Regulation and Function of the IL-1 Family Cytokine IL-1F9 in Human Bronchial Epithelial Cells

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The IL-1 family of cytokines, which now includes 11 members, is well known to participate in inflammation. Although the most recently recognized IL-1 family cytokines (IL-1F5–11) have been shown to be expressed in airway epithelial cells, the regulation of their expression and function in the epithelium has not been extensively studied. We investigated the regulation of IL-1F5–11 in primary normal human bronchial epithelial cells. Messenger (m)RNAs for IL-1F6 and IL-1F9, but not IL-1F5, IL-1F8 or IL-1F10, were significantly up-regulated by TNF, IL-1 β , IL-17 and the Toll-like receptor (TLR)3 ligand double-stranded (ds)RNA. mRNAs for IL-1F7 and IL-1F11 (IL-33) were weakly up-regulated by some of the cytokines tested. Notably, mRNAs for IL-1F6 and IL-1F9 were synergistically enhanced by the combination of TNF/IL-17 or dsRNA/IL-17. IL-1F9 protein was detected in the supernatant following stimulation with dsRNA or a combination of dsRNA and IL-17. IL-1F6 protein was detected in the cell lysate but was not detected in the supernatant. We screened for the receptor for IL-1F9 and found that lung fibroblasts expressed this receptor. We found that IL-1F9 activated mitogen-activated protein kinases and the transcription factor NF- κ B in primary normal human lung fibroblasts. IL-1F9 also stimulated the expression of the neutrophil chemokines IL-8 and CXCL3 and the Th17 chemokine CCL20 in lung fibroblasts. These results suggest that epithelial activation by TLR3 (e.g., by respiratory viral infection) and exposure to cytokines from Th17 cells (IL-17) and inflammatory cells (TNF) may amplify neutrophilic inflammation in the airway via induction of IL-1F9 and activation of fibroblasts.

Keywords: human bronchial epithelial cells; IL-1F9; human lung fibroblasts; Toll-like receptor; IL-17

Epithelial cells, which are positioned at the site of first exposure to many harmful inhaled substances and microbial pathogens, regulate both innate and adaptive immunity through the production of functional molecules and via physical interactions with immune cells (1, 2). Activation of epithelial cells can result in immediate host defense responses that exclude pathogens. However, prolonged and/or robust epithelial activation can initiate and sustain airway inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis via the release of large quantities of proinflammatory cytokines, growth factors, and chemokines that attract inflammatory cells into the airway. Cytokines from inflammatory cells and helper T cells (Th cells) are able to alter pathogen-dependent epithelial activation.

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These pathways are now considered to be involved in both host defense mechanisms and initiation of airway diseases.

The IL-1 family of cytokines, which now includes 11 members, is well known to participate in inflammation (3–5). IL-1 α (IL-1F1) and IL-1 β (IL-1F2) play important roles in both immune regulation and inflammation. IL-1 α and IL-1 β are ligands for a heterodimeric receptor consisting of the IL-1RI and IL-1R accessory protein (IL-1RAP). An IL-1R antagonist (IL-1Ra; IL-1F3) binds tightly to IL-1RI and prevents recruitment of IL-1RAP, blocking IL-1 α / β -dependent signaling. IL-18 (IL-1F4), which is a ligand for a heterodimeric receptor consisting of IL-18R and IL-18RAP, is important in both innate and adaptive immune responses. Recently, six novel members of the IL-1 family cytokines, named IL-1F5 to IL-1F10, were identified (6–8). IL-1F6, IL-1F8, and IL-1F9 are ligands for a heterodimeric receptor consisting of the IL-1R like 2 (IL-1RL2) and IL-1RAP and are expressed in skin and other epithelial tissues (8, 9). Limited information is available regarding the functions of IL-1F6, IL-1F8, and IL-1F9. All three cytokines can activate NF- κ B and the MAPKs c-Jun-N-terminal kinase (JNK) and extracellular signal-regulated kinases (ERK) in IL-1RL2-transfected cells or the mammary epithelial cell line NCI/ADR-RES (8, 9). It is also reported that IL-1F6, IL-1F8, and IL-1F9 are able to induce IL-6 production in NCI/ADR-RES cells (9), and IL-1F8 enhances the production of IL-6 and IL-8 in human synovial fibroblasts and articular chondrocytes (10). IL-1F5 acts as an IL-1RL2 receptor antagonist (8, 11). IL-1F7 is known as an anti-inflammatory mediator that can interact with the IL-18 binding protein to enhance the inhibition of IL-18-dependent IFN- γ production and can translocate into the nucleus to reduce the production of proinflammatory cytokines (12, 13). The function of IL-1F10 is not well known, but it may bind soluble IL-1RI. IL-1F11 (IL-33) was originally identified as a nuclear factor in high endothelial venules (14). IL-33 is now recognized as a ligand for a heterodimeric receptor consisting of the orphan receptor T1/ST2 (ST2, also known as IL-1RL1) and IL-1RAP (15, 16). IL-33 is reported to be expressed by fibroblasts, smooth muscle cells, epithelial cells and endothelial cells (15, 17). Although ST2 is well known to be restricted to the cell surface of Th2 cells and mast cells, several recent studies indicate that ST2 is also expressed on eosinophils, basophils, and dendritic cells (18–22). IL-33 strongly induces production of Th2 cytokines including IL-5 and IL-13 from Th2 cells and mast cells (15, 23). Although the most recently recognized IL-1 family cytokines (IL-1F5–IL-1F11) have been shown to be expressed in airway epithelial cells, the regulation of their expression and their functions in the airway have not been extensively studied.

