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Alternaria alternata-induced airway epithelial signaling and inflammatory responses via protease-activated receptor-2 expression

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Abstract

Inhalation of the fungus *Alternaria alternata* is associated with an increased risk of allergic asthma development and exacerbations. Recent work in acute exposure animal models suggests that *A. alternata*-induced asthma symptoms, which include inflammation, mucus overproduction and airway hyperresponsiveness, are due to *A. alternata* proteases that act via protease-activated receptor-2 (PAR2). However, because other active components present in *A. alternata* may be contributing to asthma pathophysiology through alternative signaling, the specific role PAR2 plays in asthma initiation and maintenance remains undefined. Airway epithelial cells provide the first encounter with *A. alternata* and are thought to play an important role in initiating the physiologic response. To better understand the role for PAR2 airway epithelial signaling we created a PAR2-deficient human bronchial epithelial cell line (16HBEPAR$^{-/-}$) from a model bronchial parental line (16HBE14o−). Comparison of *in vitro* physiologic responses in these cell lines demonstrated a complete loss of PAR2 agonist (2at-LIGRL-NH_2) response and significantly attenuated protease

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Conflicts of Interest

Drs. Kathryn A DeFea, Theodore J. Price, Gregory Dussor, Josef Vagner and Scott Boitano are all involved in PARMedics, a company that specializes in the development of protease-activated receptor-2 ligands. None of the work in this manuscript originated from the company nor is protected by the company. On behalf of my colleagues, I am stating that there is no conflict of interest in the creation and eventual publication of this manuscript.

(trypsin and elastase) and *A. alternata* responses in the 16HBEPAR$^{-/-}$ line. Apical application of *A. alternata* to 16HBE14o− and 16HBEPAR2$^{-/-}$ grown at air-liquid interface demonstrated rapid, PAR2-dependent and independent, inflammatory cytokine, chemokine and growth factor basolateral release. In conclusion, the novel human PAR2-deficient cell line allows for direct *in vitro* examination of the role(s) for PAR2 in allergen challenge with polarized human airway epithelial cells.

Keywords

Allergen; *Alternaria alternata* ; inflammatory cytokines; airway epithelium

INTRODUCTION

Alternaria alternata is a common fungus associated with allergic asthma and asthma exacerbations [1–4]. Hallmarks of allergic asthma include airway hyperreactivity, airway remodeling, mucus overproduction and inflammation [5,6]. PAR2 is a G protein-coupled receptor (GPCR) that is activated following proteolytic cleavage of the extracellular amino-terminus [7]. The resulting tethered ligand directly interacts with the receptor to initiate Gαq and β-arrestin-dependent signaling pathways [8]. Participation of *A. alternata* alkaline serine protease (AASP) and host protease-activated receptor-2 (PAR2) in the development of the asthma response have been elucidated using animal models [9–12]. Additionally, it has been suggested that alternative components from *A. alternata* can also contribute to allergen-induced response [13,14].

The airway epithelium that lines the conducting airways is uniquely positioned to encounter allergens such as *A. alternata* and thus, may provide a therapeutic target for asthma inhibition to elicit cellular responses that contribute to asthma [15–17]. It has been shown *in vitro* that airway epithelial exposure to *A. alternata* results in production of various cytokines, chemokines and inflammatory mediators that help form the innate immune response [14,18–22]. While these studies mostly suggest a role for PAR2 in the cellular response, they are limited by the numerous active signaling components in *A. alternata* [23,24] and the common use of cells such as BEAS-2B and A549 that lack key polarization features of the *in vivo* airway epithelium. The lack of commercially available, highly specific PAR2 inhibitors, and the inability to separate PAR2-dependent from PAR2-independent signaling in *in vitro* airway epithelium have been further obstacles in developing a clear understanding of PAR2-associated responses. Consequently, specific roles for airway epithelial-expressed PAR2 in allergic asthma initiation and pathophysiology are not fully understood.

Our goal is to better define the central role for human airway epithelial PAR2 in shaping the physiological and inflammatory response triggered by asthma-associated allergen exposure. In this report we address the issue of distinguishing PAR2-specific from non-specific *A. alternata*-induced airway epithelial signaling and select cytokine/chemokine/growth factor release by using CRISPR-Cas9 technology to create a human bronchial epithelial cell line devoid of PAR2 (16HBEPAR2$^{-/-}$) along with parental, PAR2-expressing cells

challenged with allergen in the presence or absence of an antagonist. Development of the 16HBEPAR2$^{-/-}$ cell line paired with antagonism studies allows for novel *in vitro* experiments in a human bronchial cell line that forms a functional, polarized epithelial layer.

