: type 1 is seen in organ development, type 2 is associated with tissue regeneration and organ fibrosis and type 3 occurs in tumor invasion and metastasis.[14–16] Recent studies suggest that type 2 EMT promotes airway remodeling in asthmatics[12,13] and that a potent *in vitro* EMT-inducer, TGF-β,[17] is associated with airway remodeling in asthma.[18] However, little is known whether or not, and how, changes in the characteristics of airway epithelial cells caused by EMT may be involved in acute exacerbation of asthma evoked by respiratory tract infections.

In the first line of defense against microbial pathogens, Toll-like receptors (TLR) recognize pathogen-associated molecular patterns leading to activation of the innate immune system.[19] It is widely accepted that unmethylated CpG motifs are recognized by TLR9 and act as pathogen-associated molecular patterns,[20–22] and synthetic oligodeoxynucleotides with species-specific CpG DNA motifs (CpG ODN) show the same effect as naturally-occurring CpG.[22] Therefore, we used CpG ODN in the present study and show that TGF-β1–induced EMT of a human lung adenocarcinoma line, A549, enhanced IL-6, IL-8 and MCP-1 production in response to CpG ODN.

Methods

Reagents

The following reagents were purchased as indicated: recombinant human TGF-β1 (PEPRO TECH, Rocky Hill, NJ, USA) (Sigma–Aldrich, St. Louis, MO, USA); ODN 2006 (B-class CpG ODN, 5′tcgtcgttttgtcgttttgtcgtt3′), control ODN (B-class ODN, 5′tgctgcttttgtgcttttgtgctt3′), ODN H154 (5′cctcaagcttgagggg3′)[22] and FAM-conjugated CpG ODN (Sigma–Aldrich); PBS and FBS (Thermo Fisher Scientific, Waltham, MA, USA); and D-MEM/Ham's F-12 medium (Wako Pure Chemical Industries, Osaka, Japan); and dexamethasone and ethanol (Wako Pure Chemical Industries).

The following antibodies were purchased as indicated: PE-conjugated mouse anti-human E-cadherin mAb (IgG1κ, clone 67A4) (BD Biosciences, San Jose, CA, USA); APC-conjugated mouse anti-human N-cadherin mAb (IgG1κ, clone 8C11), APC-conjugated rat anti-human TLR9 mAb (IgG2aκ, clone eB72-1665), APC-conjugated mouse anti-human CD14 mAb (IgG1κ, clone 61D3), PE-conjugated mouse IgG1κ (clone P3.6.2.8.1), APC-conjugated mouse IgG1κ (clone P3.6.2.8.1) and APC-conjugated rat IgG2aκ (clone eBR2a) (eBioscience, San Diego, CA, USA); PE-Cy7-conjugated mouse anti-human CD205 mAb (IgG1κ, clone HD30) (Miltenyi Biotec, Bergisch Gladbach, Germany); and PE-Cy7-conjugated mouse IgG1κ (clone MOPC-21) (BioLegend, San Diego, CA, USA).

Cell line and culture

A human lung adenocarcinoma line, A549, was cultured in D-MEM/Ham's F-12 medium containing 5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C, 5% CO_2 until the experiments.

For induction of EMT, A549 at a cell density of 5×10^4 cells/ml was seeded with TGF-β1 at 10 ng/ml in D-MEM/Ham's F-12 medium containing 5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated for 3 days at 37 °C, 5% CO_2.

Flow cytometric analysis

Expression of cell-surface E-cadherin, N-cadherin, CD14 and CD205 was analyzed by flow cytometry. After incubation with or without TGF-β1, as previously described, 1×10^5–10^6 cells were washed with FACS buffer (PBS containing 3% FBS), blocked with human IgG at 1 μg/ml and incubated with 25 μg/ml of PE-conjugated mouse anti-human E-cadherin mAb/APC-conjugated mouse anti-human N-cadherin mAb or isotype control in FACS buffer on ice for 30 min. After washing with FACS buffer, the cells were resuspended in FACS buffer and analyzed by FACSVerse (BD Biosciences). For each sample, at least 10,000 events were collected, and dot plots and histograms were generated using FlowJo (Tree Star Inc., Ashland, OR, USA).

Expression of intracellular TLR9 was analyzed by flow cytometry. After incubation with or without TGF-β1, 1×10^5–10^6 cells were washed with FACS buffer and blocked with human IgG at 1 μg/ml, followed by fixation and permeabilization using Fixation/Permeabilization Solution (BD Biosciences) according to the manufacturer's protocol. The cells were washed and then incubated with 20 μg/ml of APC-conjugated rat anti-human TLR9 mAb or isotype control in Perm/Wash buffer on ice for 30 min. Then the cells were resuspended in the FACS buffer and analyzed by FACSVerse.