IL-1 β is well known to initiate inflammatory diseases. Thus, blocking of IL-1 signaling has been extensively studied in animal models of disease as well as in humans. Recombinant IL-1Ra (anakinra) has been approved and is used clinically to treat severe rheumatoid arthritis and several autoinflammatory diseases (5). IL-33-ST2 signaling is also suspected to initiate several inflammatory diseases including asthma, pulmonary fibrosis, rheumatoid arthritis, and cardiovascular diseases (4, 5, 24). In contrast to

IL-1 β and IL-33, little information is available on the role of IL-1RL2 agonists in disease (10, 11). Blumberg and colleagues generated skin-specific IL-1F6 transgenic mice and showed that the skin phenotype was characterized by acanthosis and hyperkeratinosis (11). The skin was infiltrated by neutrophils, macrophages, and T cells, and messenger (m)RNA expressions of CXCL2, CXCL6, and IL-23 p19 were increased, suggesting that neutrophil-mediated and/or Th17-mediated inflammation might be occurring in the skin in this model (11). The same group also reported that IL-1F6 and IL-1RL2 were increased in psoriatic skin in humans. In addition to IL-1F6, elevated expression of IL-1F9 was found in the lungs of allergen-challenged mice (25, 26). Intratracheal administration of IL-1F9 induced airway hyperresponsiveness and lung neutrophil infiltration (25). Bochkov and colleagues showed that mRNA expression of IL-1F9 was elevated in bronchial epithelial cells from patients with asthma (27). Their data suggests that IL-1RL2-dependent signaling may also be involved in inflammation in humans. However, regulation and function of IL-1RL2 agonists, IL-1F6 and IL-1F9, and regulation of IL-33 are not precisely known. In the present study, we hypothesized that Th2 cytokines enhanced local Th2 inflammation of the airways via the production of IL-33, and Th17 cytokines enhanced local Th17 and neutrophilic inflammation via the production of IL-1F6 and IL-1F9 from airway epithelial cells.

MATERIALS AND METHODS

Cell Culture, Treatments, and Transfection

Recombinant cytokines and Toll-like receptor (TLR) ligands were purchased from R&D systems (Minneapolis, MN), Axxora (San Diego, CA) and InvivoGen (San Diego, CA). Primary normal human bronchial epithelial cells (NHBE) (Lonza, Walkersville, MD) were maintained in serum-free bronchial epithelial cell growth medium (BEGM, Lonza). Submerged-NHBE were stimulated by cytokines and TLR ligands. NHBE were transfected with small interfering (si)RNA or control siRNA at 5 nM using HiPerFect transfection reagent (Qiagen, Valencia, CA) as described previously (28). Rhinovirus serotype 16 (RV16) was a gift from W. Busse and E. Dick (University of Wisconsin, Madison) (28). Submerged-NHBE were infected with RV16 at a multiplicity of infection of two for 24 hours at 33°C. In some experiments, NHBE were differentiated at an air-liquid interface on 0.4 μ m pore membrane transwell plates, as previously described (29). Details are available in the online supplement.

Primary normal human lung fibroblasts (NHLF; Lonza) were maintained in fibroblast growth medium 2 (FGM2; Lonza). NHLF were transfected with siRNA against IL-1RL2, IL-1RAP or control siRNA at 20 nM using HiPerFect transfection reagent.

Real-Time PCR

Total RNA was extracted using RNeasy (Qiagen) or NucleoSpin RNA II (Clontech, Mountain View, CA) and was treated with DNase I. Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed with a TaqMan method using an Applied Biosystems 7,500 (Foster City, CA) as described previously (30). Details are available in the online supplement.

Western Blot Analysis

Thirty μ g of whole cell lysates or 20 μ l of concentrated supernatants were resolved on a 4 to 12% NuPAGE Bis-Tris Gel (Invitrogen) and then proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked and subsequently incubated with anti-human IL-1F9 mAb (clone: 278706; R&D systems) or goat anti-human IL-1F6 antibody (R&D systems). After primary antibody incubation, the membranes were labeled by infrared secondary antibody. Protein was detected on an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Details are available in the online supplement.

ELISA and Cytometric Bead Array

The concentrations of IL-1F6, IL-1F9, and IL-33 protein in cell-free supernatants were measured using sandwich ELISAs. The minimal detection limits are 62.5 pg/ml, 750 pg/ml, and 15.6 pg/ml, respectively. The concentrations of IL-8 and granulocyte colony-stimulating factor (G-CSF) were measured using a cytometric bead array assay (BD Biosciences, San Jose, CA). The minimal detection limit is 5 pg/ml.

Statistical Analysis

All data are reported as the mean $\pm$ SEM unless otherwise noted. Differences between groups were analyzed using the paired Student's *t* test and considered to be significant if *P* < 0.05.