MATERIALS AND METHODS

Materials

Alternaria alternata filtrate was purchased as a lyophilized powder from Stallergenes Greer Laboratories (Lenoir, NC). Filtrate was resuspended in Hanks Balanced Saline Solution additionally buffered with 25 mM HEPES (pH, 7.4; HBSS) and stored at 2.5 mg/mL protein at −20°C until use. Protease concentration was determined prior to use; activity of the filtrate used in these experiments was comparable to 0.5 μg/mL trypsin as determined with a commercial protease assay kit (Sigma Cat # PF-0100). Potent and specific PAR2 agonist [2-aminothiazol-4-yl-LIGRL-NH_2 (2-at-LIGRL-NH_2)] and antagonist C391 were produced in our laboratory as described [25–27]. Trypsin was purchased from Sigma (Cat#T6567) and human neutrophil elastase from Worthington Biochemical (Cat# LS003703; Lakewood, NJ). Unless listed below, all other chemicals/components were of Molecular Biology or higher grades and purchased from Fisher Scientific (Pittsburgh, PA), ThermoFisher Scientific (Watham, MA), Sigma-Aldrich (Burlington, MA), or VWR (West Chester, PA).

Cell culture

PAR2-expressing cells and the parental cells for CRISPR-Cas9 editing in this study are the 16HBE14o− cell line, SV40-transformed human bronchial epithelial cells [[28]; California Pacific Medical Center Research Institute (San Francisco, CA, USA)]. 16HBE14o− cells were passaged and grown in Minimal Essential Medium with Earle's Salts supplemented with glutamax (Life Technologies), penicillin/streptomycin and 10% FBS (MEM) as described in [25,26]. Cells were then transferred to appropriate matrix-coated culture-ware for experiments.

Guide RNA (gRNA) design for *Protease-activated receptor-2* gene (*F2RL1*) targeting

We identified and extracted the sequence of *F2RL1* from the genomic sequence of human chromosome 5 (Ensembl, Transcript ID: ENST00000296677.4) and designed a gRNA to match the 5' end of the protein sequence with the gRNA design tool (http://crispr.mit.edu) for off-target screening. The selected gRNA sequence for knockout was located in exon 1 (reverse strand GAGAGAGGCTGCTAGCAGGA; score 52; Figure 1).

Cloning of gRNAs

We used the pl-CRISPR.EFS.PAC plasmid [(Addgene Cat#57828, Cambridge, MA) [29]] that allows for simultaneous expression of the Cas9 enzyme with the gRNA and Puromycin N-acetyltransferase (PAC) for selection. The plasmid was cut using *BsmBI* restriction enzyme (Thermo Fisher Scientific Cat# FD0454, Waltham, MA, USA) according to manufacturer instructions. The digested plasmid was extracted from a 1% agarose gel (Thermo Fisher Scientific, Cat#K0691). Oligonucleotides were designed according to [30] for the cloning of exon 1 targeting gRNA

(*F2RL1* forward: 5'- CACCGAGAGAGGCTGCTAGCAGGA-3'; *F2RL1* reverse: 5'-AAACTCCTGCTAGCAGCCTCTCTC-3'; Eurofins Scientific, Brussels, Belgium). A 10 μM solution of forward and reverse oligonucleotides was annealed in a thermocycler using the following protocol: 37°C for 30 min, 95°C for 5 min with a cooling to 25°C at a rate of 5°C/min. After cooling, 100 ng of the digested pL-CRISPR.EFS.PAC plasmid was set up for ligation with 50 nM of the annealed oligonucleotides at 22°C for 5 min using Rapid DNA ligation Kit (Thermo Fisher Scientific Cat#K1422). The ligation products were transformed into *E. coli* DH5α competent bacteria (New England Biolabs Cat#C2987, Ipswich, MA) according to manufacturer instructions. The integrity of all plasmids was confirmed by Sanger sequencing (Eurofins). A 100% efficiency was observed for the insertion of gRNA sequences into the pL-CRISPR.EFS.PAC plasmid. Plasmids were then purified from *E. coli* DH5α using the NucleoBond® Xtra Maxi kit (Macherey, Cat# 740414, Nagel, Germany).

