

RESEARCH ARTICLE

Epithelial growth factor receptor tyrosine kinase inhibitors alleviate house dust mite allergen *Der p2*-induced IL-6 and IL-8Hui-Hsien Pan^{1,2,3} | Yu-Ping Hsiao^{1,3,4} | Ping-Ju Chen¹ | Yu-Ting Kang¹ | Yu-Hua Chao^{3,5} | Ji-Nan Sheu^{3,5} | Ko-Huang Lue^{1,2,3} | Jiunn-Liang Ko^{1,2,6} ¹Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan²Department of Pediatrics, Chung Shan Medical University Hospital, Institute of Allergy, Immunology, and Rheumatology, Taichung, Taiwan³School of Medicine, Chung Shan Medical University, Taichung, Taiwan⁴Department of Dermatology, Chung Shan Medical University Hospital, Taichung, Taiwan⁵Department of Pediatrics, Chung Shan Medical University Hospital, Taichung, Taiwan⁶Department of Medical Oncology and Chest Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan

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Abstract

Steroid-insensitive asthma-related airway inflammation is associated with the expression of epidermal growth factor receptor (EGFR) tyrosine kinase in asthmatic bronchial epithelium. Proinflammatory cytokines IL-6 and IL-8 are related to steroid-insensitive asthma. It is currently unknown how EGFR-tyrosine kinase inhibitors (EGFR-TKIs) affects house dust mite (HDM)-induced asthma in terms of inflammatory cytokines related to steroid-resistant asthma and further signaling pathway. Cytokine expressions and EGFR signaling pathway were performed by ELISA, reverse transcriptase PCR, real-time PCR, and Western blot in cell-line models. AMP-activated protein kinase (AMPK) pathway-related inhibitors were applied to confirm the association between EGFR-TKI and AMPK pathway. HDM induced IL-6 and IL-8 in a dose-dependent manner. Both Erlotinib (Tarceva) and Osimertinib (AZD-9291) reduced the levels of HDM-stimulated IL-6 and IL-8 levels in BEAS-2B cells. AZD-9291 was more effective than Erlotinib in inhibiting phospho-EGFR, and downstream phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and phospho-signal transducer and activator of transcription 3 (p-STAT3) pathway signaling. In addition, AMPK pathway-related inhibitor, Calcium-/calmodulin-dependent protein kinase kinase β (CaMKK β) inhibitor, down-regulated IL-8, but EGFR-TKI had no effect on AMPK pathway. Our findings highlight EGFR-TKIs, Tarceva, and AZD-9291, attenuate HDM-induced inflammatory IL-6 and IL-8 cytokines via EGFR signaling axis pathway, but not AMPK signaling pathway.

KEYWORDS

AMPK, *Dermatophagoides pteronyssinus*, EGFR-TKIs, IL-6, IL-8

1 | INTRODUCTION

Asthma, an immune-driven chronic lung disease, is characterized by airflow limitation from bronchospasm due to airway hyper-reactivity, airway smooth muscle thickening, goblet cell metaplasia, and mucus hypersecretion. Except for these defining features, asthma is recognized as a widely heterogeneous disease with multiple clinical variants and subtypes that depend on factors such as severity, responsiveness to corticosteroids, and nature of airway inflammation. Asthma that is poorly controlled by inhaled corticosteroids is associated with elevated IL-6, blood neutrophils, and eosinophils, as well as poor lung

function.¹ Significant increases in neutrophil numbers and IL-8 levels have been found in treatment-resistant severe asthma compared to difficult-to-treat-severe asthma, mild to moderate asthma, and healthy controls.²

House dust mite (HDM), the most frequent indoor aeroallergen, is an independent risk factor for the development of allergic respiratory diseases.³ The most allergenic species are *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f). Proinflammatory cytokines, such as IL-6 and IL-8, are secreted by airway-derived epithelial cells in response to HDM or other allergen.⁴ Shi J et al. found that phosphatidylinositol-3-kinase/Nuclear factor κ B (PI3K/NF κ B)

signaling pathway is involved in the induction of IL-6 and IL-8 in Der p 1-stimulated nasal epithelial cells from allergic rhinitis patients.⁵ HDM induces protease-activated receptor (PAR)-2 downstream signaling, which is dependent on epidermal growth factor receptor (EGFR) via extracellular signal-regulated kinase (ERK) pathway.⁶ Der p 2 induces dose-dependent up-regulation of granulocyte-macrophage colony-stimulating factor, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-3 α (MIP-3 α) via NF- κ B and mitogen-activated protein kinase (MAPK) activation.⁷ In this study, we confirmed that GST-Der PII₁₋₁₂₉ stimulation activates EGFR pathway via kinase activity as EGFR autophosphorylation and downstream MEK/ERK (MAPK kinase/extracellular signal-regulated kinase), PI3K/AKT (phosphatidylinositol-3-kinase/protein kinase B) and STAT (signal transducer and activator of transcription) pathway signaling.

In the asthmatic airway, epithelial cells are altered with increased expressions of EGFR and several of its ligands, leading to increased and corticosteroid-insensitive EGFR activation.^{8,9} Epithelial EGFR activation contributes to several important features of asthma including airway remodeling, mucous metaplasia, and neutrophilic inflammation.¹⁰⁻¹² Inhibition of epithelial EGFR can prevent or inhibit many of these features.^{13,14} EGFR-tyrosine kinase inhibitor (EGFR-TKI) Tarceva is a cancer drug for a subset of lung tumors dependent on the mutation status of EGFR and KRAS.¹⁵ AZD9291, another EGFR inhibitor, has been shown to be effective against both EGFR-TKI-sensitizing and T790M resistance mutations.¹⁶ In the present study, we investigated whether Tarceva or AZD-9291 is suited for the treatment of asthma, as well as the relevant signaling pathways.

2 | MATERIALS AND METHODS

2.1 | Cell culture

BEAS-2B cells, human bronchial epithelial cells, were immortalized with SV40 (American Type Culture Collection, Manassas, Virginia) and maintained in serum-free LHC-9 medium (BioSource International Inc., Nivelles, Belgium) in an incubator at 37°C and humidified atmosphere of 5% CO₂.

2.2 | Chemicals

Tarceva was purchased from OSI Pharmaceuticals (Northbrook, Illinois). AZD-9291 was obtained from MedChem Express (New Jersey). ST-609M, KN93, and U73122 were purchased from Tocris Bioscience (Ellisville, Missouri). Glaucine and chidamide were supplied by Cayman Chemical (Ann Arbor, Michigan). EGF was obtained from Peprotech (Rocky Hill, New Jersey).