RESULTS

Regulation of Epithelial Expression of IL-1F5 through IL-1F11 by Cytokines and TLR Ligands

To study the regulation of the production of the most recently recognized IL-1 family cytokine (IL-1F5–IL-1F11) in airway epithelial cells, NHBE were treated with known activating cytokines and TLR ligands for 6 hours. The most widely activated IL-1 family cytokines were IL-1F6 and IL-1F9 (Figure 1A). Messenger RNAs for IL-1RL2 agonists, IL-1F6, and IL-1F9, were significantly up-regulated by stimulation with TNF (5.4-fold and 9.5-fold, *n* = 5; *P* < 0.05), IL-1 β (9.3-fold and 7.8-fold, *n* = 5; *P* < 0.05), IL-17 (7.6-fold and 7.6-fold, *n* = 6; *P* < 0.05), double-stranded (ds)RNA (3.3-fold and 11.0-fold, *n* = 7; *P* < 0.05), LPS (1.3-fold [not significant (NS)] and 3.3-fold, *n* = 6; *P* < 0.05), flagellin (3.9-fold [NS] and 3.2-fold; *n* = 6, *P* < 0.05), and FSL-1 (4.5-fold and 4.2-fold, *n* = 6; *P* < 0.05) in NHBE (Figure 1A). Messenger RNAs for IL-1F6 and IL-1F9 were also up-regulated by Pam3CSK4 (3.8-fold and 4.7-fold, *n* = 6), but it did not reach significance. Messenger RNA for IL-1F11 (IL-33) was also significantly up-regulated, having been induced by stimulation with oncostatin M (OSM) (2.5-fold; *n* = 4, *P* < 0.05), IFN- β (2.9-fold, *n* = 5; *P* < 0.05), and IFN- γ (5.7-fold, *n* = 7; *P* < 0.05), and was significantly down-regulated by stimulation with IL-4 (0.46-fold, *n* = 5; *P* < 0.05) and IL-13 (0.46-fold, *n* = 4; *P* < 0.05) in NHBE (Figure 1A). Messenger RNAs for IL-1F7 and IL-1F8 were weakly up-regulated by stimulation with TNF (1.9-fold [NS] and 1.5-fold [NS], *n* = 5) and IL-17 (2.7-fold [*P* < 0.05] and 1.8-fold [NS], *n* = 5) in NHBE (Figure 1A). Based on the levels of induction (Figure 1A) and the levels of baseline expression (Figure 1B) in NHBE, we focused further investigation on the regulation of expression of IL-1F6, IL-1F9, and IL-33.

To further examine the details of the induction of mRNAs for IL-1F6, IL-1F9, and IL-33, we determined the concentration-dependence and time-dependence of the response in NHBE. Initial early peak levels of IL-1F6 and IL-1F9 mRNA induced by TNF, IL-1 β , IL-17, and dsRNA were observed at 3 hours (Figure 1C and data not shown). The induction of IL-1F6 by IL-17 and IL-1F9 by dsRNA was biphasic, demonstrating a second increase after 24 hours of stimulation (Figure 1C). We then assessed whether these responses involved de novo protein synthesis. NHBE were treated with DMSO (vehicle control) or the protein synthesis inhibitor cycloheximide (CHX) for 1 hour, and then stimulated with dsRNA or IL-17 for 24 hours. Up-regulation of mRNA for IL-1F6 by IL-17 and IL-1F9 by dsRNA was significantly inhibited by CHX (Figure 1F). Because we found greater induction of IL-1F6 by IL-17 and IL-1F9 by dsRNA at 24 hours of stimulation, we further examined the expression of IL-1F6, IL-1F9, and IL-33 induced by cytokines and TLR ligands at 24 hours of stimulation. Although TNF, IL-1 β , LPS, flagellin, and FSL-1 also induced the expression of IL-1F6 and/or IL-1F9, expression was not strong compared with 6 hours of stimulation (Figure 1D).

Figure 1. Regulation of newly recognized IL-1 family cytokines in human bronchial epithelial cells. Submerged primary normal human bronchial epithelial cells (NHBE) were incubated with 100 ng/ml of TNF, IL-1 β , IL-4, IL-6, IL-13, IL-17A (IL-17), OSM, IL-29, 10 ng/ml IFN- γ , 1,000 U/ml IFN- β , 1 μ g/ml Pam3CSK4, 5 μ g/ml dsRNA, 1 μ g/ml LPS, 10 ng/ml Flagellin, 1 μ g/ml FLS-1, 10 μ g/ml R-837 and 2 μ g/ml CpG-C as indicated. Messenger (m)RNA was then extracted and analyzed for mRNA expression using real-time PCR (A) 6 hours, (B) 6 hours (no-stimulation), (C) 1 to 48 hours, and (D) 24 hours. (E) NHBE were incubated for 24 hours with 0.05–25 μ g/ml double-stranded (ds) RNA or 1–100 ng/ml IL-17. (F) NHBE were preincubated with 0.01% DMSO or 1–10 μ g/ml cycloheximide (CHX) for 1 hour and then stimulated with 50 ng/ml IL-17 or 5 μ g/ml dsRNA for 24 hours. (G) NHBE were infected with RV16 at a multiplicity of infection (MOI) of 2 and then cultured for 24 hours at 33°C. (H) NHBE were transfected with small interfering (si)RNA against control RNA, RELA or IRF-3 at 5 nM for 48 h, and then stimulated with 5 μ g/ml dsRNA for 6 hours. The expression of mRNAs for IL-1F6, IL-1F9 and β -actin (ACTB) was analyzed by real-time PCR. The copy number is expressed as the number of transcripts/ng of total RNA. Results shown are mean $\pm$ SEM of three to seven independent experiments; * P < 0.05.