Transfection, positive selection and clonal selection

Cells (8×10^5) were seeded on matrix-coated 12-well plates. Confluency of ~80% was reached within 24 hrs. Cells were transfected with purified plasmid following manufacturer protocol (Thermo Fischer Scientific Cat # L3000001) and returned to the incubator for three days of growth in MEM supplemented with 4 μg/mL puromycin (Sigma-Aldrich Cat #P8833, St. Louis Missouri). At 10 days, and thereafter, cells were fed with MEM without puromycin. Once full confluency of positively transfected cells was reached, single cells/well were transferred onto a matrix-coated 96-well plate. One day later, wells with a surviving single cell were verified. Verified wells were fed and monitored until confluency was established before transfer onto T25 flasks.

In vitro impedance-based assay for functional testing and physiological response

High-capacity *in vitro* physiological response was accomplished with the xCELLigence Real Time Cell Analyzer multi-plate unit (RTCA-MP; ACEA Biosciences, San Diego, CA) and methods adapted from [31]. For reference, PAR2 activation results in an increase in impedance, represented as a Cell Index, whereas cell toxicity and/or death is associated with a decrease in Cell Index [31,32]. For our experiments, an initial background recording was taken of matrix-coated E-plates (ACEA, BioSciences) containing MEM alone. Media was replaced with 16HBE14o$^-$ or 16HBEPAR$^{-/-}$ cells in MEM with reduced FBS (5%) onto matrix-coated E-plates. Cells were allowed to grow overnight (37°C and 5% CO_2) to establish full adhesion when ligand, protease or allergen was applied to the cells and physiological responses recorded every min for up to 4 hrs. FBS-free media was used for all protease experiments. Cell responses were collected in quadruplicate. To allow for the comparison of readings, cell responses were normalized at a time just prior to treatment and data is presented without SEM (e.g., supplemental Figure S2 in [31]).

Cytokine secretion and detection

Matrix-coated 24-well Transwell filters (Corning Costar Cat #3472) were seeded with MEM (100 μL) containing 1×10^5 airway epithelial cells with 600 μL full MEM added below the filter. Apical (100 μL) and basolateral media was replaced every other day until day 5 where apical media was aspirated, and cells were grown at air-liquid interface. Upon establishment of proper transepithelial resistance (> 200 Ω*cm^2 for all filters) MEM was replaced with

serum free-MEM overnight. Cells were then apically treated with control HBSS, 10μg/100 μL *A. alternata* or *A. alternata* supplemented with 1 μmole of the PAR2 antagonist C391 [25]. Basolateral medium (100 μL) was recovered and replaced with serum-free MEM immediately prior to apical application (time 0) and at 4 (data not shown) and 24 hrs. Samples were spun at 250× g for 5 min and stored at −20°C until evaluation. Cytokine concentrations in thawed cell culture supernatants were determined using the MAGPIX® (Luminex Corp Cat #MAGPIX-XPONENT, Austin, TX) and appropriate multiplex plates following manufacturer protocols (Millipore Sigma Cat# HTH17MAG-14K, Burlington, MA and Millipore Sigma Cat# HCYTOMAG-60K). Multiplex data were analyzed with MA Analyst v5.0 (Millipore/VigeneTech) using 5-parameter (5PL) logistic curve fitting.

Statistics

A Students t-test was performed on experiments with only two groups. One way ANOVA was performed with a Tukey post-hoc test for experiments with more than 2 groups. For experiments that were monitored over time, a Two-way ANOVA was performed. For all statistical tests $p \leq 0.05$ was considered significant.

RESULTS

In vitro physiological responses to the potent, selective PAR2 agonist, 2-aminothiazol-4-yl-LIGRL-NH_2 (2at-LIGRL-NH_2) were monitored in 16HBE14o− and 16HBEPAR$^{-/-}$ cell lines (Figure 2). Responses to 2at-LIGRL-NH_2 in 16HBE14o$^-$ cells were similar to published results [26,31], with a sharp increase in Cell Index that peaked after 30 min and remained elevated throughout the experiment and a desensitizing effect at the highest concentrations of 2at-LIGRL-NH_2 tested (i.e., > 1 μM; Figure 2A). The EC_{50} [112 nM with 95% Confidence Interval (95CI) of 80 – 157 nM] and the relatively fast recovery time reflect a change in temperature to 37°C used here from the room temperature used previously [26]. In contrast, the 16HBEPAR$^{-/-}$ cells did not display a 2at-LIGRL-NH_2-induced cellular response throughout the concentration range tested (Figure 2B). The lack of a physiological response to 2at-LIGRL-NH_2 confirms the functional ablation of PAR2 signaling in this cell line.