2.3 | Western blot analysis

Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in RIPA buffer. Bradford Protein Assay (Bio-Rad Laboratories, Hercules, California) was used to measure the protein concentrations of cell lysates. We separated lysates into equivalent

amounts using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein in the gel was transferred to polyvinylidene difluoride membrane (PVDF, Pall Corporation, Port Washington, New York). Subsequently, the membrane was blocked with 5% nonfat milk at room temperature for 1 hour and then incubated overnight with anti-ACC (Cell Signaling Technology, Danvers, Massachusetts), anti-AKT (Cell Signaling Technology), anti-AMPK (Cell Signaling Technology), anti-EGFR (Cell Signaling Technology), anti-I- κ B (Cell Signaling Technology), anti-Lamin A/C (Gene Tex, San Antonio, Texas), anti-NF κ B (BD Bioscience, San Jose, California), anti-p-ACC (Cell Signaling Technology), anti-p-AKT (Cell Signaling Technology), anti-p-AMPK (Cell Signaling Technology), anti-p-EGFR(Tyr1068) (Abcam, Cambridge, UK), anti-p-EGFR (Tyr1173) (Cell Signaling Technology), anti-Erk 1/2 (Cell Signaling Technology), anti-p-p38 MAPK (Cell Signaling Technology), anti-p-STAT3 (Cell Signaling Technology), anti-STAT3 (Cell Signaling Technology), and anti- β -actin (Cell Signaling Technology).

2.4 | Purification of GST-Der PII1-129 proteins

Inoculated activated GST-Der PII1-129 strain was added to 2 L sterilized LB broth containing 50 μ g/mL ampicillin. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was the induction agent with shaking at 220 rpm in an incubator set at 25°C for 3 hours. The mixture was placed in 250 mL centrifuge tube and centrifuged at 9000g and 4°C for 20 minutes. The pellet was collected and resuspended with 30 mL resuspension buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM sodium chloride, 1 mM magnesium chloride, and 1 mM dithiothreitol. Cells were then treated with lysozyme (0.2 mg/mL) and lysed during three cycles on French press to destroy bacteria and release the target proteins. Subsequently, 40 μ L 10 mg/mL DNase and 30 μ L 10 mg/mL RNase were added and the mixture was placed in 37°C water bath for 15 minutes followed by centrifugation at 12 000 rpm and 4°C for 20 minutes. The supernatant was collected and equilibrated with 1x PBS to activate Glutathion Sepharose. Activated Glutathion Sepharose was added to the supernatant, 1x PBS and elution buffer containing 5 mM glutathione in equilibrated buffer. The flow rate was 1 mL/minute. The liquid was collected with Gilson fraction collector. Each tube contained 2 mL, with peak absorbance value of 280 nm.

Reverse transcriptase PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR).

Total RNA was extracted from cells via RareRNA (GPR02, Genepure Technology Co., Taichung, Taiwan) method. cDNA synthesis was performed with 3 μ g total RNA and High-Capacity cDNA Reverse Transcription kit (Applied BiosystemsTM, Foster City, California). PCR amplification was conducted with the following primer sequences: human IL-6 sense 5'-GCACTGGCAGAAAACAACCT-3' and antisense 5'-CAGGGGTGGTTATTGCATCT-3'; human IL-8 sense 5'-CTCTTGGCAGCCTTCCTGATT-3' and antisense 5'-TATG CACTGACATCTAAGTTCTTTAGCA-3'; human TLR2 sense 5'-GCCAAAGTCTTGATTGATTGG-3' and antisense 5'-TATACCA CAGCCCATGGAAAC-3'; human TLR4 sense 5'-AATCCCCTGAGG CATTAGG-3' and antisense 5'-CCCCATCTTCAATTGTCTGG-3'. Moreover, qRT-PCR was performed using StepOneTM Real-Time PCR System (Applied Biosystems).

2.5 | Statistical analysis

The data are presented as mean $\pm$ SE of triplicate experiments. For comparisons of biochemical and physiological parameters between treated and control groups, one-way analysis of variance (ANOVA) was conducted followed by Tukey's multiple range test using SPSS V.17 for Windows (SPSS Inc, Chicago, Illinois). $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Expressions of pro-inflammatory cytokines in GST-Der pII₁₋₁₂₉-stimulated human lung epithelial cell lines

We investigated whether GST-Der pII₁₋₁₂₉ at varying concentrations (0, 0.1, 1, 5, and 10 $\mu\text{g/mL}$) stimulates human lung epithelial cell line BEAS-2B to secrete IL-6 and IL-8. There were positive correlations between GST-Der pII₁₋₁₂₉ and expressions of IL-6 and IL-8 in conditioned medium (Figure 1A). Total cellular mRNA expressing IL-6, IL-8, and

Toll-like receptor 2 (TLR2), but not Toll-like receptor 4 (TLR4), increased with increasing concentrations of GST-Der pII₁₋₁₂₉ (Figure 1B-D).

3.2 | Tarceva inhibits inflammation in GST-Der pII₁₋₁₂₉-stimulated human lung epithelial cell lines

BEAS-2B cells were co-treated with Tarceva (4, 20, 100, and 250 nM) or dexamethasone (1 μM) for 2 hours followed by exposure to GST-Der pII₁₋₁₂₉ (1 $\mu\text{g/mL}$) for 24 hours. There was negative correlation between the concentration of Tarceva and the expressions of IL-6 and IL-8 in conditioned medium (Figure 2A). Total cellular mRNA expressing IL-6 and IL-8 decreased with increasing concentration of Tarceva on RT-PCR (Figure 2B) and real-time PCR (Figure 2C,D).

3.3 | Tarceva affects signal expression in EGFR signaling pathway

Le Cras et al.¹⁴ demonstrated that EGFR signaling, especially in the airway epithelium, plays an important role in mediating airway