In addition, we could not detect greater induction of IL-33 by cytokines and TLR ligands at 24 hours of stimulation (Figure 1D). Messenger RNA for IL-1F6 and IL-1F9 was dose-dependently

induced by concentrations of IL-17 as low as 1 ng/ml and dsRNA as low as 50 ng/ml (Figure 1E). In contrast, the expression of IL-33 was best induced by IFN- γ (Figure 1A)

and reached the peak level at 6 hours and then returned to baseline by 24 hours after stimulation (Figure 1C).

Double-stranded RNA is a surrogate for viral RNA and viral replication and is sensed by TLR3, the cytosolic RNA helicases RIG-I and MDA5, and the classical dsRNA recognition enzyme PKR (28, 31). To test the effect of virus infection on the induction of IL-1F9 in airway epithelial cells, NHBE were infected with RV16. Messenger RNA for IL-1F9 was significantly up-regulated by RV16 (Figure 1G). Activation of TLR3, RIG-I, or MDA5 by dsRNA and viruses transduces its signals to NF- κ B and IRF-3 (28, 31). To better understand the mechanism of IL-1F9 induction by dsRNA, we knocked down the transcription factors NF- κ B and IRF-3. NHBE were transfected with siRNA against control RNA, V-rel reticuloendotheliosis viral oncogene homolog A (RELA; NF- κ B p65) or interferon regulatory factor 3 (IRF-3), and then stimulated with dsRNA for 6 h. Control experiments demonstrated that target molecules were significantly suppressed by siRNA against RELA and IRF-3 but not by control siRNA, and siRNA did not inhibit expression of the housekeeping gene ACTB (see Figure E1 in the online supplement). Induction of IL-1F9 by dsRNA was significantly inhibited by siRNA against RELA but not by siRNA targeting IRF-3 or control siRNA (Figure 1H). These results suggest that IL-1F9 is induced by dsRNA in airway epithelial cells, and that the response is mediated via NF- κ B-dependent pathways.

Effect of Combinations of Cytokines and dsRNA on the Expression of IL-1F6 and IL-1F9

It is well known that levels of TLR3-dependent and virus-dependent gene expression are altered by stimulation with

inflammatory cytokines, TNF, and IL-1 β as well as Th cytokines including IFN- γ , IL-4, and IL-17 in airway epithelial cells (28). Vos and colleagues have shown that the combination of TNF and IL-1 β induces the expression of IL-1F9 in airway epithelial cells, although they did not show the effect of individual cytokines (32). We therefore examined the effect of combined stimulation with cytokines, TNF, IL-1 β , IFN- γ , IL-4, and IL-17, and the TLR3 ligand, dsRNA, on the expression of IL-1F6 and IL-1F9 in NHBE. We found that dsRNA and TNF synergistically and significantly enhanced IL-17-dependent IL-1F6 mRNA expression (2.8-fold and 3.6-fold, $n = 6$; $P < 0.05$, 24-h stimulation) (Figure 2A–2C). In contrast, IFN- γ significantly reduced dsRNA-dependent IL-1F6 mRNA expression in NHBE (0.22-fold, $n = 5$; $P < 0.05$, data not shown). In addition, IL-17 synergistically and significantly enhanced dsRNA-dependent IL-1F9 mRNA expression in NHBE (2.2-fold, $n = 6$; $P < 0.05$, 24-h stimulation) (Figures 2D and 2F). The combination of TNF and IL-17 also synergistically and significantly enhanced IL-1F9 mRNA expression in NHBE (6–13-fold, $n = 6$; $P < 0.05$, 24-h stimulation) (Figures 2E and 2F). The Th1 cytokine IFN- γ , and the Th2 cytokine IL-4 did not affect dsRNA-dependent IL-1F9 mRNA expression in NHBE ($n = 5$, data not shown). Messenger RNA for IL-33 was not synergistically enhanced by the combination of cytokines and dsRNA, and secretion of IL-33 was not induced by any stimulus that we tested in NHBE (data not shown).

Detection of IL-1F6 and IL-1F9 Protein

To confirm epithelial expression of IL-1F6 and IL-1F9 proteins, we measured the concentration in the culture supernatant using a specific ELISA. Significant concentrations of IL-1F9 were

Figure 2. Effect of the combination of TNF, IL-17, and dsRNA on the expression of IL-1F6 and IL-1F9 in human bronchial epithelial cells. Submerged-NHBE were incubated for 24 hours (A, B, D and E) or 6 to 48 hours (C and F) with 100 ng/ml TNF, 100 ng/ml IL-17, and 5 μ g/ml dsRNA or their combination. Expression of mRNAs for IL-1F6 and IL-1F9 was analyzed by real-time PCR. The copy number is expressed as the number of transcripts/ng of total RNA. Results shown are mean $\pm$ SEM of six independent experiments; * $P < 0.05$.

