Synergism Between Interleukin (IL)-17 and Toll-like Receptor 2 and 4 Signals to Induce IL-8 Expression in Cystic Fibrosis Airway Epithelial Cells

Shota Mizunoe1,2,†, Tsuyoshi Shuto1,†, Shingo Suzuki1, Chizuru Matsumoto1, Kenji Watanabe1,2, Keiko Ueno-Shuto1, Mary Ann Suico1, Kouhei Onuki1,2, Dieter C. Gruenert4,5,6,7,8,9,10, and Hirofumi Kai1,*

1Department of Molecular Medicine, Graduate School of Pharmaceutical Sciences, Global COE “Cell Fate Regulation Research and Education Unit”, Kumamoto University, Kumamoto 862-0973, Japan
2The Japan Society for the Promotion of Science (JSPS), Tokyo 102-8472, Japan
3Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto 860-0082, Japan
4Department of Otolaryngology - Head and Neck Surgery, 5Department of Laboratory Medicine, 6Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research, 7Helen Diller Family Comprehensive Cancer Center, 8Institute for Human Genetics, 9Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA 94115, USA
10Department of Pediatrics, University of Vermont College of Medicine, Burlington, VT 05405, USA

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Abstract. Cystic fibrosis (CF) is the most common lethal inherited disorder and is caused by mutations in the gene encoding the CF transmembrane regulator (CFTR). The CF lung expresses a profound proinflammatory phenotype that appears to be related to a constitutive hypersecretion of interleukin (IL)-8 from airway epithelial cells in response to microbial infection. Since overproduction of IL-8 in CF contributes to massive bronchial infiltrates of neutrophils, identification of the pathways underlying IL-8 induction could provide novel drug targets for treatment of neutrophil-dominated inflammatory diseases such as CF. Here, we show that IL-17A synergistically increases IL-8 production induced by a toll-like receptor (TLR) 2 agonist, peptidoglycan (PGN), or TLR4 agonist, lipopolysaccharide (LPS), in a human CF bronchial epithelial cell line (CFBE41o-). A strong synergism was also observed in primary human CF bronchial epithelial cells, but not in human non-CF cell lines and primary cells. Notably, despite the induction of nuclear factor-κB and MAP kinases during TLR2 or TLR4 activation in CFBE41o-, IL-17A-dependent synergism appears to be the result of enhanced PGN- or LPS-induced phosphorylation of p38. Taken together, these studies provide evidence that IL-17A is a critical factor in increasing IL-8 expression in bacteria-infected CF airways via a pathway that regulates p38 phosphorylation.

Keywords: interleukin (IL)-17, toll-like receptor 2 and 4 (TLR2/TLR4), IL-8, cystic fibrosis (CF), p38

Introduction

Cystic fibrosis (CF) is the most common lethal inherited disorder in the Caucasian population and is caused by mutations in the gene encoding the CF transmembrane regulator (CFTR) (1). CF is characterized by recurrent pulmonary infections and chronic inflammation that ultimately lead to death from respiratory failure (1, 2). Defects in expression of CFTR in the airway epithelial cells induces chronic airway inflammation that appears to be related to an intrinsic defect in regulation of the immune response and/or a hyperresponsiveness to infection by bacterial strains such as *Pseudomonas*, *Staphylococcus*, *Haemophilus*, *Aspergillus*, and *Burkholderia* species. This hyperresponsiveness correlates with im-
paired Cl⁻ conductance, Na⁺ hyperabsorption, mucus hypersecretion, and impaired mucociliary clearance in CF airways (2). CF airway epithelial cells produce the proinflammatory cytokine interleukin (IL)-8 that strongly regulates the neutrophil-dominated inflammatory process characteristic of CF airways (2, 3) and have led to numerous studies evaluating the mechanisms underlying IL-8 induction in CF airways.

IL-17A is a cytokine that was recently shown to be primarily produced by a CD4⁺ T cell subset, T helper 17 induction in CF airways. Numerous studies evaluating the mechanisms underlying IL-8 expression in CF airways (2, 3) and have led to numerous studies evaluating the mechanisms underlying IL-8 induction in CF airways.