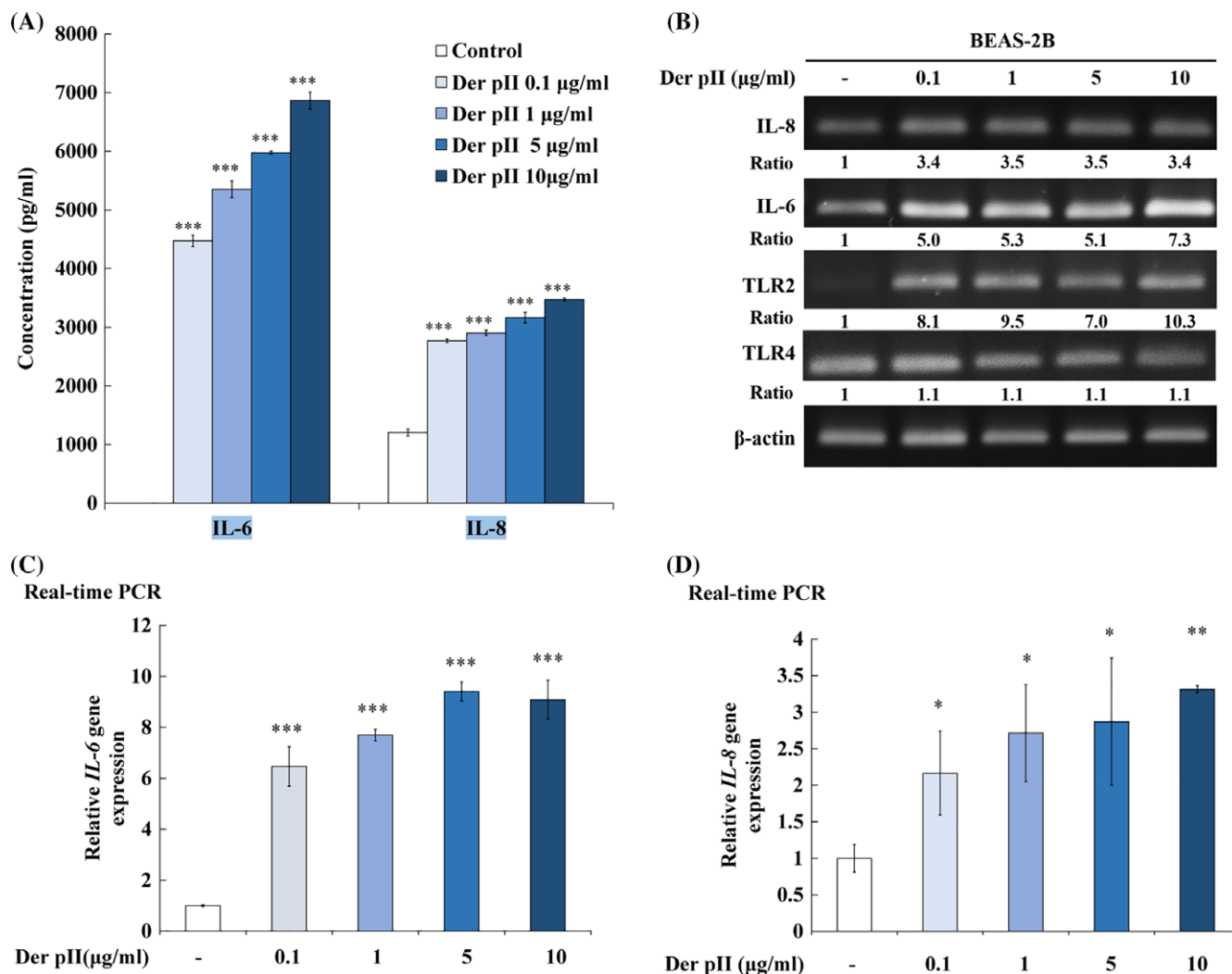


FIGURE 1 Expressions of IL-6 and IL-8 in GST-Der pII₁₋₁₂₉-stimulated human lung epithelial cells. BEAS-2B cells (1×10^6 cells/6 cm dish) were treated with varying concentrations of GST-Der pII₁₋₁₂₉ (0, 0.1, 1, 5, and 10 $\mu\text{g/mL}$) for 24 hours. (A) IL-6 and IL-8 in conditioned medium were measured on ELISA. Total cellular mRNA expressing IL-6 and IL-8 was measured on (B) RT-PCR and (C, D) real-time PCR. Data are expressed as mean $\pm$ SD. Differences were analyzed with one-way ANOVA. (*) indicates significant difference as compared with control * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ [Color figure can be viewed at wileyonlinelibrary.com]

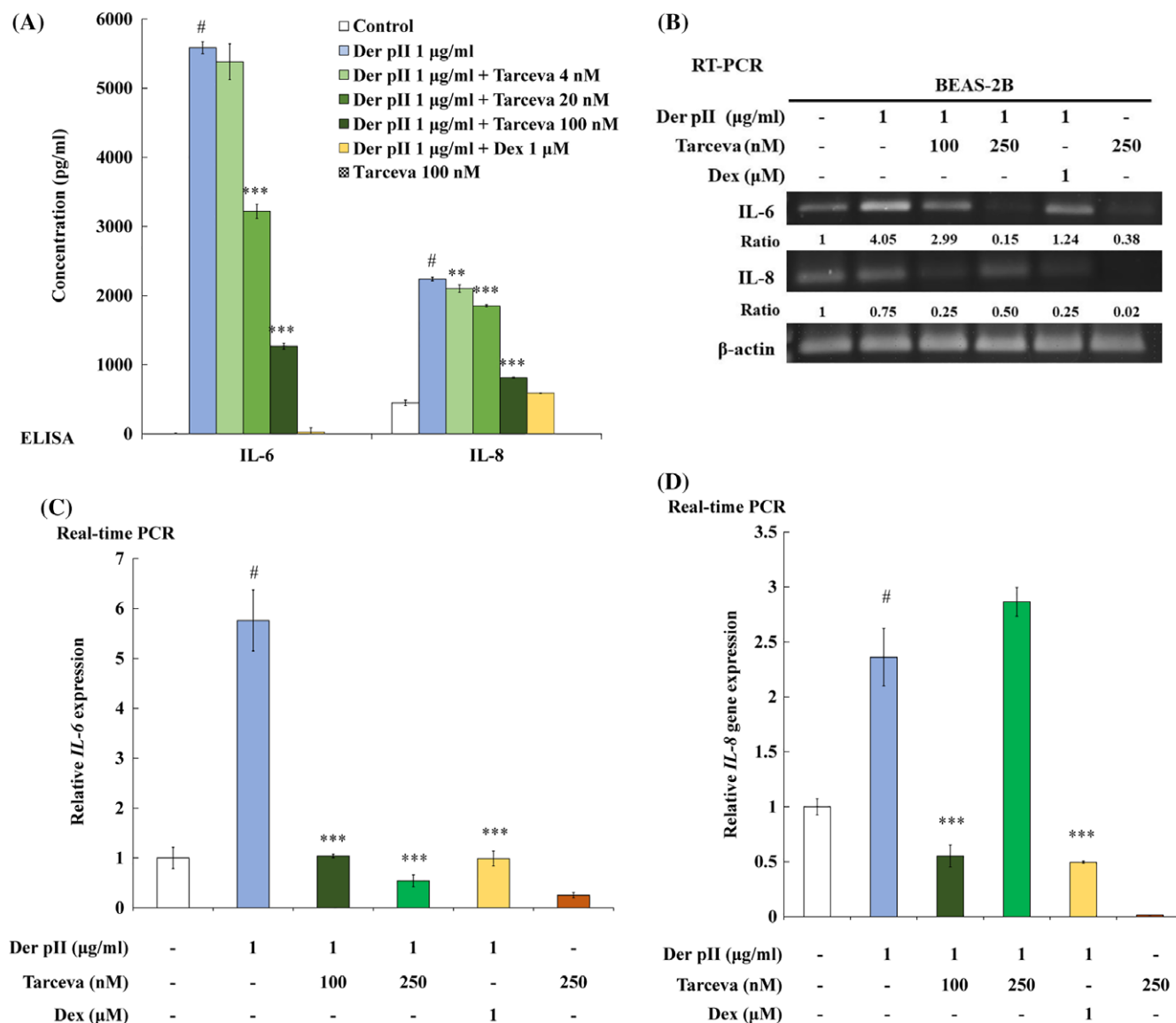


FIGURE 2 Effects of Tarceva on GST-Der pII1-129-induced inflammation in human lung epithelial cells. BEAS-2B cells pretreated with Tarceva (4, 20, 100, and 250 nM) or dexamethasone (1 µM) for 2 hours were stimulated with Der p II (1 µg/mL) for 24 hours. (A) IL-6 and IL-8 in conditioned media were measured on ELISA. Total cellular mRNA expressing IL-6 and IL-8 was measured on (B) RT-PCR and (C,D) real-time PCR. Data are expressed as mean ± SD. Differences were analyzed by one-way ANOVA. (#) Indicates significant differences when compared with control, and (*) when compared with Der pII alone. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ [Color figure can be viewed at wileyonlinelibrary.com]