To analyze the cellular uptake of CpG, EMT-induced cells were incubated with FAM-conjugated CpG ODN or unconjugated CpG ODN at 10 μg/ml in D-MEM/Ham's F-12 medium for 24 h at 37 °C and shielded from light. Then 1×10^5–10^6 cells were washed with FACS buffer and analyzed by FACSVerse.

Quantitation of A549-derived cytokines

EMT-induced cells in D-MEM/Ham's F-12 medium were incubated with CpG ODN or control ODN at 1, 3, 10 and 30 μg/ml for 48 h for concentration analysis, or at 10 μg/ml for 6, 12, 24 and 48 h for time-course analysis. The levels of IL-6, IL-8 and MCP-1 in the culture supernatants were measured by Cytometric Bead Assay (CBA; BD Biosciences) according to the instructions of the manufacturer. The supernatants were stored at −80 °C until assay. For inhibition assay, EMT-induced cells were incubated with CpG ODN at 10 μg/ml and ODN H154 at 0, 1, 3, 10, 30 and 100 μg/ml or dexamethasone at 0, 1, 10, and 100 pM for 24 h, and the culture supernatants were subjected to CBA.

Real-time quantitative PCR analysis

EMT-induced cells were incubated with CpG ODN or control ODN at 10 μg/ml for 4 h. Then the total RNA was extracted from the cultured cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted mRNA was reverse–transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was performed using primers and Taqman probes designed by Thermo Fisher Scientific, Inc. Data were calculated by the ΔΔCt method, using the cDNA and, as a reference, β-actin cDNA. The relative quantitation (RQ) values were calculated using the following equation: $RQ = 2^{-\Delta\Delta Ct}$.

Statistics

Statistical analysis was performed using an unpaired two-tailed t-test for pair-wise comparisons or by ANOVA with Tukey's test for multiple comparisons. Statistical significance was defined as a P value of less than 0.05. All data are expressed as the mean ± SD of 3–6 independent experiments.

Results

TGF-β1 induced EMT in A549 cells

EMT induction by TGF-β1 in A549 alveolar epithelial cells has been established.[16] Using our materials, after incubation with TGF-

β1 the cells showed the typical morphology of the mesenchymal phenotype: cell elongation, loss of cell polarity and reduced cellular adhesion (data not shown). Additionally, we used flow cytometry to analyze for E-cadherin and N-cadherin cell-surface markers, which are negative and positive markers, respectively, of the mesenchymal cell-type.[17,23,24] As reported, A549 cells cultured with TGF-β1 showed reduced E-cadherin expression and increased N-cadherin expression (Fig. 1A) compared with the TGF-β1–unstimulated control cells, and the percentage of E-cadherin-low, N-cadherin-high cells quantitated using dot plots was significantly increased (Fig. 1B). These results indicate that TGF-β1 successfully induced EMT in A549 cells.

EMT induction enhanced CpG ODN-induced production of IL-6, IL-8 and MCP-1 by A549 cells

Next, we investigated the effect of EMT on the responsiveness of A549 cells to CpG ODN. As shown in Figure 2A, IL-6, IL-8 and MCP-1 production was significantly increased with CpG ODN at 3–30 μg/ml in A549 cells that had undergone EMT, but not in those that had not (Fig. 2A). Time-course analysis showed that the difference in production of those cytokines reached a maximum at 48 h after treatment with CpG ODN (Fig. 2B). Similarly, expression of mRNA for those cytokines by CpG ODN in EMT-induced A549 cells started to increase within 1 h after the addition of CpG ODN (data not shown), and it became statistically significant at 4 h (Fig. 2C). Such enhancement of mRNA expression by CpG ODN was not observed in A549 cells without EMT induction (Fig. 2C). We have to note that the baseline cytokine/chemokine production was upregulated only by EMT induction, even without CpG ODN stimulation, although it was not statistically significant (Fig. 2A,C). However, interaction between TGF-β1 treatment and CpG ODN stimulation was statistically significant for each cytokine/chemokine at the protein (Fig. 2A, $P < 0.001$) and mRNA (Fig. 2C, $P < 0.01$) levels, indicating a synergistic effect of EMT induction and CpG ODN stimulation, even when we take into consideration the upregulation of baseline cytokine/chemokine production by EMT induction. Therefore, we conclude that EMT induction enhanced CpG ODN-induced production of IL-6, IL-8 and MCP-1 by A549 cells at the transcriptional level.