Because PAR2 can be activated by several trypsin-like serine proteases [8], and this type of activation is what occurs *in vivo*, we repeated the sensitive *in vitro* physiological assays with trypsin or elastase to evaluate concentrations that could provide PAR2-specific responses when apically exposed to airway epithelial cells (Figure 2C, 2E). Both trypsin (EC_{50} = 14 nM; 95CI = 11 – 17 nM) and elastase (EC_{50} = 300 nM, 95CI: 240 – 370 nM) displayed concentration-dependent activation of 16HBE14o− cells. The responses to trypsin and to elastase were largely absent in 16HBEPAR2$^{-/-}$ cells, although the elastase did induce a small physiological response (Figure 2D, 2F). These experiments demonstrate that low concentrations of trypsin and elastase primarily act at PAR2 to initiate signaling and subsequent *in vitro* physiological responses in airway epithelial cells.

While we have shown that *Alternaria* alkaline serine protease (AASP) is an important driver of asthma-like symptoms in animal models [11,12]. *A. alternaria* filtrates contain a variety of defined and undefined components [23,24] that may also contribute to cellular signaling

(e.g., [14,33]). The comparison of *A. alternata* filtrate-induced signaling in 16HBE14o− and 16HBEPAR2$^{-/-}$ cells with the extremely sensitive RTCA-MP *in vitro* physiological output allowed us to more directly investigate PAR2-dependent and PAR2-independent signaling in human bronchial epithelial cells. Addition of *A. alternata* filtrate to 16HBE14o− cells resulted in an initial decrease in Cell Index followed by an increase that was similar to the PAR2 agonist and protease responses shown above, albeit on a reduced scale (Figure 3A). The full agonist response for *A. alternata* occurred at the lowest concentration tested, 30 ng/mL. Higher *A. alternata* concentrations elicited a secondary loss of Cell Index response indicative of PAR2 desensitization, interference with PAR2 signaling and/or activation of alternative signaling pathways. The physiological response induced by *A. alternata* filtrate in 16HBEPAR$^{-/-}$ cells resulted in a much different pattern. The initial dip in Cell Index was significantly reduced and the distinct peak in Cell Index induced by *A. alternata* in 16HBE14o− cells was largely absent across all concentrations measured (Figure 3B). Increases in Cell Index elicited by *A. alternata* were still evident, showing that the filtrate contains a PAR2-independent signaling component to establish the physiological response.

An advantage of the 16HBE14o− cell line over more commonly used human airway epithelial cell lines (e.g., A549 or BEAS-2B) is the ability to grow at air-liquid interface in an epithelial layer that separates apical from basolateral membrane and thus, better represents the *in vivo* condition [34–36]. To better understand how PAR2 signaling contributes to *A. alternata*-induced inflammation, we compared *A. alternata*-induced basolateral secretions in 16HBE14o− and 16HBEPAR$^{-/-}$ cells in combination with a potent PAR2 antagonist, C391 [12,25]. *A. alternata* filtrate induced significant IL-6 and IL-8 secretion in 16HBE14o− cells at 24 hr and this secretion was significantly reduced by PAR2 antagonism with C391 (Figure 3A – B). In the absence of PAR2, secreted IL-6 and IL-8 was significantly lower after the serum starvation step and did not significantly increase with 24 hr exposure to *A. alternata*. Apical *A. alternata* exposure resulted in a similar increase in RANTES and IP-10 secretion in 16HBE14o− cells, with significant increases at 24 hrs that were significantly reduced by C391 (Figure 3C – D). However, the 16HBE14o− cells also showed significant increase in these cytokines in the control HBSS application, suggesting an additional constitutive secretion pathway. This was apparent in the 16HBEPAR2$^{-/-}$ cells where secretion of both RANTES and IP-10 was increased at the 24 hr time point, albeit with a much lower final concentration than that observed in the 16HBE14o− cells. TNF-α exhibited a similar PAR2-dependent and independent secretion with the additional reduction PAR2-dependent secretion in response to overnight serum removal (Figure 4E). Growth factor (VEGF and PDGF) secretion was also affected by PAR2 (Figure 4F – G). In the PAR2-expressing 16HBE14o− cells, both VEGF and PDGF showed increased secretion at 24 hrs that was fully inhibited by C391. While *A. alternata* did not alter VEGF secretion, PDGF was increased at the 24 hr time point in the 16HBEPAR2$^{-/-}$ cells. These results suggest that PAR2 signaling in the airway epithelium is required for full cytokine/chemokine/growth factor release following *A. alternata* exposure and that it plays an essential role coordinating an acute inflammatory response to allergen exposure.