hyper-responsiveness and remodeling in HDM-induced chronic allergic asthma model. BEAS-2B cells (1×10^6 cell/6 cm dish) were treated with GST-Der pII₁₋₁₂₉ (1 µg/mL) or EGF (10 ng/mL) for varying time periods (10, 30, 60, and 180 minutes). Protein levels were determined on Western blot with upregulation of p-EGFR(Tyr1068), p-EGFR(Tyr1137), p-STAT3, p-AKT, and p-p38 MAPK after GST-Der pII₁₋₁₂₉ stimulation. EGFR pathway was activated 60 minutes after GST-Der pII₁₋₁₂₉ stimulation, and 10 minutes after EGF stimulation. (Figure 3A) We also evaluated whether Tarceva affects GST-Der pII₁₋₁₂₉-induced activation of EGFR signaling pathway. BEAS-2B cells pretreated with Tarceva (20, 100, and 250 nM) or dexamethasone (1 µM) for 2 hours were stimulated with GST-Der pII₁₋₁₂₉ (1 µg/mL) for 10 minutes. Protein levels were determined on Western blot with down-regulation of p-EGFR (Tyr1068) and p-AKT (Figure 3B).

3.4 | AZD-9291 inhibits inflammation in GST-Der pII₁₋₁₂₉-stimulated human lung epithelial cell lines

AZD 9291, a third-generation EGFR-TKI, produces an impressive response in patients with centrally confirmed EGFR T790 M, which is highly related to the resistance to first-generation EGFR-TKI. BEAS-2B cells were co-treated with AZD-9291 (4, 20, 100, and 500 nM) or dexamethasone (1 µM) for 2 hours followed by exposure to GST-Der pII₁₋₁₂₉ (1 µg/mL) for 24 hours. There was negative correlation between the concentration of Tarceva and the expressions of IL-6 and IL-8 in conditioned medium (Figure 4A). Total cellular mRNA expressing IL-6 and IL-8 decreased with increasing concentration of AZD-9291 on RT-PCR (Figure 4B) and real-time PCR (Figure 4C, D).

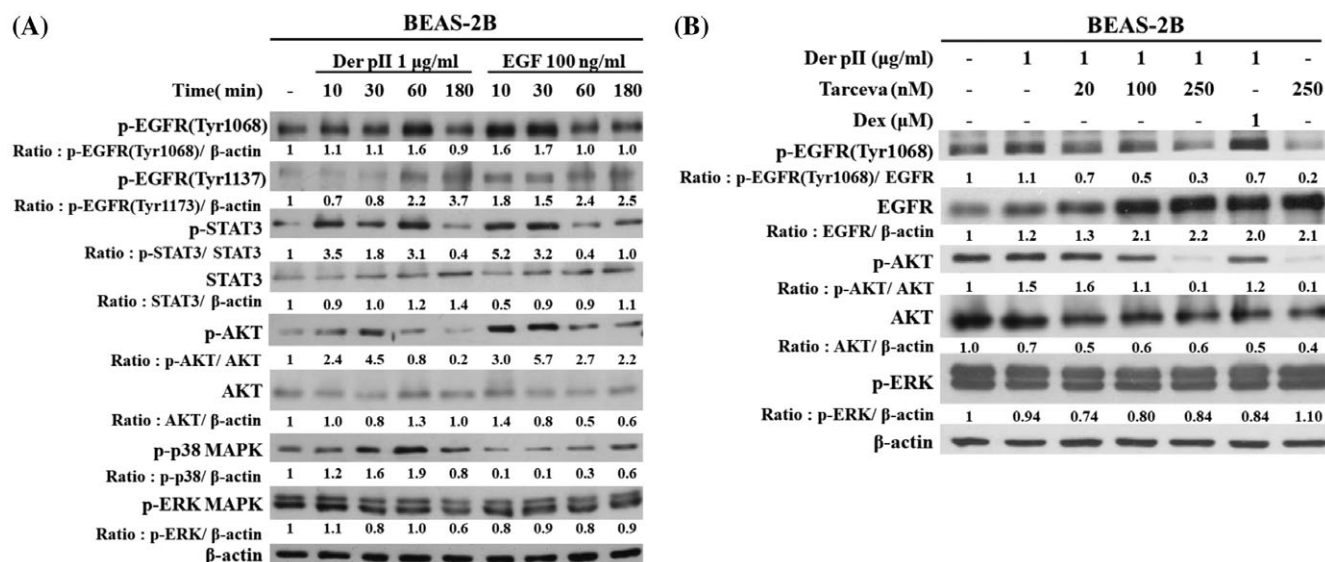


FIGURE 3 Signal expressions in EGFR signaling pathway in GST-Der pII1-129-stimulated human epithelial cells with/without Tarceva treatment. (A) BEAS-2B cells (1×10^6 cell/6 cm dish) were treated with Der pII (1 µg/mL) or EGF (10 ng/mL) for varying time periods (10, 30, 60, and 180 minutes). Protein levels of p-EGFR(Tyr1068), p-EGFR(Tyr1137), p-STAT3, STAT3, p-AKT, AKT, p-p38 MAPK, and p-ERK MAPK were analyzed on Western blot. β-actin served as internal control. (B) BEAS-2B cells (1×10^6 cell/6 cm dish) pretreated with Tarceva (20, 100, and 250 nM) or dexamethasone (1 µM) for 2 hours were stimulated with Der p II (1 µg/mL) for 10 minutes. Protein levels of p-EGFR(Tyr1068), EGFR, p-AKT, AKT, and p-ERK MAPK were analyzed on Western blot. β-actin was used as an internal control

3.5 | AZD-9291 affects signal expression in EGFR signaling pathway

BEAS-2B cells pretreated with AZD-9291 (4, 20, and 100 nM) or dexamethasone (1 µM) for 2 hours were stimulated with GST-Der pII₁₋₁₂₉ (1 µg/mL) for 10 minutes. Protein levels were determined on Western blot with down-regulation of p-STAT3, p-AKT, and p-EGFR (Tyr1068), and up-regulation of p-EGFR(Tyr1173) (Figure 5A). We also confirmed the effects of AZD-9291 on EGFR signaling pathway with EGF. BEAS-2B cells pretreated with AZD-9291 (4, 20, and 100 nM) for 2 hours were stimulated with EGF(10 ng/mL) for 10 minutes. Protein levels were determined on Western blot with down-regulation of p-STAT3 and p-AKT, but up-regulation of p-EGFR (Tyr1068) and p-EGFR(Tyr1173) (Figure 5B). Moreover, AZD-9291 suppressed activation of NF-κB (Figure 5C).