ODN H154, a DNA inhibitor for TLR9, suppressed production of IL-6, IL-8 and MCP-1 by EMT-induced A549 cells in response to CpG ODN

TLR9 is a receptor for CpG DNA that mediates the cellular response to CpG DNA[20–22] and also recognizes synthetic ODNs that contain species-specific CpG motif signaling.[22] Therefore, we next used ODN H154, a selective DNA inhibitor for TLR9,[25,26] to elucidate

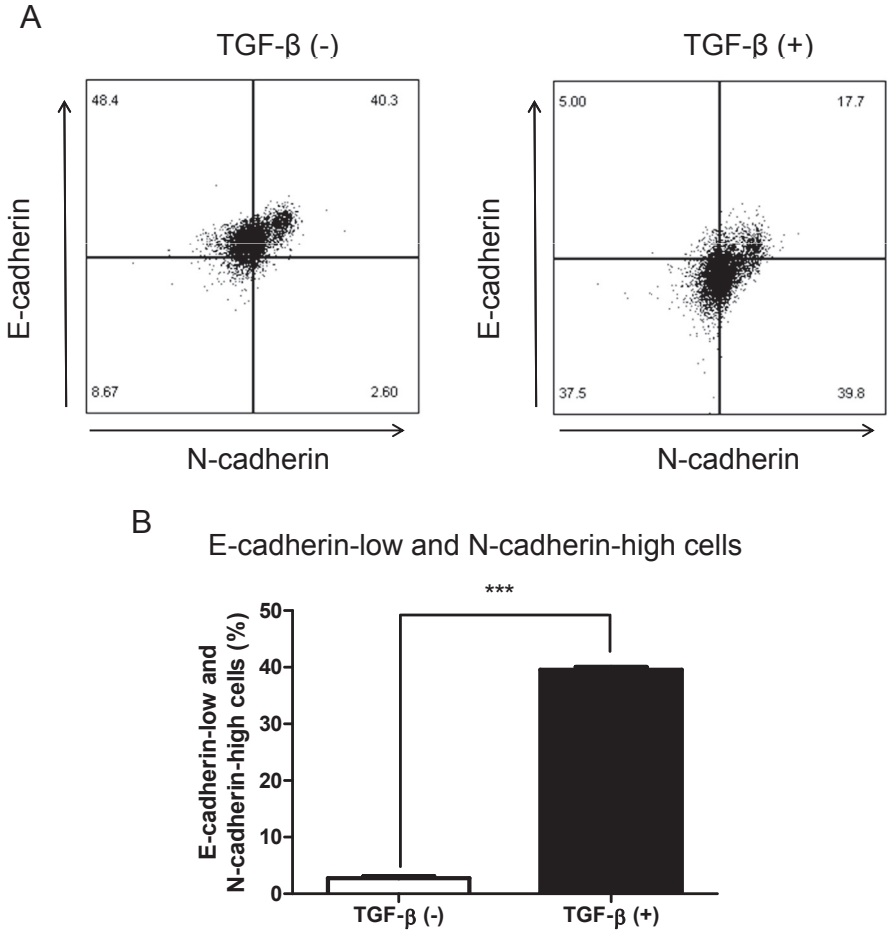

Fig. 1. Induction of EMT in A549 cells by TGF-β1. **A**: After incubation with (right dot plot) or without TGF-β1 (left dot plot) at 10 ng/ml, A549 cells were stained with PE-conjugated anti-E-cadherin antibody and APC-conjugated anti-N-cadherin antibody. Representative two-dimensional dot plots of cells stained with anti-E-cadherin antibody and anti-N-cadherin antibody from three independent experiments are shown, with the gates for analyzing E-cadherin-low, N-cadherin-high cells shown in **B**. The number in each gate represents the percentage of cells in each quadrant. **B**: The percentages of E-cadherin-low, N-cadherin-high cells determined using the gates in **A** are shown. *Bars* represent the SD (n = 3). ***$P < 0.001$ vs. cells incubated without TGF-β1.