Figure 3. Detection of IL-1F6 and IL-1F9 protein in human bronchial epithelial cells. Submerged-NHBE were incubated for (A) 48 to 72 hours, (B) 72 hours, or (C) 48 hours with 100 ng/ml of TNF, 100 ng/ml IL-17, and 5 μ g/ml dsRNA or their combination. (D) NHBE were stimulated with 5 μ g/ml dsRNA and 50 ng/ml IL-

17 for 48 hours and treated with 4 mM ATP during the last hour. Protein expression of IL-1F6 and IL-1F9 in supernatant was analyzed by (A, B, and D) ELISA and (C) Western blot, and (C) protein expression in cell lysate was analyzed by Western blot using rat anti-human IL-1F9 mAb or biotinylated goat anti-human IL-1F6 antibody. (A) Results shown are mean $\pm$ SEM of four to seven independent experiments. (C) The results are representative of four separate experiments.

detected in the supernatant only after stimulation with dsRNA in NHBE (1.4 ± 0.6 ng/ml, $n = 7$ (48 h); 3.4 ± 1.2 ng/ml, $n = 5$, 72 h) (Figure 3A). In addition, IL-17 synergistically enhanced dsRNA-dependent IL-1F9 production in NHBE (2.3 ± 0.8 ng/ml, $n = 7$, 48 h; 6.8 ± 2.6 ng/ml, $n = 5$, 72 h) (Figure 3A). Production of IL-1F9 was dose-dependently induced by dsRNA and the reaction was enhanced by IL-17 as low as 1 ng/ml (Figure 3B). However, IL-1F6 protein was not detected in the supernatant of NHBE after stimulation with any of the tested cytokines, dsRNA or their combination ($n = 5$, data not shown). We further confirmed this finding by Western blot. IL-1F6 protein was found in cell lysates after stimulation with TNF, IL-17, and dsRNA (Figure 3C). In addition, expression of IL-1F6 in cell lysates was further enhanced when NHBE were stimulated by the combination of IL-17 and dsRNA or IL-17 and TNF (Figure 3C). However, IL-1F6 protein was not detected in the concentrated supernatants of stimulated and nonstimulated NHBE (Figure 3C). We confirmed this finding using a second mouse anti-human IL-1F6 mAb (clone; 278,706) (data not shown). In contrast, IL-1F9 protein was inducible and was detected in both cell lysates and supernatants in a pattern similar to that observed at the mRNA level and by ELISA (Figure 3C).

Martin and colleagues showed that murine IL-1F6-transfected bone marrow-derived macrophages did not constitutively release IL-1F6 into the medium, and IL-1F6 was released by treatment with LPS followed by ATP (33). We therefore examined whether treatment with 4 mM ATP induced the release of IL-1F6 and IL-1F9 in NHBE. NHBE were stimulated with dsRNA and IL-17 for 48 hours, and were treated with ATP during the last hour. However, IL-1F6 protein was still undetectable in culture supernatants (data not shown) and production of IL-1F9 was not enhanced by treatment with ATP (Figure 3D), although morphology of NHBE was altered by ATP.

To further investigate the regulation of IL-1F6 and IL-1F9 in differentiated-human bronchial epithelial cells, NHBE were differentiated at an air-liquid interface. Messenger RNAs for IL-1F6 and IL-1F9 were significantly up-regulated by stimulation with TNF (1.9-fold [NS] and 3.8-fold [$P < 0.05$]), IL-17 (3.1-fold and 3.6-fold; $P < 0.05$) and dsRNA (3.5-fold [NS] and 12.9-fold [$P < 0.05$]) but were not enhanced by IL-4 and IFN- γ ($n = 8$) (Figure 4A; data not shown). To investigate the polarity of IL-1F6 and IL-1F9 production, we washed the apical side of cells with PBS and collected supernatants from the basolateral side after 48 hours of stimulation. We found that differentiated-NHBE produced IL-1F9 protein mainly on the basolateral side

of the monolayer after stimulation with dsRNA (5.3 ± 1.7 ng/ml, $n = 6$; $P < 0.05$) (Figure 4B). However, IL-1F6 protein was not detected on either the apical or basolateral side after stimulation ($n = 6$, data not shown). We further tested the expression of proteins by Western blot. Both IL-1F6 and IL-1F9 proteins were found in cell lysates of differentiated-NHBE after stimulation with TNF, IL-17, and dsRNA (Figure 4C). These results suggest the regulation of IL-1F6 and IL-1F9 in submerged-human and differentiated-human bronchial epithelial cells is similar.