3.6 | Expressions of pro-inflammatory cytokines via AMPK signaling pathway

Several studies have suggested that AMPK activation exerts significant anti-inflammatory and immunosuppressive effects.^{17,18} In addition to changes in AMP or ADP levels, activation of AMPK is triggered by alternative activating pathway that involves Calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) in response to increases in cell Ca²⁺.¹⁹ AMPK suppresses the activation of NF-κB system, which regulates innate immunity and inflammation.²⁰ We treated BEAS-2B cells (1×10^6 cell/6 cm dish) with AMPK pathway-related inhibitors, 10 µM KN93 (CaM kinase II inhibitor), 10 µM STO-609 (CaMKKβ inhibitor), 20 µM U73122 (phospholipase C inhibitor) and 10 µM glaucine (calcium channel blocker) for 2 hours, followed by stimulation with GST-Der pII₁₋₁₂₉ (1 µg/mL) for 24 hours. IL-8 in conditioned media, as measured on ELISA, decreased after addition of 10 µM STO-609 and 20 µM U73122, but

not after the addition of KN93 or glaucine. The protein levels of Acetyl-CoA carboxylase (ACC), AMPK, p-ACC, and p-AMPK were analyzed on Western blot. We found that GST-Der pII₁₋₁₂₉ inhibits the expression of p-AMPK, but not p-ACC. STO-609 further inhibited the expression of p-AMPK. However, KN93 or glaucine increased the expressions of p-AMPK and p-ACC.

For further confirmation of the effects of CaMKKβ inhibitor, we pretreated BEAS-2B cells with CaMKKβ inhibitor STO-609 (2.5, 5, 10 µM) for 2 hours, followed by stimulation with GST-Der pII₁₋₁₂₉ (1 µg/mL) for 24 hours. IL-6 and IL-8 in conditioned media were measured on ELISA. With increasing concentrations of STO-609, protein levels determined on ELISA with down-regulation of IL-8 but remaining the same as for IL-6.

3.7 | Tarceva or AZD-9291 affects signal expression in AMPK signaling pathway

BEAS-2B cells were pretreated with Tarceva (20, 100, and 250 nM) or AZD-9291 (4, 20, and 100 nM) for 2 hours and stimulated with GST-Der pII₁₋₁₂₉ (1 µg/mL) for 30 minutes. Protein levels were determined on Western blot with no upregulation of p-AMPK (Figure 6E, F). Neither Tarceva nor AZD-9291 inhibited the proinflammatory cytokines stimulated by GST-Der pII₁₋₁₂₉ via activation of AMPK signaling pathway.

4 | DISCUSSION

In the present study, we detected the expressions of pro-inflammatory cytokines in human bronchial epithelial cells stimulated by GST-Der pII₁₋₁₂₉ and treated with EGFR-TKI. We found that increases in IL-6 and IL-8 correlate well with increases in concentration of GST-Der

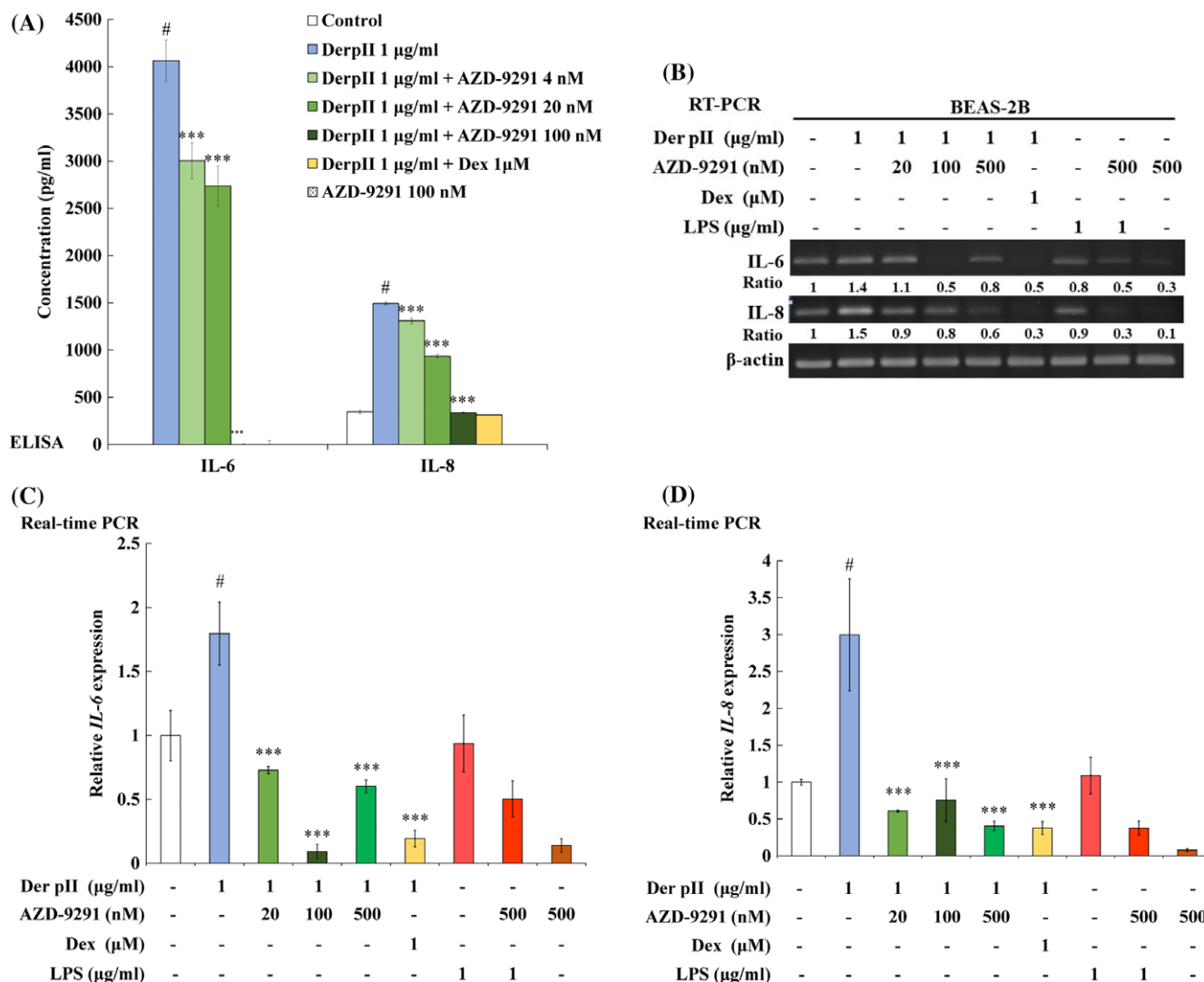


FIGURE 4 Effects of AZD-9291 on GST-Der pII1-129-induced inflammation in human lung epithelial cells. BEAS-2B cells pretreated with AZD-9291 (4, 20, 100, and 500 nM) or dexamethasone (1 µM) for 2 hours were stimulated with GST-Der pII1-129 (1 µg/mL) for 24 hours. (A) IL-6 and IL-8 in conditioned media were measured on ELISA. Total cellular mRNA expressing IL-6 and IL-8 was measured on (B) RT-PCR and (C,D) real-time PCR. Data are expressed as mean $\pm$ SD. Differences were analyzed by one-way ANOVA. (#) Indicates significant difference when compared with control, and (*) when compared with Der pII alone. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ [Color figure can be viewed at wileyonlinelibrary.com]

pII1-129 and reductions in EGFR inhibition, with AZD9291 more effective than Tarceva. GST-Der *pII1-129* did not affect the expression of AMPK signaling pathway in human lung epithelial cell line. However, the regulation of IL-8 was associated with CAMKK β inhibitor. Both Tarceva and AZD9291 affected HDM-induced allergic inflammation via EGFR signaling pathway, but not AMPK signaling pathway.