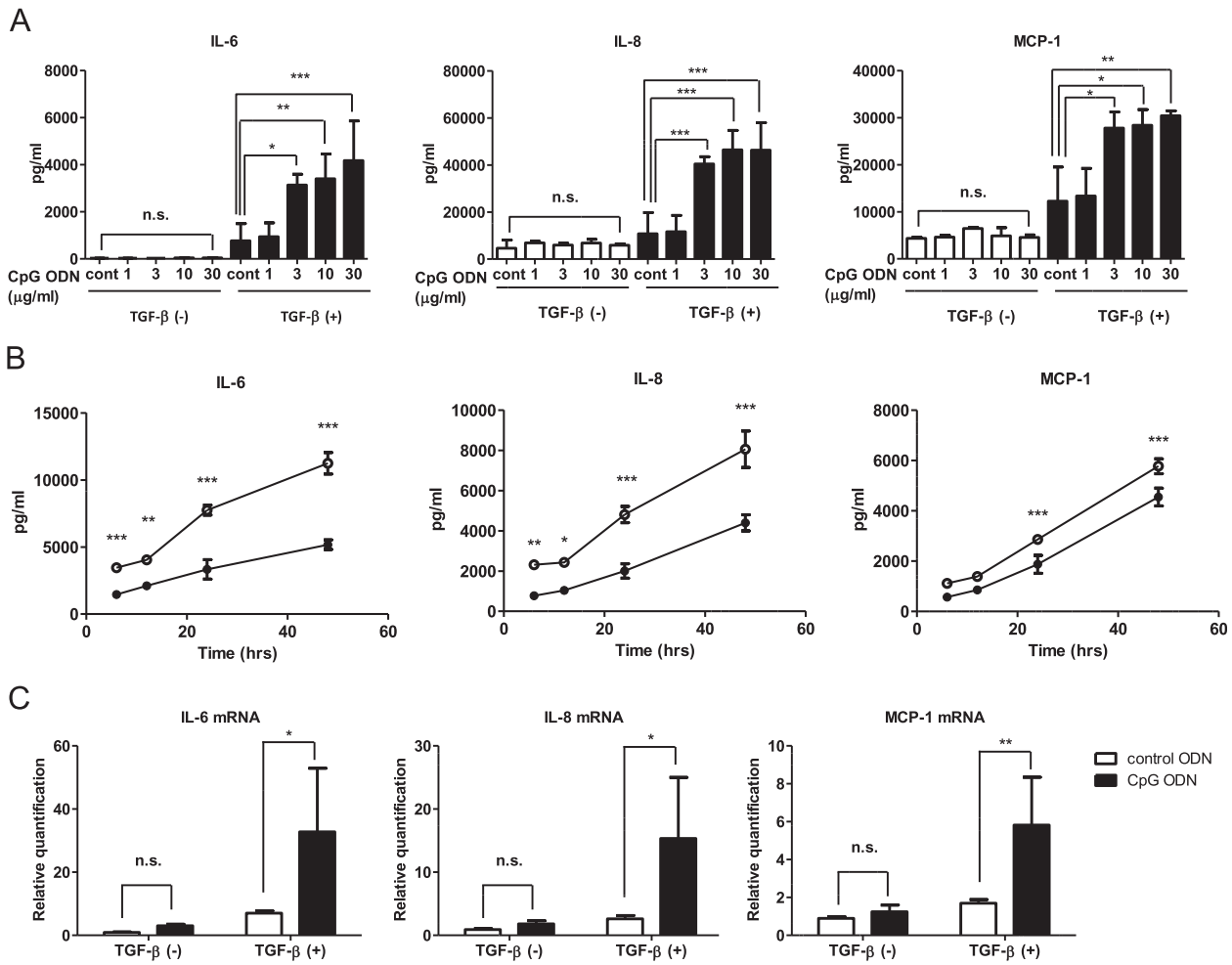

Fig. 2. **A**: Levels of IL-6, 8 and MCP-1 in the A549 cell culture supernatant after stimulation with CpG ODN at 1, 3, 10 and 30 μg/ml or control ODN at 30 μg/ml for 48 h following incubation with (■) or without (□) TGF-β1 at 10 ng/ml. *Bars* represent the SD (n = 3). n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. cells stimulated with control ODN. **B**: Levels of IL-6, 8 and MCP-1 in the A549 cell culture supernatant after stimulation with CpG ODN (○) or control ODN (●) at 10 μg/ml for 6, 12, 24 and 48 h following EMT induction. *Bars* represent the SD (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. cells stimulated with control ODN. **C**: Expression levels of IL-6, 8 and MCP-1 mRNA after stimulation of A549 cells with CpG ODN (■) or control ODN (□) at 10 μg/ml for 4 h following incubation with or without TGF-β1 at 10 ng/ml. *Bars* represent the SD (n = 4). n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.01$ vs. cells stimulated with control ODN.

the role of TLR9 in the mechanism of the enhanced sensitivity to CpG ODN caused by EMT. Addition of ODN H154 to the culture significantly suppressed production of IL-6, IL-8 and MCP-1 by EMT-induced A549 cells in response to CpG ODN (Fig. 3A). The levels of IL-6 and IL-8 were almost completely suppressed to the levels of control ODN. These results indicate that the cytokine production evoked by CpG ODN is mediated by TLR9 and that TLR9 is essential in the mechanism for enhanced sensitivity to CpG ODN caused by EMT.

Similarly, dexamethasone significantly suppressed the cytokine production by EMT-induced A549 cells to the control levels (Fig. 3B).

TLR9 expression was decreased by EMT

Next, we examined the expression level of intracellular TLR9 by flow cytometry. Flow cytometry found the TLR9 expression level to be slightly lower in A549 cells that had undergone EMT compared to those that had not (Fig. 4A–C). Real-time PCR found that expression of mRNA for TLR9 was also significantly decreased in cells incubated with TGF-β1 for 48 h compared to those without TGF-β1 (Fig. 4D). Collectively, although TLR9 is important in the mechanism of enhanced responsiveness of EMT-induced A549 cells to CpG ODN, the basic expression level of TLR9 was not upregulated by that EMT induction.