Recently, increased expression of IL-17A was shown in bronchoalveolar lavage (BAL) fluid and sputum from bacterially infected CF patients (21, 22). Moreover, Roussel et al. (23) showed that IL-17A enhances P. aeruginosa filtrate (PAF)-induced IL-8 induction in the CuFi CF airway epithelial cell line apparently through the up-regulation of a NOD-like receptor (NLR) family protein, nucleotide-binding oligomerization domain 1 (NOD1). These studies suggest that IL-17A may contribute to IL-8-dependent CF pathogenesis. In the present study, the effect of IL-17A stimulation on the CF airway epithelial cell response to two major bacterial components, Gram-positive bacterial PGN, a TLR2 agonist, and Gram-negative bacterial LPS, a TLR4 agonist, was evaluated to further understand the physiological relevance of IL-17A in CF pathogenesis. The studies showed a strong synergism between IL-17 and TLR2 and TLR4 induction of IL-8 expression in a CF airway epithelial cell line and in primary patient-derived CF primary bronchial epithelial cells. The IL-17A-dependent synergy appeared to be the result of enhanced PGN- or LPS-induced phosphorylation of p38 MAPK. Overall, the data suggest that IL-17A is a critical factor in increasing IL-8 expression in bacteria-infected CF airway, possibly via a pathway that regulates p38 MAPK phosphorylation.

Materials and Methods

Reagents and antibodies

Recombinant human IL-17A was purchased from R&D Systems (Minneapolis, MN, USA). LPS (from *E. coli* 0111:B4), SB203580, and actinomycin D were purchased from Sigma (St. Louis, MO, USA). SP600125 was purchased from Biomol (Plymouth Meeting, PA, USA). PGN from *Staphylococcus aureus* was purchased from Fluka (Buchs, Switzerland). PD98059 and anisomycin were purchased from Calbiochem (La Jolla, CA, USA). Anti-phospho-JNK (Thr183/Tyr185), anti-phospho-p38 (Thr180/Tyr182), anti-phospho-ERK1/2 (Thr202/Tyr204), and anti-phospho IκBα (Ser32) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and treatment

16HBE14o- (24) and CFBE41o- (25) cells were generated as described previously and grown in a fibronectin/vitrogen/BSA-coated flask in MEM (19). CFBE41o- is a well-characterized cell line that was generated by transformation of CF tracheo-bronchial cells with SV40 and was reported to be homozygous for the ΔF508 mutation (ΔF508/ΔF508). Because CFBE41o- cells retain many aspects of human CF bronchial epithelial cells, it is mainly used in this study. Calu-3 cells were purchased from ATCC (Manassas, VA, USA) and cultured in DMEM/F12 (26). All cell lines were cultured in the medium supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Primary normal (NHBE) and primary diseased human bronchial epithelial cells derived from a patient CF (DHBE-CF) were purchased from Takara Biomedical Co. (Otsu) and were maintained in BEBM supplemented with BEGM.
SingleQuots including Bovine Pituitary Extract, insulin, hydrocortisone, GA-1000, retinoic acid, transferrin, triiodothyronine, epinephrine, and human epidermal growth factor (Takara Biomedical Co.) according to the manufacturer’s instruction. All cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C. For the detection of IL-8 induction, all cell lines were subjected to serum starvation 12 h prior to the treatment with PGN or LPS to reduce the background activation of IL-8 expression, while primary cells were cultured in BEBM containing only insulin, GA-1000, transferrin, and triiodothyronine 12 h prior to the treatment with PGN or LPS.

Quantitative real-time RT-PCR analysis
Quantitative real-time RT-PCR for human IL-8, TLR2, TLR4, and 18s ribosomal RNA (18srRNA) was carried out using the primers shown in Table 1 as previously described (27). Briefly, total RNA from the cells was isolated using RNAisoPlus and synthesis of cDNA was performed using PrimeScript RT regent kit (Takara Biomedical Co.). Real-time quantitative RT-PCR analysis was performed using SYBR Premix Ex Taq (Takara Biomedical Co.). The relative quantity of target genes’ mRNA was normalized using 18srRNA as the internal control and expressed as the relative quantity of target genes’ mRNA (fold induction).

SDS-PAGE and western blotting
Cells grown to 90% – 100% confluence on 6-cm plates were used to collect the cell protein lysates. The protein lysates were analyzed by SDS-PAGE and western blotting as described previously (28).