Effect of IL-1F9 on the Activation of MAPKs and Transcription Factors in Human Lung Fibroblasts

IL-1F9 is known as a ligand for a heterodimeric receptor consisting of IL-1RL2 and IL-1RAP. It has been previously reported that expression of IL-1RL2 is restricted to the skin and other epithelial tissues, whereas IL-1RAP is ubiquitously expressed (9). In addition, reports on the function of IL-1F9 in primary human cells have been limited. Therefore, we first screened for the expression of IL-1RL2 in several types of human primary cells. We found that IL-1RL2 was expressed in the resident cells (human primary fibroblasts, skin keratinocytes, and lung epithelial cells) rather than in the immune cells (human peripheral blood T cells, B cells, monocytes, and eosinophils) (data not shown). Magne and colleagues reported that IL-1F8 enhanced the production of IL-6 and IL-8 in human synovial fibroblasts (10). It has been reported that bronchial epithelial cells can interact with fibroblasts, via the production of recruiters, activators, and differentiation factors for fibroblasts (34). To our knowledge, no one has reported a function of IL-1F9 in human lung fibroblasts. We therefore focused on the potential effects of IL-1F9 in lung fibroblasts. We first screened for the activation and phosphorylation of MAPKs and transcription factors by IL-1F9 in lung fibroblasts. NHLF were harvested at the indicated time points after exposure to IL-1F9 or IL-1 β and then phosphorylation of the kinases and transcription factors was determined by Western blot. We found that MAPKs, JNK, ERK, and p38 and transcription factors, NF- κ B and CREB, were all phosphorylated after stimulation with IL-1F9, although the activation of MAPKs was weaker than with IL-1 β stimulation (*see* Figure E2 in the online supplement). Activation of JNK, ERK, p38, and CREB by IL-1F9 appeared within 15 minutes after stimulation with a return to baseline levels at 60 minutes (Figure E2). This data suggests that IL-1F9 can activate human lung fibroblasts via activation of MAPKs and transcription factors, NF- κ B and CREB.

Figure 4. Induction of IL-1F6 and IL-1F9 in differentiated human bronchial epithelial cells. NHBE were differentiated at an air-liquid interface and then stimulated with 50 ng/ml of TNF, 50 ng/ml IL-17, and 2.5 μ g/ml dsRNA or their combination for (A) 24 hours and (B and C) 48 hours. (A) The expression of mRNAs

for IL-1F6 and IL-1F9 was analyzed by real-time PCR, (B) protein expression of IL-1F9 in supernatant was analyzed by ELISA and (C) protein expression in cell lysate was analyzed by Western blot. Results shown are mean $\pm$ SEM of (A) eight and (B) six independent experiments. (C) The results are representative of three separate experiments.

IL-1F9 Induces Cytokines and Chemokines that Recruit and Activate Neutrophils

The above results demonstrate that IL-1F9 was induced by stimulation with a TLR3 activator, inflammatory cytokines and Th17 cytokines and was released on the basolateral side of epithelial cells (Figures 2 and 4), and that the IL-1F9 receptor, IL-1RL2, was expressed on fibroblasts (Figure E2). These results suggest that epithelial-derived IL-1F9 might enhance Th17 responses and neutrophilic inflammation via the activation of fibroblasts in the airway. To test the possible role of IL-1F9 in fibroblasts, NHLF were treated with different concentrations of IL-1F9. IL-1F9 dose-dependently and significantly enhanced

expression of mRNA for neutrophil chemokines IL-8 (50.5-fold, $n = 3$; $P < 0.05$) and CXCL3 (16.6-fold, $n = 3$; $P < 0.05$), the neutrophil activator and the proliferation and differentiation factor for the progenitor cells G-CSF (37.0-fold, $n = 3$; $P < 0.05$), and the dendritic cell and Th17 chemokine CCL20 (65.4-fold, $n = 3$; $P < 0.05$) in NHLF (Figure 5A). In addition, IL-1F9 was also capable of inducing mRNA expression for IL-1 β , IL-6, GM-CSF, CCL2, CCL5, and CXCL10 in NHLF (data not shown). To confirm these responses at the protein level, we measured the production of IL-8 and G-CSF by CBA. IL-1F9 dose dependently induced the production of IL-8 (control; 105 ± 13 pg/ml, 500 ng/ml IL-1F9-treated; $7,982 \pm 993$ pg/ml,

Figure 5. IL-1F9 induces the expression of cytokines and chemokines via its receptor complex, IL-1RL2 and IL-1RAP in human lung fibroblasts. NHLF were incubated for (A) 3 hours or (B) 24 hours with 10–500 ng/ml of IL-1F9. (C) Primary normal human lung fibroblasts (NHLF) were incubated with 500 ng/ml of heat-inactivated IL-1F9 or polymyxin B (PMB)-treated IL-1F9 for 3 hours. (D, E) NHLF were transfected with small interfering RNA (siRNA) against control RNA, IL1RL2, or IL1RAP at 20 nM for 48 hours and then stimulated with 250 ng/ml IL-1F9 or 1 ng/ml IL-1 β for 3 hours. (A, C, D and E) Levels of mRNA expression were determined by real-time PCR and (B) protein concentration was determined by cytometric bead array. Results shown are mean $\pm$ SEM of four to five independent experiments; * $P < 0.05$, ** $P < 0.005$.

$n = 4$; $P < 0.05$) and G-CSF (control; 5 ± 1 pg/ml, 500 ng/ml IL-1F9-treated; 209 ± 48 pg/ml, $n = 4$; $P < 0.05$) in NHLF (Figure 5B). To eliminate the possible role that endotoxin contamination played in the activation of fibroblasts, recombinant IL-1F9 was heat-inactivated at 95°C for 5 minutes or treated with 100 $\mu\text{g/ml}$ polymyxin B (PMB). Heat-inactivation of IL-1F9 completely abolished its ability to induce IL-8 in NHLF (Figure 5C). In contrast, PMB did not affect the induction of IL-8 by IL-1F9 in NHLF (Figure 5C) indicating that the effects of IL-1F9 were not due to endotoxin contamination.