CpG ODN uptake was increased by EMT

Finally, we examined the possibility that increased CpG ODN uptake by EMT-induced A549 cells was involved in the cells' enhanced sensitivity to CpG ODN. Flow cytometry found that CpG uptake was increased in A549 cells incubated with TGF-β1 compared to the control cells (Fig. 5A–C). However, EMT induction caused no change in cell-surface expression of CD14 or CD205, two CpG receptors reported to be important in CpG uptake and known to be expressed in macrophages, dendritic cells, respectively[27,28] (data not shown).

Discussion

Today, although most asthmatics' symptoms are well-controlled by the use of inhaled corticosteroids, the existence of severe asthmatics remains a major problem. Airway remodeling is a major histological characteristic in severe asthmatics and has been shown

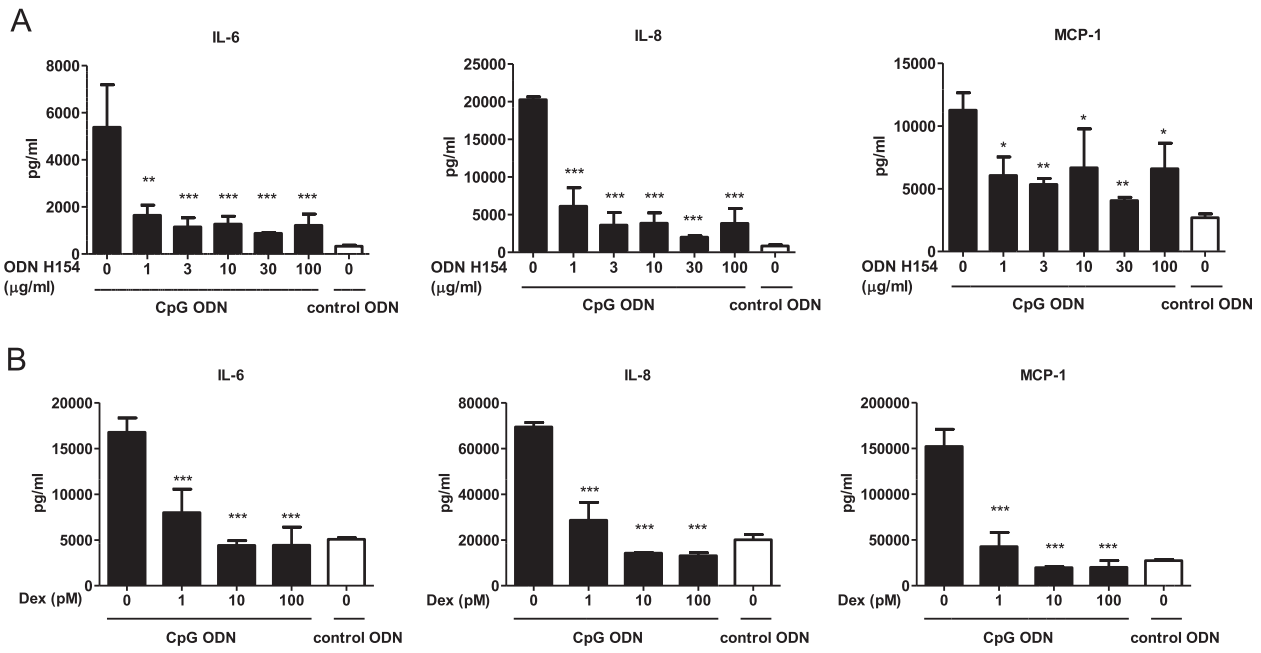

Fig. 3. A: Levels of IL-6, 8 and MCP-1 in the A549 cell culture supernatant after incubation with ODN H154 at 0, 1, 3, 10, 30 and 100 μg/ml in addition to CpG ODN or control ODN at 10 μg/ml for 24 h following induction of EMT. *Bars* represent the SD (n = 3). n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. cells stimulated with CpG ODN but without ODN H154. **B**: Levels of IL-6, 8 and MCP-1 in the A549 cell culture supernatant after incubation with dexamethasone (Dex) at the indicated concentrations and CpG ODN at 10 μg/ml for 24 h following induction of EMT. Control samples were incubated with control ODN at 10 μg/ml and ethanol. *Bars* represent the SD (n = 3). n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. cells stimulated with CpG ODN and ethanol but without dexamethasone.

to determine the severity of symptoms.[10,11] EMT has been proposed as one origin of airway remodeling.[12,13] Therefore, in the present study we used A549 cells and CpG ODN to elucidate the effect of TGF-β-induced EMT on cytokine production by airway epithelial cells in response to microbial stimuli by investigating cytokine production and the underlying mechanism(s).