HDM is one of the most common allergens associated with symptomatic allergic airway diseases. Two common species, Der. p and Der. f, are widespread globally. More than 20 proteins from HDM have been identified as allergens. Der p2, MD-2-related lipid recognition domain, predominantly promotes release of IL-13 and IL-4 Th2 cytokines from peripheral blood mononuclear cells of allergic donors.²¹ Der p1 and Der p2 release pro-inflammatory cytokines such as IL-6 and IL-8.^{5,7} IL-8, a chemoattractant for eosinophil, contributes to neutrophilia in the airways in asthma exacerbation or severe asthma. In the pathogenesis of severe asthma, IL-8 directly facilitates airway remodeling by increasing bronchial smooth muscle cell migration and proliferation.²² A significant increase in

IL-8 levels has been found in patients with treatment-resistant severe asthma compared to difficult-to-treat-severe asthma, mild to moderate asthma, and healthy controls.² IL-6 is a pleiotropic cytokine produced by many cell types in response to multiple inflammatory stimuli and cytokines.²³ IL-6 is found in mixed eosinophilic-neutrophilic bronchitis patients. IL-6 directly correlates with asthma severity and forced expiratory volume in 1 second (FEV₁).²⁴ Increased concentrations of IL-6 have been found in bronchoalveolar lavage and sputum samples from corticosteroid-resistant smokers or nonsmokers with asthma.^{25,26} Furthermore, genome-wide association study has identified polymorphisms in IL-6R as asthma risk loci.²⁷

EGFR is a tyrosine kinase receptor. Protein tyrosine kinases are necessary for the activation and proliferation of inflammatory cells. EGFR signaling pathway is important for initiating and determining clinical outcomes in many respiratory diseases. Aberrant EGFR pathway is associated with early-stage pathogenesis of lung fibrosis, cancer and numerous airway hypersecretory diseases, including COPD,

