

RESEARCH ARTICLE

German cockroach extract prevents IL-13-induced CCL26 expression in airway epithelial cells through IL-13 degradation

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Abstract

Inhaled aeroallergens can directly activate airway epithelial cells (AECs). Exposure to cockroach allergens is a strong risk factor for asthma. Cockroach allergens mediate some of their effects through their serine protease activity; protease activity is also a major contributor to allergenicity. The Th2 cytokine interleukin-13 (IL-13) induces upregulation of the eosinophil chemotactic factor CCL26. CCL26 induces eosinophil migration in allergic inflammation. In this work, we studied the effect of cockroach proteases on IL-13-induced effects. Immersed cultures of the human bronchial epithelial cell line BEAS-2B and air-liquid interface (ALI) cultures of primary normal human bronchial epithelial (NHBE) cells were stimulated with IL-13, *Blattella Germanica* cockroach extract (CE), or both. IL-13-induced genes were analyzed with qRT-PCR. IL-13 induced upregulation of CCL26, periostin, and IL-13R α 2 in bronchial epithelial cells which were decreased by CE. CE was heat-inactivated (HICE) or pre-incubated with protease inhibitors. HICE and CE preincubated with serine protease inhibitors did not prevent IL-13-induced CCL26 upregulation. CE-degraded IL-13 and specific cleavage sites were identified. CE also decreased IL-4-induced CCL26 upregulation and degraded IL-4. Other serine proteases such as bovine trypsin and house dust mite (HDM) serine proteases did not have the same effects on IL-13-induced CCL26. We conclude that CE serine proteases antagonize IL-13-induced effects in AECs, and this CE effect is mediated primarily through proteolytic cleavage of IL-13. IL-13 cleavage by cockroach serine proteases may modulate

Abbreviations: ALI, air-liquid interface; AP, activating peptide; BCE, boiled cockroach extract; CCL26, C-C motif chemokine ligand 26; CCR3, C-C motif chemokine receptor 3; CE, cockroach extract; CP, control peptide; CX₃CR1, C-X3-C motif chemokine receptor 1; GAPDDH, glyceraldehyde-3-phosphate dehydrogenase; HDM, house dust mite; HICE, heat inactivated; IL-13, interleukin-13; IL-13R α 1, IL-13 receptor subunit alpha 1; IL-13R α 2, IL-13 receptor subunit alpha 2; IL-4, interleukin 4; IL-4R α , interleukin 4 receptor subunit alpha; IL-8, interleukin 8; ILC2, type 2 innate lymphoid cells; NHBE, normal human bronchial epithelial; PAR-2, protease-activated receptor-2; SBTI, Soybean Trypsin Inhibitor; TAD, transactivation domain; Th2, T helper 2.

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CCL26-mediated effects in allergic airway inflammation by interfering directly with the pro-inflammatory effects of IL-13 in vivo.

KEYWORDS

allergy, bronchial epithelial cells, cockroach extract, inflammation, interleukin 13, serine proteases

1 | INTRODUCTION

The airway epithelium has an essential and highly regulated role as the physical innate barrier that interacts with and clears inhaled particles and irritants to maintain normal airway function.¹ Growing evidence suggest that airway epithelium dysfunction orchestrates airway inflammatory diseases.² Airborne allergens, such as house dust mite, cockroach, fungal, or pollen allergens, can interact and activate the airway epithelium resulting in the release of pro- and anti-inflammatory factors, such as cytokines, chemokines, antioxidants, and antimicrobial peptides.^{3–6} In turn, these pro-inflammatory factors may also enhance the production of allergen-specific IgE to the inhaled allergens.⁷ Allergic airway inflammation is characterized by eosinophilic infiltration of the lung tissues and airways^{8–10} and the presence of T helper 2 (Th2) cytokines, such as interleukin 4 (IL-4), IL-13, and interleukin 5 (IL-5).^{11,12}

Increased levels of IL-13 have been detected in blood, sputum, and bronchoalveolar lavage (BAL) fluid of subjects with asthma compared to healthy individuals.^{13–15} IL-13 is secreted by multiple cell types including, but not limited to, activated Th2 cells, B lymphocytes, mast cells, and type 2 innate lymphoid cells (ILC2s).^{16–19} IL-13 has a central role in asthma pathogenesis through its multiple effects. IL-13 regulates B cell class switching to IgE,²⁰ facilitates inflammatory cell migration into the lung and other tissues through upregulation of adhesion molecules and chemotactic factors,²¹ promotes goblet cell proliferation and mucus production,²² and stimulates airway remodeling²¹ and airway hyperresponsiveness.²³