ELISA analysis
The IL-8 concentration in the culture supernatant was assessed by an ELISA kit for human IL-8 (CXCL8/IL-8 Duo Set) from R&D Systems (Minneapolis, MN, USA) according to the manufacturer’s instruction. Briefly, 100 μl of sample was added to each well coated with a murine monoclonal antibody against IL-8. To be within range, supernatants from cell cultures were optimally diluted in serum-free medium. A biotinylated goat anti-human IL-8 was added, and samples were incubated for 2 h, followed by the incubation with a streptavidin conjugated to horseradish-peroxidase. After three washes between each step, substrate solution was added for 20 min. Absorbance was measured on a microplate reader at 450 nm, and IL-8 concentration was determined in each sample by comparison to a standard curve.

Statistical analysis
The data were analyzed by one-way ANOVA with the Tukey-Kramer multiple comparison test (JMP software; SAS Institute, Cary, NC, USA) as indicated in each figure legend.

Results

IL-17A-dependent IL-8 induction in human CFBE41o- cells
To confirm the effect of IL-17A on the regulation of IL-8 gene expression in CF airways, CFBE41o- cells were first treated with IL-17A and the IL-8 mRNA levels were assessed by quantitative real-time RT-PCR. As shown in Fig. 1A, after the treatment with IL-17A, the IL-8 mRNA expression in CFBE41o- cells was slightly but significantly up-regulated. A significant increase in IL-8 gene expression was observed at IL-17A concentrations in the range 10 – 100 ng/ml. IL-17A-dependent up-regulation of IL-8 protein was also observed by ELISA (Fig. 1B). To further elucidate the signaling pathways involved in IL-8 up-regulation, NF-κB activation and the MAPKs signaling pathways in CFBE41o-cells were assayed because they are downstream signals of IL-17A activation in numerous cell types (8). Exposure to IL-17A results in phosphorylation of the JNK, p38, and ERK pathways (Fig. 1C), but not in IκBα, an activator of NF-κB. The role of these MAPK signaling pathways in the regulation of IL-17A-dependent IL-8 induction in CFBE41o-cells was elucidated with specific inhibitors of JNK, p38, and ERK pathways. SB203580 and PD98059, but not SP600125, significantly suppressed IL-17A-dependent IL-8 induction (Fig. 1D). These data suggest that p38 and ERK affect the IL-17A-dependent signaling pathway for the IL-8 induction in

Table 1. Primers used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Human mRNA</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>CTGGCCGTGGCTCTCTTG</td>
<td>CCTTGGA...</td>
</tr>
<tr>
<td>TLR2</td>
<td>GGGCGGAAATTTACCTCTTG</td>
<td>AGGCCG...</td>
</tr>
<tr>
<td>TLR4</td>
<td>CTGCCAATGGATCAAGGACCA</td>
<td>TTATCTG...</td>
</tr>
<tr>
<td>18s ribosomal RNA</td>
<td>CGGCTACCACATCCAAGGAA</td>
<td>GCTGGAATTACCCGCGCT</td>
</tr>
</tbody>
</table>
IL-17A Enhances TLR2/4 Signals in CF

**IL-17A synergizes with PGN and LPS to promote IL-8 induction in human CF airway epithelial cell lines and primary cells**

Since IL-17A has been shown to affect other signaling pathways (11), the modulatory role of IL-17A in signaling during bacterial infection in CF airways. The effect of co-treatment with IL-17A and Gram-positive bacterial PGN was initially assayed. PGN, a TLR2 agonist, alone up-regulates IL-8 in CFBE41o- cells (Fig. 2: A and B). There was a notable synergy in the induction of IL-8 gene and protein expressions when PGN was used in combination with IL-17A (Fig. 2: A and B). Similar analyses performed using non-CF airway epithelial cell lines, 16HBE14o- and Calu-3 cells, showed no synergism with the combination of IL-17A and PGN in either cell line (Fig. 2C). To exclude the possibility that effect of IL-17A is only observed in cell lines, the effect of IL-17A on the IL-8 gene expression was evaluated in NHBE and DHBE-CF cells. A low of IL-8 gene induction and no synergy was observed when IL-17A and PGN were used together for IL-8 induction in NHBE cells. However, there was significant synergy in IL-8 gene and protein induction in DHBE-CF cells when IL-17A and PGN were used together (Fig. 2: D and E).