DISCUSSION

In this study, we present a newly developed PAR2-deficient human bronchial epithelial cell line (16HBEPAR$^{-/-}$) as a novel tool to better define the role of PAR2 in the initial physiological responses to acute *A. alternata* exposure. Our studies are in line with several others that demonstrate *A. alternata*-induces cytokine/chemokine response in human airway epithelial cells [18,20–22,33,37,38]. 16HBE14o− cells have been used to highlight the need for polarized epithelium to study *A. alternata in vitro* (e.g., [22]), the accompanying polarized 16HBEPAR2$^{-/-}$ can provide further distinct advantage in the study of physiologic properties that require epithelial layers. The noted findings that serum starvation resulted in altered baseline secretions between 16HBE14o− and 16HBEPAR2$^{-/-}$ cells also point to an ill-defined role for airway epithelial associated proteases in PAR2 signaling (e.g., [39]). Human airway epithelial PAR2 signaling has also been associated with ion channel function [40–42] and cell migration [43,44]; 16HBEPAR2$^{-/-}$ cells provide a novel tool to better define the role for PAR2 in these physiologic endpoints in concert with, or distinct from, *A. alternata* exposure.

Several allergens have been shown to express PAR2-cleaving proteases and induce asthma-associated inflammatory responses in animal and cellular models. These include house dust mite [45,46]; German cockroach frass [47–50] in addition to *A. alternata* [11,12,51]. Like *A. alternata*, these asthma-associated allergens contain a variety of microbe-associated molecular factors (e.g., glycoproteins, lipopolysaccharides, peptidoglycans and double-stranded DNA) that can initiate cellular signaling in the airway via host pattern recognition receptors (PRRs). In the case of *A. alternata*, it has been suggested by some researchers that the inflammatory response is independent of PAR2 [13,14]. Significantly we and others have noted the variation of protease in both homemade and commercially available *A. alternaria* extracts; similar to *in vitro* studies where PAR2-independent responses were noted (e.g., [14,33]), differences in protease activity can partially explain these results. As noted (e.g., [51]) and demonstrated with the 16HBEPAR2$^{-/-}$ cells in this report, even with protease-expressing *A. alternata*, there is distinct signaling and cytokine secretion that is independent of PAR2 expression. Nevertheless, our data shows a clear action on human PAR2 by *A. alternata* using multiple assays.

We conclude that PAR2 in the airway epithelium is an important receptor to initiate physiologic changes invoked by *A. alternata*, and thus is a viable target for PAR2 drug development in the reduction of inflammation associated with allergic asthma. The 16HBEPAR2$^{-/-}$ cells developed here will be a useful tool for characterizing how other allergens may act via human PAR2 to promote airway responses that are involved in asthma pathology.

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Highlights:

- *Alternaria alternata* actions on human airway epithelial cells depend on protease-activated receptor-2 (PAR2) signaling
- *Alternaria alternata* cytokine, chemokine and growth factor is shaped by PAR2 activation
- PAR2-deficient human airway epithelial cells create a model for studying PAR2 in airway disease

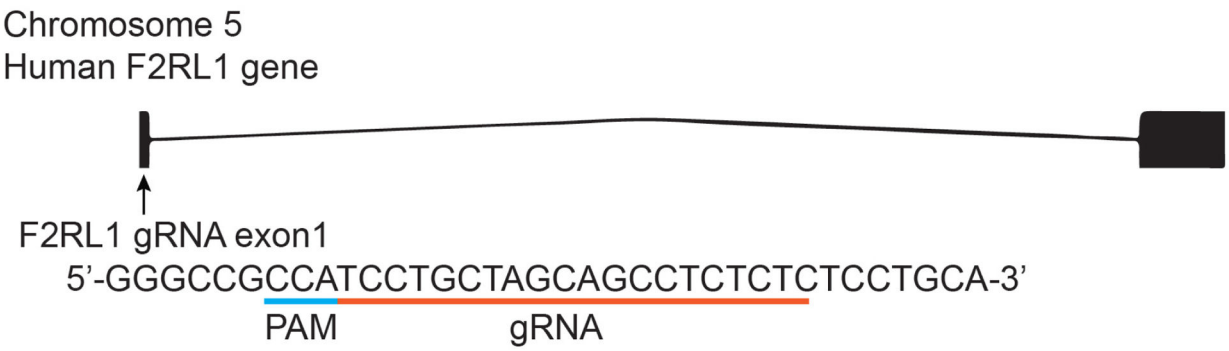

Figure 1: Design of the CRISPR strategy to target the *F2RL1* gene.