Regarding eosinophilic inflammation, IL-13 promotes recruitment and activation of eosinophils in the airways through the release of eotaxins from the airway epithelium.²⁴ Among eotaxins, Eotaxin-3/CCL26 has been shown to be the most effective chemoattractant for eosinophils in asthma through CC motif chemokine receptor 3 (CCR3) ligation.^{25,26} CCL26 has been shown recently to also be a ligand for C-X3-C motif chemokine receptor 1 (CX₃CR₁) expressed on myeloid cells.^{27,28} CCL26 is the highest expressed eotaxin in cultured airway epithelial cells obtained from healthy donors upon stimulation with IL-13 or IL-4.²⁹ CCL26 is also the most potent eotaxin for chemotaxis of eosinophils from patients with asthma and

induces both CCR3-dependent and CCR3-independent eosinophil chemotaxis.²⁵

Exposure to cockroach allergens is one of the strongest risk factors for the development of allergic asthma in low-income urban populations.^{30–32} Cockroach allergens have been detected in dust of 85% of homes in inner-city US; exposure to high levels of cockroach allergens is associated with higher risk of asthma-related health problems and development of allergic asthma in cockroach-sensitized patients.³⁰ Multiple cockroach allergens and other proteins of cockroach origin have been identified to possess proteolytic activity,^{33,34} which is believed to be a major contributor to their allergenicity.³⁵ We have shown that inhibition of cockroach enzymatic activity by heat inactivation, or specific inhibitors, prevents mucosal sensitization in a murine model of allergic airway inflammation³⁶; the same has been shown for cockroach allergens by others,³⁷ as well as for other allergens, such as house dust mite and aspergillus allergens.^{38,39} Some of the pro-inflammatory effects of cockroach allergens may be the result of direct activation of airway epithelial cells by these allergens.^{40–42}

IL-13 and cockroach allergens both have significant pro-inflammatory effects on the airway epithelium that may increase the risk to develop asthma or increase the severity of the disease. However, there is little information on the combined effects of inhaled allergens and Th2 cytokines on airway epithelial cells. In this manuscript, we tested whether CE alters the ability of IL-13 to activate airway epithelial cells. We show that, contrary to what would be expected, CE prevents IL-13-mediated airway epithelial cell activation and abbreviates IL-13-induced CCL26 effects through a mechanism that is serine protease dependent and likely the result of IL-13 degradation by cockroach serine proteases.

2 | METHODS

2.1 | Cell culture and activation

The virus-transformed human airway epithelial cell line BEAS-2B was purchased from American Type Cell Collection (ATCC, Manassas, VA, USA). BEAS-2B cells were cultured in multi-well plates pre-coated overnight

with 30 µg/mL bovine collagen type 1 (Life Technologies, Grand Island, NY, USA), 10 µg/mL bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA), and 2 µg/mL human fibronectin (Life Technologies) dissolved in Bronchial Epithelial Basal Media (BEBM) (Lonza, Walkersville, MD, USA). BEAS-2B cells were cultured in Bronchial Epithelial Growth Media (BEGM) consisting of BEBM supplemented with bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, and triiodothyronine (components of the Single Quots kit from Lonza) and the antibiotics penicillin G/streptomycin 100 U/mL and 100 µg/mL, respectively (Life Technologies). BEAS-2B cells used were cultured for a maximum of 20 passages from the initial thaw. All cell cultures were tested negative for mycoplasma using the MycoAlert Kit (Lonza).

Primary Normal Human Bronchial Epithelial (NHBE) cells from two different normal donors were purchased from Lonza. NHBE cells were cultured for a maximum of five passages with BEGM in multi-well plates pre-coated overnight with 30 µg/mL bovine collagen type I dissolved in BMEM and used for experiments during passage four and five only. NHBE cells were cultured with BEGM (as described above) with the addition of gentamicin, amphotericin, and retinoic acid (Lonza SingleQuots). Air-liquid interface (ALI) cultures of NHBE were done as described.⁴³

BEAS-2B and NHBE cells were grown in cell incubators (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO₂ and 90% humidity. When BEAS-2B and NHBE cell cultures reached 90% confluency, they were growth factor deprived (cultured in BEBM supplemented with penicillin/streptomycin) for 24 h. Cells were then stimulated with different concentrations of recombinant human (rh)IL-13 (R&D Systems, Minneapolis, MN, USA), German cockroach extract (CE) (*Blattella Germanica*) (Greer Laboratories, Lenoir, NC, USA), or both. CE comes as lyophilized frozen whole body that was at time of use reconstituted in 0.9% NaCl of sterile saline solution at a concentration of 6 mg/mL of total protein and stored at 4°C until used.