We showed that EMT induction enhanced production of IL-6, IL-8 and MCP-1 by A549 cells at both the mRNA and protein levels in response to CpG ODN (Fig. 2A–C). IL-6 is a very well-known pro-inflammatory cytokine and is reported to promote Th2 effector cell expansion. By suppressing the function of CD4+CD25+ regulatory T cells IL-6 inhibits peripheral tolerance,[29] which may lead to worsening of asthmatic symptoms. In mice, overexpression of IL-6 in the airways induced airway remodeling with subepithelial fibrosis.[30] From our present study, we can speculate that epithelial cells that had undergone EMT in severe asthmatics are at least partially responsible for overexpressed IL-6 in the airways at the time of acute exacerbations related to respiratory tract infections, and this upregulation of IL-6 may accelerate Th2 responses as well as airway remodeling under the circumstances. On the other hand, IL-8 and MCP-1, also known as Chemokine (C–C motif) ligand 2 (CCL2), are well-known chemotactic factors for neutrophils,[31] monocytes,[32] mast cells,[33] fibrocytes[34] and Th17 cells[35] in the lung. It is widely accepted that a high neutrophil count in the sputum is a predictive factor for steroid-resistant, severe asthma,[36] and an increased neutrophil count and elevated IL-8 level in induced sputum correlated closely with the severity of asthma.[37] Together with our present results, IL-8 produced by EMT-induced airway epithelial cells during respiratory tract infections may play an important role in steroid-resistant severe asthma by recruiting neutrophils and other inflammatory cells. MCP-1 is more than just a chemoattractant, since it induces mast cell degranulation[38] and airway hyperreactivity.[38,39] It is noteworthy that EMT induction itself by 72-h culture with TGF-β promoted the basic production of cytokines even without CpG ODN stimulation. EMT-promoted upregulation of mRNA for IL-6 was previously reported using a similar method to ours with A549 cells and TGF-β,[40] but we showed here that EMT induction alone also elevated other cytokines, i.e., IL-8 and MCP-1, at both the mRNA and protein levels. Taken together, these mediators, namely, IL-6, IL-8 and MCP-1, that were upregulated by EMT induction itself and by EMT-enhanced responsiveness to stimulation with CpG ODN, may exacerbate—at least in part—asthmatic symptoms related to respiratory tract infections. The protein levels of other mediators including those directly related to Th2 inflammation, e.g., IL1-β, IL-4, IL-5, IL-13, IFN-α, TNF-α, RANTES, periostin, IL-25, IL-33 and GM-CSF, were not significantly increased in EMT-induced A549 cells by exposure to CpG ODN.

In spite of down-regulation of TLR9 expression in EMT-induced A549 cells (Fig. 4A–D), we showed that an inhibitory DNA for TLR9, ODN H154,[25] significantly suppressed production of IL-6, IL-8 and MCP-1 by EMT-induced A549 cells in response to CpG ODN (Fig. 3), indicating that TLR9 is essential for the enhanced response to CpG ODN brought about by EMT induction. TLR9 is a receptor that recognizes unmethylated CpG motifs, which are widely found in bacterial DNA and known to activate the innate immune system.[19–22] Although the precise frequency and impact of bacterial infections on exacerbations of asthma have not been fully examined, respiratory infections are the biggest cause of acute exacerbation of asthma.[5,41] In addition, histopathological changes in the airway, such as airway remodeling, can impair mucociliary clearance,[7,42] a change that may increase bacterial infections in asthmatics.[43] Thus, our present finding of upregulation of the TLR9-mediated response to a microbial component, CpG ODN, in EMT-induced A549 cells might partially explain the mechanism(s) of increased susceptibility of severe asthmatics' airways to extrinsic pathogens.

Our finding of increased CpG uptake due to EMT induction (Fig. 5A–C) may explain one mechanism of EMT-induced A549 cells' enhanced sensitivity to CpG ODN. However, the expression