The designed gRNA from the reverse strand sequence targeted on exon 1 in *F2RL1* is indicated by the green line oriented from 5' to 3'. The protospacer adjacent motif (PAM) sequence is indicated by a blue line. The gRNA pairs with its DNA target, located 37 bp downstream of the translation initiation codon ATG, followed by a 5'NGG sequence (PAM). Cas9 catalyzes a double stranded cleavage on the genomic DNA 3 bp before the PAM sequence.

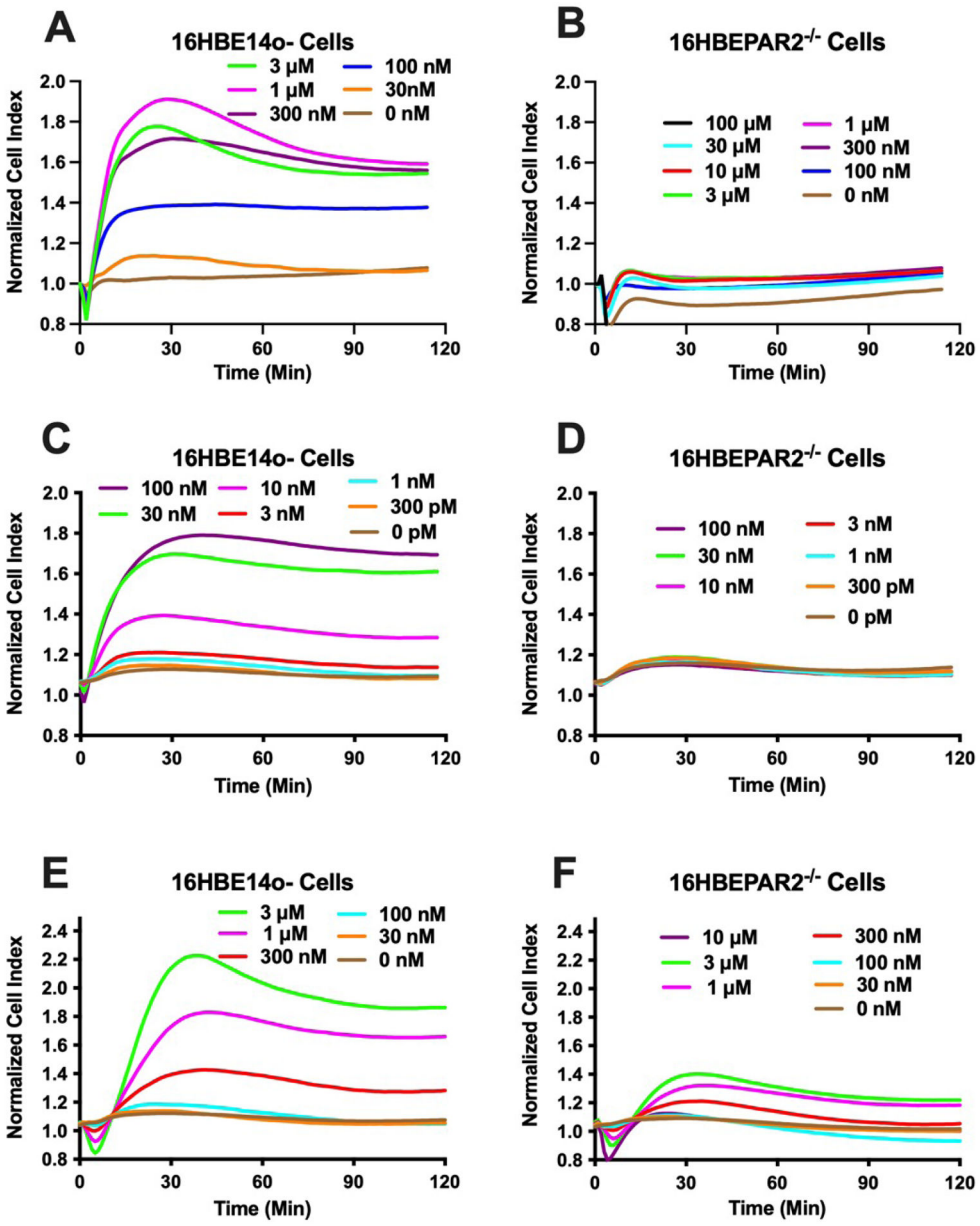

Figure 2: The *in vitro* physiological responses of human bronchial epithelial cells following addition of select PAR2 activators.