To inhibit CE proteases, CE was heat-inactivated (HICE) at 65°C for 30 min or incubated for 30 min at room temperature with each one of the following protease inhibitors separately: E-64 (Sigma) a cysteine protease inhibitor at a final concentration of 10 µM, aprotinin (Roche Applied Science, Mannheim, Germany) a serine protease inhibitor and very effective against trypsin-like proteases at a final concentration of 2 µg/mL, or pepstatin A (Sigma) an acid proteases inhibitor at a final concentration of 1 µg/mL. CE protease activity was also inhibited using trypsin inhibitor from glycine max (soybean trypsin inhibitor SBTI) at a final concentration of 0.5–1 µg/mL

(Sigma). The concentrations of inhibitors used were selected based on the effective working concentration recommended by the manufacturer of each inhibitor and based on previous studies.^{44–46} To study PAR-2 activation, the PAR-2 activating peptide (PAR-2 AP) SLIGRL-NH₂ and the control peptide (PAR-2 CP) LRGILS-NH₂ were used at a concentration of 50 µM for both, as described previously.^{47,48} PAR-2 peptides were generously provided by Dr. M Hollenberg, University of Calgary.

For comparative studies, different concentrations of trypsin from bovine pancreas (Sigma) or house dust mite (HDM) extract (Greer Laboratories) were used with IL-13 instead of CE. Recombinant human interleukin-4 (rhIL-4) (R&D Systems) was also substituted for IL-13 in some experiments. Cell viability was evaluated 24 h after stimulation using a 0.4% trypan blue (Life Technologies) exclusion test.⁴⁹ All the conditions presented in all the figures of this manuscript are done in duplicates in each independent experiment except for the experiments with ALI cultures.

2.2 | Reverse transcription and quantitative polymerase chain reaction

Airway epithelial cell total RNA was isolated using either the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) or TRIzol (Life Technologies). 0.5 µg total RNA was reverse transcribed using 0.5 µg/reaction of Oligo(dT) 12–18 as primer (Invitrogen, Waltham, MA, USA) and 200 U/reaction of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) in a 20 µL reaction volume.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs02658911_g1), CCL26 (Hs00171146), and interleukin 13 receptor subunit alpha 2 (IL-13Rα2) (Hs00152924_m1) gene expression assays (Thermo Fisher Scientific) were used to perform qRT-PCR. Amplification was performed for 40 cycles of 95°C for 15 s, and 60°C for 1 min. Samples were analyzed in triplicate using Applied Biosystems QuantStudio Real-Time PCR System (Thermo Fisher Scientific). The mRNA levels of tested genes were normalized to the housekeeping gene GAPDH and analyzed using the 2^{−ΔΔCT} method⁵⁰ and expressed as fold change of stimulated over unstimulated cells.

2.3 | Enzyme-linked immunosorbent assay (ELISA)

CCL26 protein was measured in cell supernatants using the DuoSet ELISA kit for human CCL26/Eotaxin-3 (R&D Systems) according to the manufacturer's instructions. Seven-point serial 2-fold dilution standard curve of

recombinant human CCL26 with highest concentration of 4000 and lowest 62.5 pg/mL was used to calculate concentrations in cell supernatants. IL-8 protein was measured using the DuoSet ELISA kit for human IL-8 (R&D Systems) according to the manufacturer's instructions. Seven-point serial 2-fold dilution standard curve of recombinant human IL-8 with highest concentration of 2000 and lowest 31.3 pg/mL was used to calculate concentrations in cell supernatants. Optical density was measured using a Power Wave XS (BioTek, Winooski, VT, USA) plate reader.

2.4 | Quantification of CE trypsin-like activity

Trypsin-like activity was measured using the fluorogenic peptide substrate butoxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin (AMC)-HCl (QAR-AMC) (Bachem, King of Prussia, PA, USA). The assay was performed as described previously.³⁶ Trypsin from bovine pancreas with known N-benzoyl-L-arginine ethyl ester (BAEE) U/mg activity was used to make a five-point 5-fold dilution standard curve to calculate serine protease activity of unknown samples. Activity of protease solutions is shown as BAEE U/mg.

2.5 | Detection of human IL-13R α 1 by flow cytometry

Resting and CE-activated BEAS-2B cells were harvested with trypsin, centrifuged for 5 min at 300g, and resuspended (1×10^6 cells/tube) in 2 mL of FACS buffer (0.5% BSA, 0.1% NaN₃ and 3% FBS in 1x PBS). Mouse IgG control antibody (50 μ g/ 10^6 cells) (Invitrogen) and human FcR blocking reagent (1 μ g/ 10^6 cells) (Miltenyi Biotec, Auburn, CA, USA) were added to minimize non-specific binding of antibodies. Cells were then incubated with monoclonal mouse anti-human IL-13R α 1 APC-conjugated antibody (Monoclonal Mouse IgG_{2B} Clone # 419718) or APC-conjugated mouse IgG2b isotype control (both from R&D Systems) at concentration 0.5 μ g/ 10^6 cells. Flow cytometry was performed using LSR Fortessa-II (BD Bioscience, Franklin Lakes, NJ, USA). Results were analyzed using FlowJo (TreeStar, Ashland, OR, USA).