To clarify the mechanism of cytokine and chemokine production by recombinant IL-1F9 in NHLF, we knocked down the IL-1F9 receptor complex. NHLF were transfected with siRNA against control, IL-1RL2, and IL-1RAP, and were then stimulated with IL-1F9 or IL-1 β for 3 hours. Control experiments demonstrated that target molecules were significantly suppressed by siRNA against each target molecule but not by control siRNA, and siRNA did not inhibit expression of the housekeeping gene, ACTB (Figure 5D; data not shown). Induction of IL-8 by IL-1F9 was significantly inhibited by siRNA against IL-1RL2 and IL-1RAP but not by control siRNA (Figure 5E). In contrast, induction of IL-8 by IL-1 β was significantly inhibited by siRNA against IL-1RAP but not by siRNA targeting IL-1RL2 or control siRNA (Figure 5E). These results suggest that IL-1F9 activates lung fibroblasts via a receptor-mediated mechanism.

DISCUSSION

The IL-1 family of cytokines has recently been expanded by the discovery of seven new members, IL-1F5–11. All of the agonistic IL-1 family cytokines have been reported to share similar signaling pathways. Therefore, the most recently identified agonistic IL-1 family cytokines, IL-1F6, IL-1F8, IL-1F9, and IL-33, are also believed to be involved in inflammation. Initial studies of these latest IL-1 family cytokines suggested that mRNAs for these molecules were expressed in the lungs and in the epithelium. However, regulation and function of IL-1F5–11 in the airway have not been extensively studied. In the present study, we determined the expression profiles of the latest IL-1 family cytokines in primary human bronchial epithelial cells. This study provides the first demonstration that the proinflammatory cytokines TNF and IL-1 β , the Th17 cytokine IL-17, and the TLR3 agonist dsRNA induce the expression of both IL-1F6 and IL-1F9 in airway epithelial cells. We have also shown that fibroblasts are primary target cells for IL-1F9, and that IL-1F9 can induce the production of neutrophil activators, neutrophil chemoattractants, and Th17- and dendritic cell-attracting chemokines in lung fibroblasts.

It has been reported that the combination of TNF and IL-17 synergistically enhances the production of neutrophil chemokines IL-8 and CXCL1 in airway epithelial cells (35). We have recently reported that the Th2 cytokine IL-4 synergistically enhances dsRNA-dependent expression of the dendritic cell activator TSLP in airway epithelial cells (28). We therefore tested whether combinations of TNF, IL-17, and dsRNA might further enhance the expression of IL-1F6 and IL-1F9 in airway epithelial cells. We found that the combination of TNF and IL-17 or dsRNA and IL-17 synergistically enhanced the expression of IL-1F6 and IL-1F9 (Figure 2). In contrast, combinations of cytokines including TNF, IL-1 β , IL-17, and other TLR ligands showed minimal effects or only additive effects on expression of IL-1F6 and IL-1F9 (data not shown). This suggests that respiratory viral infections that lead to activation of TLR3 may strongly enhance the expression of IL-1F6 and IL-1F9 in the presence of IL-17-producing cells (Th17 cells) in the airway.

Although the other TLR ligands that we tested, including Pam3CSK4, LPS, and flagellin, did not strongly activate epithelial IL-1F6 and IL-1F9; these TLR ligands are well known to activate monocytes, macrophages, and dendritic cells to induce production of TNF and IL-1 β , which would be expected to activate epithelial production of these cytokines during bacterial infections *in vivo*.

We demonstrate for the first time the production of IL-1F6 and IL-1F9 protein in human primary cells. Surprisingly, IL-1F6 protein was not detected in culture supernatants of activated NHBE, despite detection of the protein in cell lysates (Figures 3 and 4). In contrast, IL-1F9 protein was detected in both cell lysates and supernatants, although levels of the protein were higher in cell lysates (Figures 3 and 4). As is the case with IL-1 β and IL-18, IL-1F6 and IL-1F9 lack signal peptides and may require additional signaling for secretion. The mechanism of IL-1 β production and secretion has been studied extensively in many laboratories (see Reference [36]). The TLR-NF- κ B pathway is well known as an initial signal for the large accumulation of pro-IL-1 β in the cytosol. A second signal for IL-1 release is the activation of the nucleotide-binding oligomerization domain–leucine-rich repeats containing pyrin domain 3 (NLRP3) inflammasome. Activation of NLRP3 triggers the processing of procaspase-1 into mature active caspase-1, which then catalyzes IL-1 β processing and release. ATP is known to induce the activation of NLRP3 and the secretion of mature IL-1 β via P2X7 receptor-dependent signaling in monocytes. Secretion of IL-18 also involves a similar mechanism (37). Most recently, Martin and colleagues have shown that secretion of IL-1F6 in murine macrophages also requires two signals, TLR activation and ATP stimulation, using IL-1F6-transfected bone marrow-derived macrophages (33). We investigated whether ATP is capable of inducing the secretion of IL-1F6 and IL-1F9 in NHBE but found that it did not affect IL-1F6 and IL-1F9 protein secretion after stimulation with dsRNA, IL-17, and their combination in NHBE (Figure 3D and data not shown). This suggests that the caspase-1-inflammasome pathway may not be involved in the secretion of IL-1F6 and IL-1F9 in human epithelial cells. Future study is required to identify species and/or cell specific differences in the mechanisms of secretion of IL-1F6 and IL-1F9.