The effect of IL-17A co-treatment with a Gram-negative bacterial LPS was then assessed and showed a significant synergistic induction of IL-8 gene and protein observed with the combined exposure to IL-17A and LPS, a TLR4 agonist (Fig. 3: A and B). Moreover, no synergy was observed when the IL-17A and LPS combination was used to treat the non-CF cell lines, 16HBE14o- and Calu-3 cells (Fig. 3C). As was observed in the primary cells treated with PGN, the expression of IL-8 by co-treatment with IL-17A and LPS was dramatically higher in DHBE-CF cells when compared to that in NHBE cells (Fig. 3: D and E). Taken together, these data suggest an immunostimulatory role for IL-17A during bacterial infection of CF airway.

**p38 MAPK activation is involved in the synergistic induction of IL-8 in CF cells**

While having demonstrated the synergy between IL-
17A and PGN or LPS in promoting IL-8 expression in CF cells, the mechanisms that underlie IL-17A-based synergy is still undefined. Initial studies evaluating the role of TLR2 and TLR4 expression in the IL-17A-based synergistic increases in PGN- or LPS-dependent IL-8 induction showed that neither TLR2 nor TLR4 gene expression was affected by IL-17A treatment (Fig. 4).

Analysis of intracellular signaling pathways activated by IL-17A treatment was assessed in CFBE41o- cells (Fig. 1C). PGN and LPS phosphorylate IkBa, p38, and JNK, while IL-17A phosphorylates p38 and ERK (Fig. 5: A and B). When the cells were co-treated with PGN or LPS and IL-17A, only p38 phosphorylation was significantly increased (Fig. 5: A – C). The role of p38 in the IL-17A-mediated synergistic effect on PGN- or LPS-dependent IL-8 induction was evaluated by treating CFBE41o- cells with anisomycin, a p38 MAPK agonist (29). As shown in Fig. 5D, anisomycin strongly phosphorylates p38 MAPK in CFBE41o- cells. This increase in p38 MAPK phosphorylation was accompanied by a concomitant increase of IL-8 expression upon co-treatment with PGN or LPS (Fig. 5E) and implies that IL-17A modulation of p38 MAPK may be responsible for the synergistic increase in IL-8 expression in CF cells. Although a previous study showed increased IL-8 mRNA stability by IL-17A through TNFα-related mechanism (30), it is unlikely that the IL-17A-mediated synergy observed here is caused by enhanced IL-8 mRNA stabil-
**IL-17A Enhances TLR2/4 Signals in CF**

Fig. 3. IL-17A-dependent synergistic enhancement of LPS-induced expression of IL-8 in transformed human CF and primary airway epithelial cells. A: CFBE41o- cells were treated with LPS (10 μg/ml) alone or in combination with IL-17A (100 ng/ml) for 4 h. Real-time quantitative RT-PCR (Q-PCR) was then performed to determine IL-8 mRNA expression levels. IL-8 mRNA levels were normalized to the level of 18s ribosomal RNA (internal control). B: CFBE41o- cells were treated with LPS (10 μg/ml) alone or in combination with IL-17A (100 ng/ml) for 24 h, and then IL-8 protein levels in conditioned media were determined by ELISA analysis. C: 16HBE14o- and Calu-3 were treated with LPS (10 μg/ml) alone or in combination with IL-17A (100 ng/ml) for 4 h, followed by Q-PCR. D: Primary NHBE and DHBE-CF cells were treated with LPS (10 μg/ml) alone or in combination with IL-17A (100 ng/ml) for 4 h and then analyzed by Q-PCR. E: Primary DHBE-CF cells were treated with LPS (10 μg/ml) alone or in combination with IL-17A (100 ng/ml) for 48 h. IL-8 protein levels in condition media were then determined by ELISA analysis. Results represent the mean ± S.D. from three independent experiments. ANOVA using the Tukey-Kramer protocol was implemented to assess the significance of the differences in expression between LPS-treated and LPS / IL-17A–treated CFBE41o-, NHBE, and DHBE-CF cells (*P < 0.05, **P < 0.005, ***P < 0.0005; n.s., not significant).

Fig. 4. Expression of TLR2 and TLR4 in CFBE41o- cells with IL-17A treatment. CFBE41o- cells were stimulated with IL-17A (1, 10, 100 ng/ml) for 4 h. Real-time quantitative RT-PCR (Q-PCR) was used to assess TLR2 or TLR4 mRNA expression and normalized to the level of 18s ribosomal RNA (internal control).