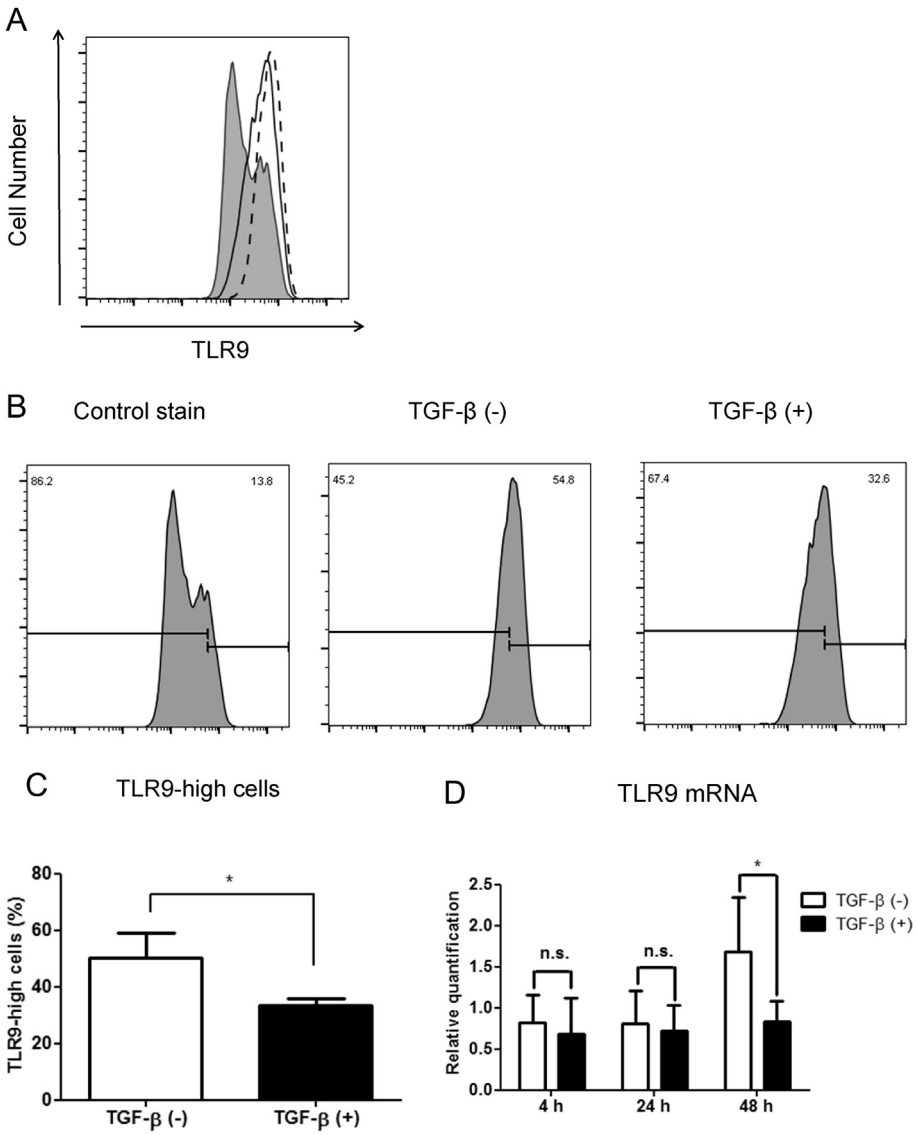

Fig. 4. Expression of TLR9 by A549 cells. **A**: After incubation with (solid line) or without (dashed line) TGF-β1 at 10 ng/ml, A549 cells were fixed, permeabilized and stained with APC-conjugated anti-TLR9 antibody. Expression of intracellular TLR9 was analyzed by flow cytometry. Representative histograms from four independent experiments are shown. The shaded area indicates cells stained with the control antibody. **B**: The histograms in *A* are shown separately, with the gates for analyzing TLR-9-high cells in **C**. The numbers shown in each gate are the percentages of cells belonging to each gate. **C**: The percentages of TLR-9-high cells determined using the gates in **B** are shown. *Bars* represent the SD (n = 4). $^{*}P < 0.05$ vs. cells incubated without TGF-β1 (□). **D**: After incubation with (■) or without (□) TGF-β1 for 4, 24 and 48 h, the total RNA was extracted from the cultured cells and the relative quantitation values of mRNA for TLR9 were calculated. *Bars* represent the SD (n = 3–6). n.s., not significant; $^{*}P < 0.05$ vs. cells incubated without TGF-β1.

levels of CD14 and CD205, two known receptors for CpG ODN expressed in macrophages and dendritic cells, respectively,[27,28] were not altered on EMT-induced A549 cells. There may be a yet-unknown receptor for CpG ODN uptake on A549 cells, but the mechanism underlying this increased uptake of CpG ODN by A549 cells needs to be further elucidated.

Airway remodeling was reported to be directly associated with airway hyperresponsiveness by a physiological mechanism: the remodeled airway lumen can be easily narrowed or occluded without excessive muscle shortening compared to the normal airway.[9] Our present data suggest that airway remodeling induced by EMT might promote reactivity to extrinsic pathogens by inducing cytokine production that would lead to prolonged and exaggerated airway inflammation. Thus, our data add a new mechanism(s) by which severe asthmatics' airways with remodeling are more susceptible to exacerbation induced by respiratory tract infections. We must note that while bacterial DNA containing CpG motifs is recognized by TLR9, rhinovirus, the most common pathogen found in acute exacerbation of asthma,[44,45] is recognized by TLR2, 7 and 8.[46] Therefore, the effect of EMT on reactivity of these receptors needs to be further studied to strengthen our hypothesis. In addition, our study investigated only B-class ODN but not other classes, and we used a human lung adenocarcinoma line, A549, in which EMT induced by TGF-β has been established and well-studied. In order to strengthen the case that our hypothesis is universal in human biology, our results need to be reproduced using human primary bronchial epithelial cells with other ODN classes as well.