Each panel (**A - F**) represents the impedance responses (Cell Index) measured each minute following the addition of PAR2 agonist. Agonist concentrations were chosen to reflect full responses in 16HBE14o− cells and reduced in ½ log steps Traces represent the average of four experiments. Concentration-dependent response to 2at-LIGRL-NH_2 for 16HBE14o− **(A)** or 16HBEPAR2$^{-/-}$ **(B)** cells. Concentration-dependent response to trypsin for 16HBE14o− **(C)** 16HBEPAR2$^{-/-}$ **(D)** cells. Concentration-dependent response to elastase for 16HBE14o− **(E)** 16HBEPAR2$^{-/-}$ **(F)** cells. The lack (2at-LIGRL-NH_2; trypsin)- or severely reduced (elastase)-induced physiological responses in the 16HBEPAR2$^{-/-}$ cell line demonstrate a necessity for PAR2 expression for *in vitro* physiological response.

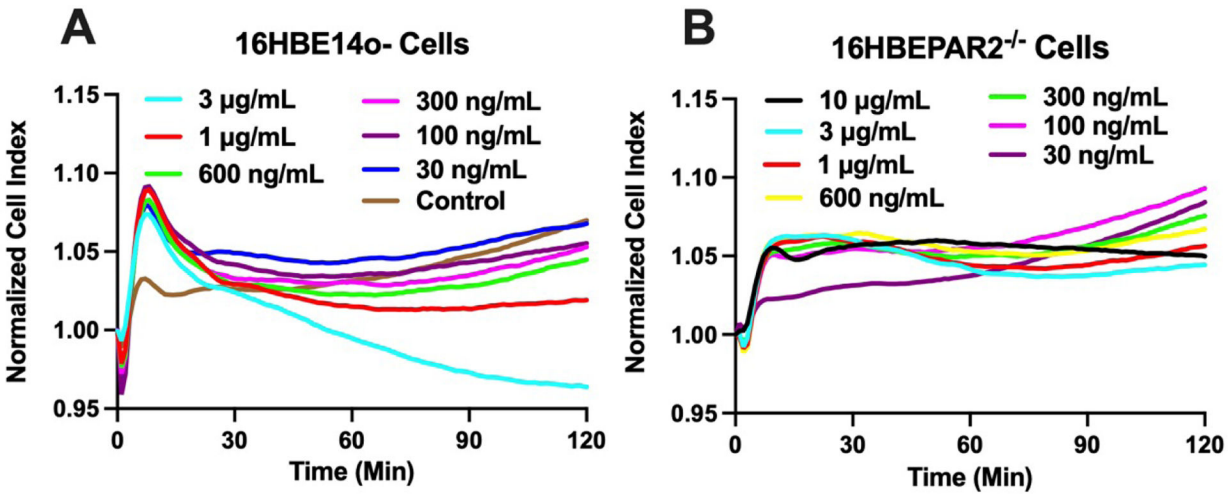

Figure 3: PAR2-dependent *in vitro* physiological response to *A. alternata*.

16HBE14o− **(A)** or 16HBEPAR$^{-/-}$ cells **(B)** were treated with 30 ng/mL – 10 µg/mL *A. alternata* filtrate and Cell Index was measured every minute for 2 hrs. *A. alternata* induced a distinctly different response in the PAR2-expressing parental cell line (16HBE14o−). Notably, the initial dip in signaling followed by a rapid peak and recovery was missing in the PAR2-ablated cell line (16HBEPAR2$^{-/-}$). Traces are mean, normalized values from 6 experiments.