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Discussion

There has been an increasing interest on the biological activity of members of the IL-17 family, especially IL-17A, because of the diversity in its expression and its functional pleiotropism. Numerous pre-clinical and clinical studies identified IL-17A as a critical component in host defense responses and inflammatory diseases and suggest that IL-17A might be a promising therapeutic target (8–10). In CF, the elevated levels of IL-17A in the sputum of adult patients are thought to contribute to the bacterial infection related respiratory exacerbations that decrease with antibiotic therapy (6, 21, 22). The results presented here are the first to indicate that IL-17A is a critical factor in the increase in IL-8 expression resulting from TLR2- and TLR4-dependent activation in...
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CF airway. These studies are consistent with a previous clinical report showing that patients chronically infected with *P. aeruginosa* have higher IL-17A mRNA expression levels when compared to patients who were not chronically infected (21). The fact that those chronically infected patients had a trend towards a lower forced expiratory volume in 1 s (FEV1) suggests that the IL-17A – TLR2/4 – IL-8 axis may be physiologically important. Moreover, preliminary studies showing IL-17A up-regulation after intratracheal exposure of PGN or LPS resulted in subsequent increases in keratinocyte chemoattractant (KC) (an IL-8 homologue) expression and in neutrophil recruitment to the lungs in Cfr mutant (ΔF508/ΔF508) mice also support the physiological relevance of IL-17A – TLR2/4 – IL-8 axis (S. Mizunoe et al., unpublished data).

It is widely accepted that the p38 MAPK pathway is activated by IL-17A signalling; although, it is still not clear how p38 MAPK modulates other signaling pathways. Henness et al. showed that IL-17A augments TNFα-induced secretion of IL-8 from human airway smooth muscle cells via a p38 MAPK-dependent post-transcriptional pathway (30). Our results suggest that there are likely other IL-17A-dependent mechanisms in airway epithelial cells that modulate p38 MAPK (Fig. 5F). Although the possibility that the mechanism may involve the enhanced activation of MKK3/6, an immediate upstream regulator of p38 MAPK in various types of signals as well as the IL-17A signal (31 – 33), with IL-17A co-treatment cannot be excluded. An interesting recent report by Valente et al., relevant to the studies presented here, showed that a unique signaling molecule MAPK phosphatase-1 (MKP-1), a negative regulator of p38 MAPK, is down-regulated by IL-17A in adult mouse primary cardiac fibroblast (34). This suggests that IL-17A-dependent MKP-1 suppression will contribute to the enhanced p38 MAPK signaling. In the context of the studies presented here, one can envision a mechanism where IL-17A-mediated inhibition of MKP-1 contributes to the synergism between IL-17A and TLR2/4 signals by enhancing phosphorylation of p38 MAPK in CF airway epithelial cells.

While it is still unclear whether TLR2 and TLR4 signaling pathways are solely responsible for modulating inflammation in CF airways, IL-17A has been shown to prime CF airway epithelial cells (Cufi cells) to increase IL-8 expression in response to bacterial infection through the up-regulation of one of the pattern recognition receptors (PRRs), NOD1 (23). Furthermore, there are numerous reports that demonstrate IL-17A synergistic enhancement of pro-inflammatory responses when in combination with other cytokines such as TNFα, IL-1β, and interferon-γ (11) that are associated with inflammation in CF (35). Thus, there may be other inflammatory signaling pathways that can be modulated by IL-17A. Further study will be important to dissect the mechanisms whereby IL-17A enhances CF airway inflammatory pathogenesis.

Whether p38 MAPK can be a potential therapeutic target in CF is an important issue that will require further study. Increasing evidence suggests that the p38 MAPK signal is activated by various pro-inflammatory and stress-related stimuli under abnormal pulmonary conditions such as asthma, COPD, and CF (36, 37). Since p38 MAPK up-regulates the expression of factors that are critically involved in the pathogenesis of these pulmonary diseases such as inflammatory cytokines, mucins (38), proteases (39), and reactive oxygen species (3), it is a plausible therapeutic target for the treatment of CF. Clearly, further study using in vivo models is necessary to evaluate the potential of targeting IL-17A or p38 for the treatment of CF airway inflammation. The data from the present study suggest that IL-17A is a critical factor in increasing IL-8 expression in bacteria-infected CF airway possibly via p38 MAPK activation.

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