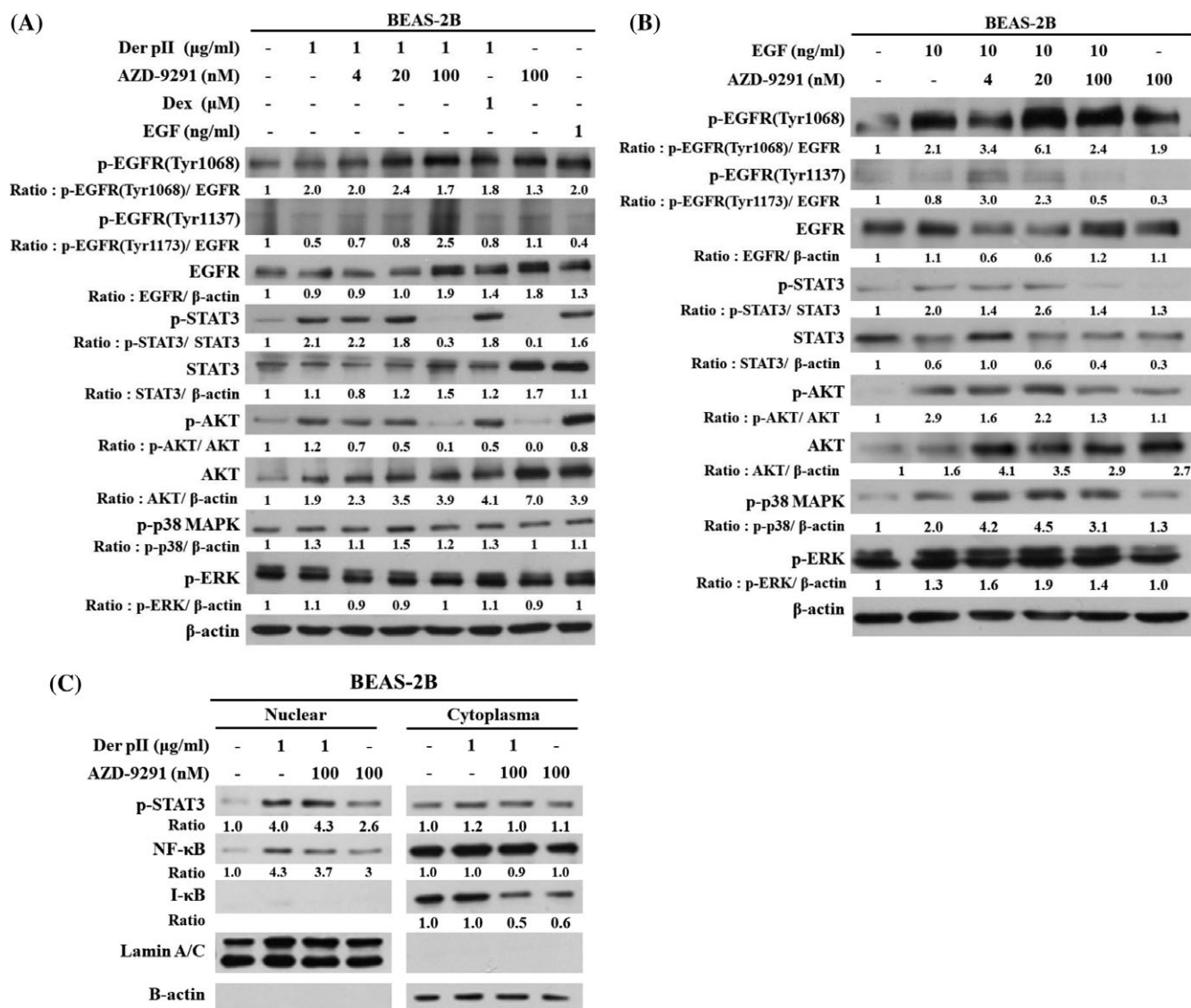


FIGURE 5 Signal expressions in EGFR signaling pathway in GST-Der pII1-129-stimulated human epithelial cells with/without AZD-9291 treatment. BEAS-2B cells pretreated with AZD-9291 (4, 20, and 100 nM) or dexamethasone (1 μM) for 2 hours were stimulated with GST-Der pII1-129 (1 μg/mL) for 10 minutes (A) protein levels of p-EGFR(Tyr1068), p-EGFR(Tyr1137), EGFR, p-STAT3, STAT3, p-AKT, AKT, p-p38 MAPK, and p-ERK MAPK were analyzed on Western blot. β-actin was used as an internal control. (B) BEAS-2B cells pretreated with AZD-9291 (4, 20, and 100 nM) or dexamethasone (1 μM) for 2 hours were stimulated with EGF (10 ng/mL) for 10 minutes. The protein levels of p-EGFR(Tyr1068), p-EGFR(Tyr1137), EGFR, p-STAT3, STAT3, p-AKT, AKT, p-p38 MAPK, and p-ERK MAPK were analyzed on Western blot. β-actin was used as an internal control. (C) Extracted nucleus and cytoplasmic parts of the same amounts were pretreated with AZD-9291 (100 nM) for 2 hours, and then stimulated with GST-Der pII1-129 (1 μg/mL) for 30 minutes. Protein levels of NF-κB and IκB were analyzed on Western blot. Lamin A/C and β-actin were nuclear and cytoplasmic internal controls. Data are expressed as mean ± SD. Differences were analyzed by one-way ANOVA. (#) Indicates significant difference when compared with control, and (*) when compared with GST-Der pII1-129 alone. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

asthma, and cystic fibrosis.^{10,28} Epithelial EGFR activation contributes to airway remodeling, mucous metaplasia, neutrophilic inflammation, and secretion of IL-33 with subsequent activation of type 2 inflammatory responses.^{11,29} A previous study has shown strong correlation between EGFR and IL-8 expression in severe asthma. Lack of effect of dexamethasone on primary epithelial cells is marked for EGF-stimulated IL-8 release in H292 cells.¹¹ Several receptors (EGFR, EGR1, ESR2, and PGR), transcription factors (MYC, JAK) and cytokines (IL-8, IL-6, and IL-1B) have been found to be associated with inflammatory environment and steroid resistance in asthma.³⁰ The insensitivity of proinflammatory responses of EGFR to corticosteroids may be important in severe asthma, which is characterized by overexpression of EGFR, increased

tyrosine phosphorylation, and neutrophilic inflammation.⁸ Previous studies showed that EGFR inhibitor not only reduces AHR, goblet cell metaplasia, airway collagen deposition, eosinophil influx, and proinflammatory cytokine levels (IL-4 and IL-13) in acute asthma models but also attenuates HDM-triggered epithelial resistance and improves restoration of epithelial junctions.^{6,13,31} A clinical trial for EGFR inhibitor BIBW2948 in patients with COPD has demonstrated inhibition of EGFR activation and reduced mucous metaplasia.³² Le Cras et al. have shown reduced airway hyper-reactivity and airway smooth muscle thickening after treatment with another EGFR inhibitor, Tarceva, in mice exposed to HDM for 6 weeks. In atopic dermatitis animal models, IL-6 and IL-1β expressions have been shown to be attenuated in the