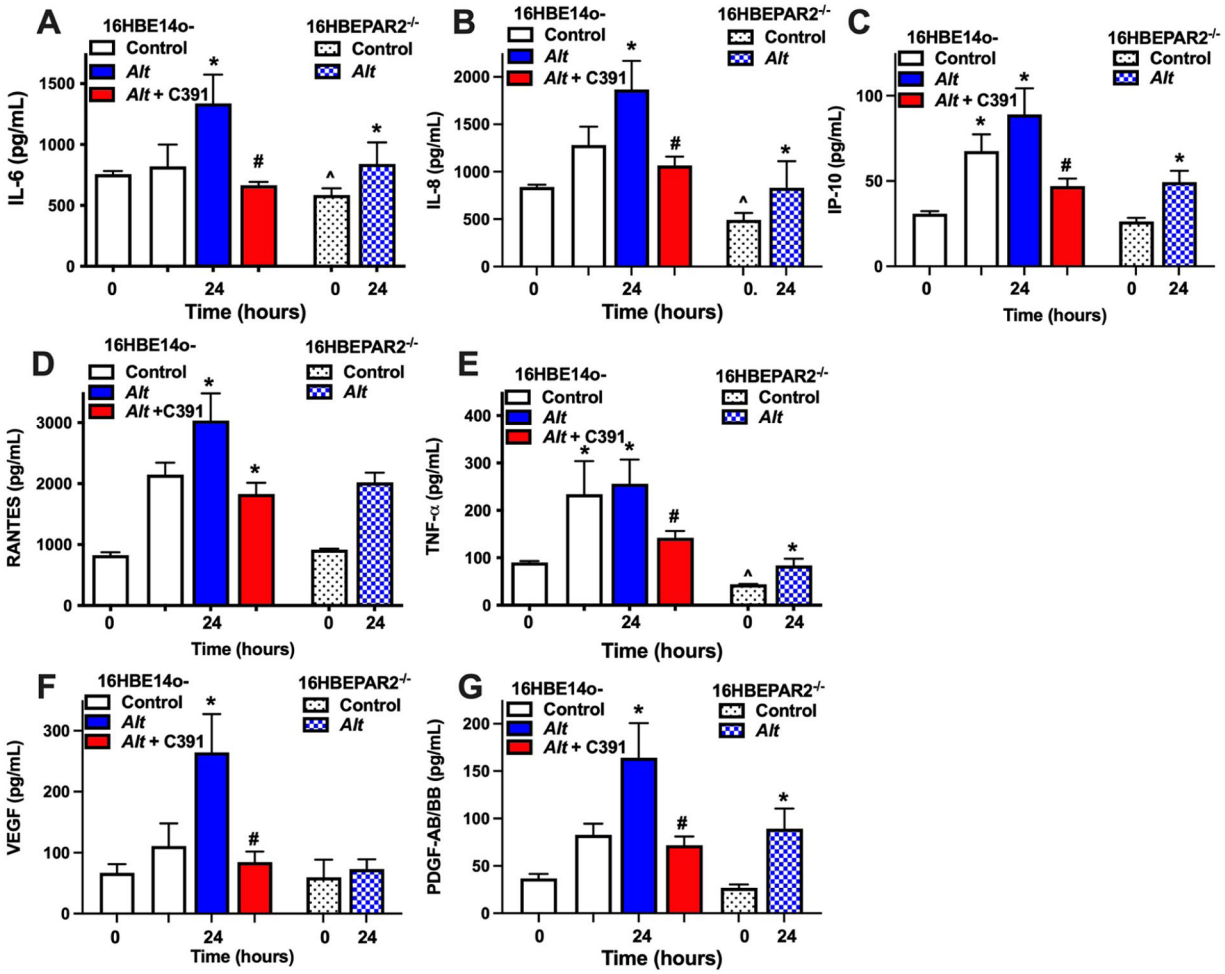

Figure 4: PAR2-mediated *A. alternata*-induced secretion.

(A - G) Polarized human bronchial epithelial cells expressing PAR2 (16HBE14o−; solid columns) or lacking PAR2 (16HBEPAR2$^{-/-}$; shaded columns) were grown at air-liquid interface, serum-starved overnight (time 0) and then apically challenged with HBSS (control, white columns), *A. alternata* (blue columns), or *A. alternata* with the PAR2 antagonist C391 (red columns). Basolateral cytokine concentration was measured just before apical treatment (t = 0) and at 24 hrs post-addition. PAR2 is necessary for full response to *A. alternata* (see text for details). Statistical differences ($p < 0.05$; one-way ANOVA with a Tukey post-test) are as follows: * indicates difference from corresponding time 0; # indicates difference with C391 treatment to matching time point; ^ indicates difference between 16HBE14o− and 16HBEPAR2$^{-/-}$ at time 0. Experiments with 16HBE14o− cells $n = 4 \leq n \leq 11$; experiments with 16HBEPAR2$^{-/-}$ n = 3.