In conclusion, EMT induction in A549 cells enhanced CpG ODN uptake and subsequent production of IL-6, IL-8 and MCP-1. These results suggest that EMT is closely related to airway remodeling, especially in severe asthmatics, and may play an important role in

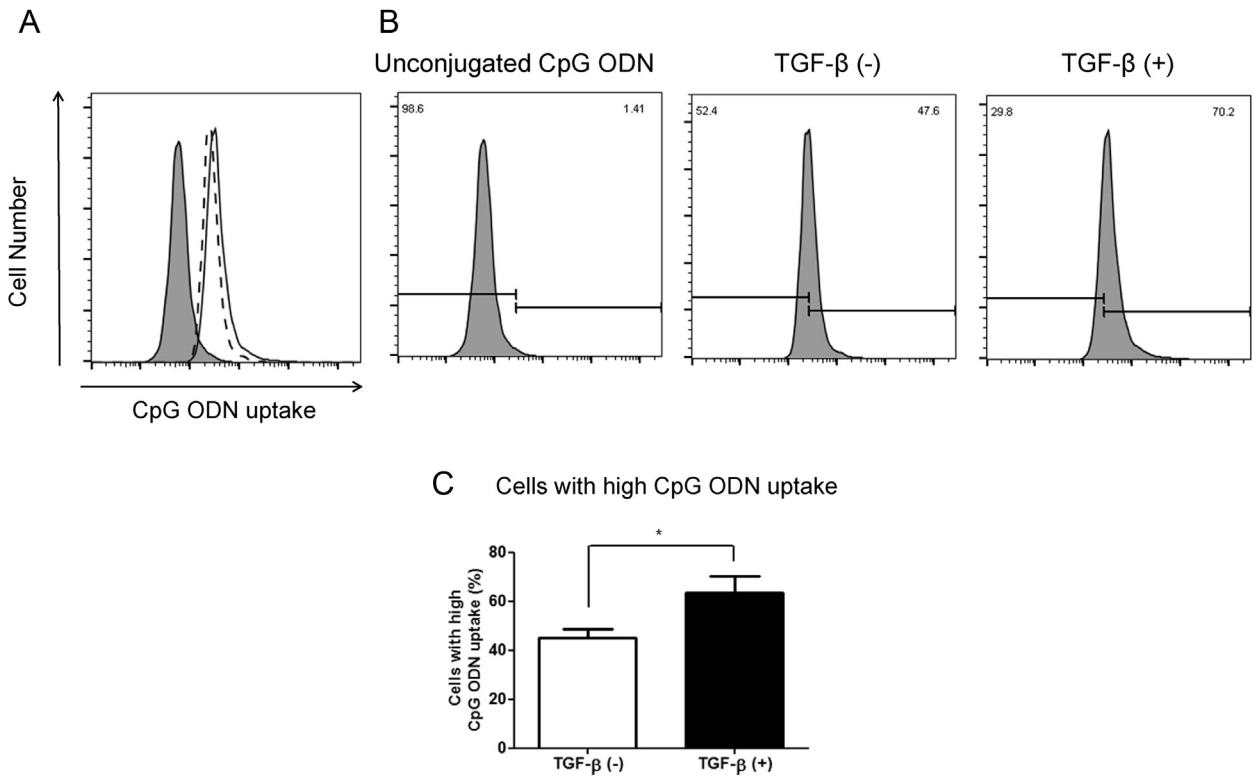

Fig. 5. CpG ODN uptake by A549 cells. **A**: After incubation with (solid line) or without (dashed line) TGF-β1 at 10 ng/ml, A549 cells were incubated with FAM-conjugated CpG ODN at 10 μg/ml for 24 h and then analyzed by flow cytometry. A representative histogram from three independent experiments is shown. The shaded area indicates cells incubated with unconjugated CpG ODN. **B**: The histograms in **A** are shown separately, with the gates for analyzing cells that showed high CpG ODN uptake. The numbers in each gate are the percentages of cells belonging to the gate. **C**: The percentages of cells that showed high CpG ODN uptake determined using the gates in **B** are shown. *Bars* represent the SD (n = 3). $^{*}P < 0.05$ vs. cells incubated without TGF-β1.

exacerbation in those patients by enhancing sensitivity to extrinsic pathogens. EMT is reported to play important roles not only in airway remodeling in asthmatics, but also in other major respiratory diseases such as lung fibrosis and lung cancer. Our present findings suggest the existence of novel mechanism(s) by which respiratory diseases are exacerbated in response to extrinsic pathogens.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

KK, MS performed data collection and the statistical analysis, and wrote the manuscript. KKoy, SI contributed to data collection. KKoy, MS, AH, TN, KO designed the study and contributed to the interpretation of the results. All authors read and approved the final manuscript.

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