A high concentration of IL-1F9 protein was detected in epithelial supernatants, especially after stimulation with dsRNA and IL-17. We next investigated the potential function of IL-1F9 in the airway. We found IL-1RL2 was expressed on epithelial cells and fibroblasts. Although airway epithelial cells expressed IL-1RL2 and responded to IL-1F9, the response was much weaker than in fibroblasts (data not shown). The fact that airway epithelial cells released IL-1F9 protein to the basolateral side of the monolayer (Figure 4), suggested a potential role for epithelial cell–derived IL-1F9 in the activation of subepithelial cells such as fibroblasts. To this effect we found that IL-1F9 induced the activation of several MAPKs and transcription factors, NF- κ B and CREB, in primary human lung fibroblasts (Figure E2). This suggests that epithelial cells may interact with lung fibroblasts via production of IL-1F9 and that the signaling pathway by IL-1F9 in fibroblasts is similar to the signaling pathways used by other IL-1 family cytokines.

Although recombinant IL-1F9 activated lung fibroblasts, it required high concentrations to induce a significant amount of IL-8 production (Figure 5B). To exclude the possibility that this function was the result of contamination by endotoxin or other cytokines, we performed a knock-down experiment for the IL-1F9 receptor. SiRNA against IL-1RL2, which is a specific receptor for IL-1F9, significantly suppressed IL-1F9-dependent IL-8 expression but it did not inhibit IL-1 β -dependent IL-8 ex-

pression (Figure 5E). In contrast, siRNA against IL-1RAP, which is a shared receptor for IL-1F9 and IL-1 β , significantly suppressed both IL-1F9-dependent and IL-1 β -dependent IL-8 expression in fibroblasts (Figure 5E). In addition, heat-inactivated IL-1F9 did not activate the induction of IL-8 and PMB did not affect IL-1F9-dependent IL-8 expression in NHLF (Figure 5C). These data suggest that IL-1F9 activates fibroblasts via an IL-1F9 receptor-mediated mechanism. Although the concentration of IL-1F9 required for the response was higher (10–500 ng/ml) than that required for IL-1 β (Figure 5), it is in agreement with recent studies showing that recombinant IL-1F9-dependent activation of the NCI/ADR-RES cell line requires 500 ng/ml (9), and recombinant IL-1F8-dependent activation of synovial fibroblasts requires 5 μ g/ml (10). In the present study, we showed that epithelial cells were able to produce more than 1 ng/ml of IL-1F9 (2.7–16.7 ng/ml). Because the concentration of IL-1F9 in culture supernatants is diluted, the concentration of IL-1F9 in the proximity of the epithelial cells must be significantly higher. Thus, it is possible that production of IL-1F9 from airway epithelial cells is sufficient to activate fibroblasts in the airway, especially myofibroblasts in the epithelial trophic unit immediately adjacent to the lamina reticularis (38).

We found that IL-17 enhanced the production of IL-1F9 in the presence of TNF or dsRNA in airway epithelial cells (Figure 3). Th17 and IL-17 are well known to mediate immunity to extracellular bacteria and initiate neutrophilic inflammation (35, 39). IL-1F8, which shares the receptor complex with IL-1F9, has been reported to induce production of the neutrophil chemokine IL-8 in human synovial fibroblasts (10). Therefore, we tested whether IL-1F9 also contributes to neutrophilic inflammation. We found that IL-1F9 induced neutrophil chemokines, IL-8 and CXCL3, and the neutrophil and progenitor cell activator, G-CSF, in lung fibroblasts (Figure 5). Interestingly, IL-1F9 was also able to induce fibroblasts to produce the chemokine CCL20, which is known as a dendritic cell chemokine and is now also accepted to be a Th17-attracting chemokine. These results suggest that the production of IL-1F9 by epithelial cells and the activation of fibroblasts by IL-1F9 involve feed forward mechanisms to amplify Th17 inflammation and neutrophilic inflammation in the airway. Very recently, Ramadas and colleagues have reported that IL-1F9 was elevated in the lungs of allergen-challenged mice, and intratracheal administration of IL-1F9 induced airway hyperresponsiveness, chemokine production, and neutrophil influx in the lungs of mice (25). Their data supports that IL-1F9 contributes to the neutrophilic inflammation in the airway. The expression of IL-1F9 in airway epithelial cells from patients with neutrophilic airway inflammatory diseases is therefore worthy of investigation.

In summary, in this study we report that expression of IL-1F9 was induced in airway epithelial cells by stimulation with inflammatory cytokines including IL-17, the prototypic Th17 cytokine, and the TLR3 ligand dsRNA and that these two stimuli acted synergistically to induce expression of the protein. IL-1F9 in turn induced the production of neutrophil-activating and neutrophil-recruiting cytokines and chemokines in lung fibroblasts. Our findings suggest that infection of the airways with respiratory viruses and bacteria along with the recruitment of Th17 cells may greatly amplify neutrophilic inflammation in the airways via the production of IL-1F9 and the activation of fibroblasts.

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