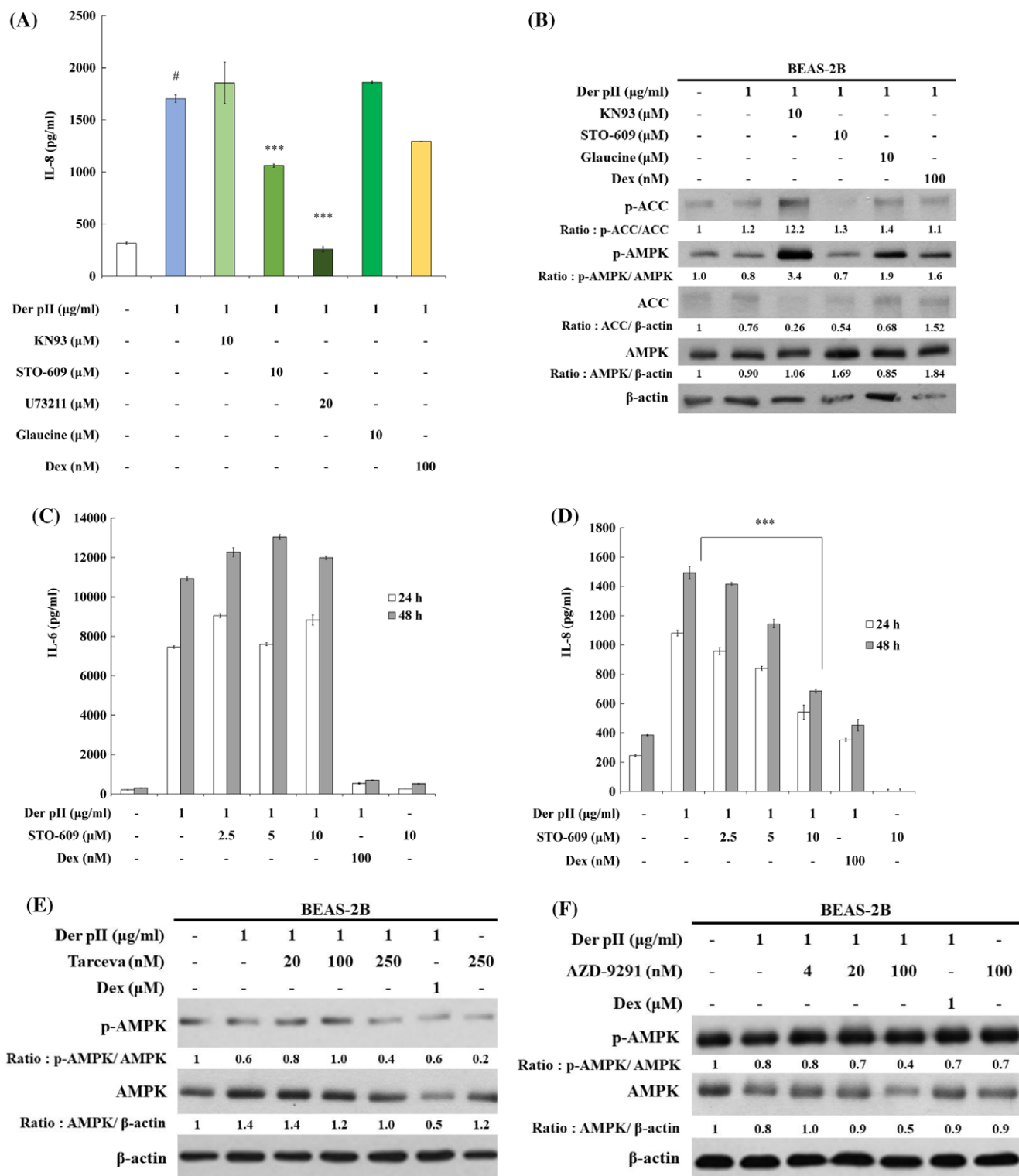


FIGURE 6 Effects of AMPK pathway-related inhibitors on GST-Der pII1-129-induced inflammation and Tarceva/AZD-9291 on GST-Der pII1-129-stimulated AMPK pathway signaling in human lung epithelial cells. BEAS-2B cells (1×10^6 cell/6 cm dish) pretreated with AMPK pathway inhibitors (KN93, STO-609, U73122, and Glaucine) for 2 hours were stimulated with GST-Der pII1-129 (1 µg/mL) for 24 hours. (A) IL-8 in conditioned media was measured on ELISA. (B) Protein levels of p-ACC, p-AMPK, ACC, and AMPK were analyzed on Western blot. β-actin served as internal control. Relative ratios of p-ACC/β-actin, p-AMPK/β-actin, ACC/β-actin, and AMPK/β-actin are shown. BEAS-2B cells pretreated with CaM-KKβ inhibitor, STO-609 (2.5, 5, and 10 µM), for 2 hours were stimulated with GST-Der pII1-129 (1 µg/mL) for 24 hours. (C) IL-6 and (D) IL-8 in conditioned media were measured on ELISA. BEAS-2B cells (1×10^6 cell/6 cm dish) pretreated with (E) Tarceva (20, 100, and 250 nM) or dexamethasone (1 µM) (F) AZD9291 (4, 20, and 100 nM) or dexamethasone (1 µM) for 2 hours were stimulated with GST-Der pII1-12 (1 µg/mL) for 30 minutes. Protein levels of p-AMPK and AMPK were analyzed on Western blot. β-actin served as internal control. Relative ratios of p-AMPK/β-actin and AMPK/β-actin are shown. Data are expressed as mean $\pm$ SD. Differences were analyzed by one-way ANOVA. (*) when compared with Der pII alone. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ [Color figure can be viewed at wileyonlinelibrary.com]

skin of EGF-treated mice, and EGF treatment suppresses allergen-induced IL-6 production by keratinocytes.³³ In H292 cells, EGF increases IL-8 release, and this is suppressed by the EGFR-selective tyrosine kinase inhibitor, AG1478, but only partially by dexamethasone.¹¹ In the present study, we found that the expression of HDM-stimulated IL-6 and IL-8 in BEAS-2B cells decreases as the concentration of either Tarceva or AZD 9291 increases. Liu et al. have shown that the expressions of pro-allergic cytokines (IL-4, IL-5, IL13, and GM-CSF) and pro-inflammatory cytokines (IL-6, IL-8, and MCP-1) are inhibited in response to Der p2 through NF- κ B activation.³⁴ EGFR phosphorylation leads to the activation of NF- κ B,³⁵ which induces the upregulation of IL-8 gene expression.¹¹ We also showed that AZD-9291 inhibits the activation of NF- κ B.

EGFR activates one or more downstream effectors including MEK/ERK, PI3K/AKT, and STAT, as well as mammalian target of rapamycin (mTOR) pathways through receptor autophosphorylation and cytoplasmic protein binding.¹² Previous studies have shown that EGFR-TKI inhibits allergic inflammation by blocking tyrosine kinase signaling cascade, while gefitinib inhibits EGFR and PI3K/Akt activation with decreases in IL-4 and IL-13 in a dose-dependent manner.^{13,36} Furthermore, PAR-2 downstream signaling is dependent on EGFR, as PAR2-induced p-ERK-response is suppressed by EGFR inhibitor AG1478 (Figure 2B).⁶ From the results of this study, Tarceva inhibits the expression of kinase activity EGFR autophosphorylation and downstream PI3K/AKT pathway signaling, while AZD-9291 inhibits the expression of kinase activity EGFR autophosphorylation and downstream PI3K/AKT and STAT pathway signaling.

AMPK, a crucial regulator of energy and metabolic homeostasis, is a serine/threonine kinase which consists of a heterotrimeric complex including catalytic α subunit and regulatory β and γ units.¹⁷ It is activated via increased AMP concentration and by the phosphorylation of α subunit via the upstream kinases serine/threonine kinase 11 (LKB1), CaMKK β , and transforming growth factor- β -activated kinase 1 (TAK1). Previous studies have indicated that activation of AMPK bidirectionally modulates inflammation. AMPK activation increases IL-6 production in cardiac fibroblasts via p38 MAPK-dependent pathway.³⁷ Tang et al. found that AMPK inhibitors ara-A and compound C antagonize adiponectin-mediated potentiation of IL-6 expression, suggesting that AMPK activation is an obligatory event in adiponectin-induced IL-6 expression in human synovial fibroblasts.³⁸ Pretreatment with compound C, an AMPK inhibitor, and siRNA for gene knockdown of AMPK significantly reduce cigarette smoke extract (CSE)-induced increase in IL-8 production.³⁹ In contrast, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), an AMPK activator, decreases CSE-induced IL-8 production while Compound C increases it, as does pretreatment with AMPK α 1-specific small interfering RNA.⁴⁰ Administering AICAR to mice results in activation of AMPK in the lungs and decreased severity of LPS-induced lung injury, as determined by diminished neutrophil accumulation in the lungs, reduced interstitial pulmonary edema, and diminished levels of TNF- α and IL-6 in bronchoalveolar lavage fluid.⁴¹ Metformin, an AMPK agonist, reduces systemic inflammation by decreasing the levels of C-reactive protein and IL-6 in mild metabolic syndrome.⁴² In the present study, we found that STO-609 remains the same as IL-6, and down-regulation of IL-8 is induced by HDM. Response to Tarceva and protein profiles in before and after treatment nonsmall cell lung cancer (NSCLC) models have suggested

that AMPK is a putative target protein, while AMPK activation status impacts EGFR targeted drug response.⁴³ No previous study has discussed the impact of EGFR-TKI on AMPK pathway. From the results of this study, EGFR-TKI has no effect on AMPK pathway.

A previous study found that some severe asthmatic subjects with increase of EGFR expression are steroid refractory, which is not a consequence of corticosteroid treatment.⁴⁴ EGFR-TKI has been found to decrease AHR, goblet cell metaplasia, airway collagen deposition, eosinophil influx, and proinflammatory cytokine levels (IL-4 and IL-13), and improve restoration of epithelial junctions in asthma models. In this study, we also found that EGFR-TKI decreases the levels of proinflammatory cytokines (IL-6 and IL-8) highly associated with corticosteroid-resistant asthma. EGFR-TKI (Tarceva or AZD9291) may be an alternative for treating steroid-resistant asthma. However, adverse effects of EGFR-TKIs include enhanced skin rash and inflammation, which should be taken into account. EGFR-TKI, when used in the treatment of lung cancers, may induce acquired resistance, limiting its application to less life-threatening chronic diseases. Further studies, especially clinical trials in steroid-resistant asthmatic subjects, are needed.

In summary, EGFR-TKI, either Tarceva or AZD-9291, inhibits GST-Der p11-129-induced inflammation via EGFR autophosphorylation and downstream PI3K/AKT and STAT pathway signaling, as well as reduces proinflammatory cytokines such as IL-6 and IL-8.

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AUTHOR CONTRIBUTION

PHH, CPJ, LKH, and KJL conceived and designed the experiments; HYP, CPJ, KYT, CHY and SJN performed the experiments; PHH and CPJ analyzed the data and wrote the paper; LKH and KJL reviewed and edited the paper.

CONFLICT OF INTEREST

The authors declared no conflicts of interest.

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