2.6 | Western blot

Cell lysates were separated on 10% SDS-PAGE Mini-Protean Precast gels (Bio-Rad, Hercules, CA, USA) and

wet transferred to nitrocellulose membranes at 400 mA for 60 min at 4°C. Total STAT6 and phosphorylated STAT6 (pSTAT6) Tyr⁶⁴¹ were detected using a monoclonal mouse anti-human STAT6 (BD Bioscience) or a polyclonal rabbit anti-human pSTAT6 (Cell Signaling, Danvers, MA, USA) antibodies, respectively. Membranes were then blotted with IRDye-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (LI-COR, Lincoln, NE, USA) antibodies, respectively.

To detect IL-13 and IL-13 cleavage products, 10 μ g/mL of IL-13 or IL-13 with 2.5 mg/mL of CE or CE preincubated with aprotinin or E64 was prepared. Three microliters of each sample was loaded on in-house made 16% SDS-PAGE gels and wet transferred as described above. IL-13 was then detected using a polyclonal Goat anti-human IL-13 antibody (R&D Systems) followed by an IRDye-conjugated donkey anti-goat IgG antibody (LI-COR). IL-4 cleavage was also detected as described for IL-13 and 10 μ g/mL of IL-4 or IL-4 with 2.5 mg/mL of CE was used.

All western blot membranes were imaged using Odyssey Infrared Imager (LI-COR) and analyzed using Image Studio software (LI-COR).

2.7 | IL-13 digestion, N-terminal labeling, and mass spectrometry (MS) analysis

Cleavage of IL-13 by CE was tested by incubating 10 ng of IL-13, IL-13 + 10 μ g of CE, or IL-13 + CE preincubated with 10 μ M aprotinin. All samples were loaded into a pre-cast gel (4–20%), resolved for ~0.5 cm, and stained with Coomassie blue silver. Fragments of IL-13 were N-terminally labeled with an in-gel acetylation protocol based on established work.⁵¹ The addition of this chemical modification at the N-termini of the full-length protein and protein fragments is to facilitate the unambiguous identification of the cleavage events generated by CE. Gel bands were excised and cut into smaller pieces (~1 mm) and transferred to a round bottom 96-well plate. Gel pieces were de-stained twice with 100 mM NH₄HCO₃ containing 50% acetonitrile (ACN) at 37°C for 10 min. Reduction was performed by incubation with 10 mM 2-mercaptoethanol for 30 min at 37°C, followed by alkylation with 50 mM iodoacetamide in the same conditions. Gel fragments were dehydrated with 100% ACN and recovered in 100 μ L of acetic acid and 20 μ L of acetic anhydride. Samples were incubated for 5 h at room temperature. Excess labeling reagents was removed, and gel pieces were rinsed with deionized water. The gel was dried down with ACN to remove the excess of derivatizing reagents. It was washed

again with deionized water and titrated with 200 mM NH_4HCO_3 to a pH of 8. Extra washes were done until the pH was around 7.0. Samples were incubated with ACN until fully dried and in-gel digestion was performed by swelling the dry gel with 0.5 μg of trypsin in 100 mM NH_4HCO_3 and overnight incubation at 37°C. Trypsin at this step will generate more peptides, but the newly generated peptides are not labeled by acetylation. The supernatant was recovered, and serial extractions were done by incubating the gel fragments with extraction buffer 1 (MS H_2O with 2% (v/v) ACN and 1% (v/v) formic acid) and extraction buffer 2 (50% extraction buffer 1 and 50% (v/v) ACN) for 1 h at 37°C. Supernatants were combined, dried down in a speed vac (Genevac), and stored at -20°C .

For analysis, the peptides were recovered in MS-compatible H_2O with 2% (v/v) ACN and 1% (v/v) formic acid. Peptides were separated using a nanoflow-HPLC (Thermo Scientific EASY-nLC 1200 System) coupled to an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific). Reverse phase separation of the peptides was done on a trap column (5 μm , 100 $\text{\AA}$, 100 $\mu\text{m} \times 2\text{ cm}$, Acclaim PepMap 100 nanoViper C18; Thermo Fisher Scientific) and an analytical column (2 μm , 100 $\text{\AA}$, 50 $\mu\text{m} \times 15\text{ cm}$, PepMap RSLC C18; Thermo Fisher Scientific) using a 60 min linear gradient from 3.85% to 36.8% ACN in 0.1% formic acid. Data was analyzed using ProteinProspector (v5.22.1) against the human IL-13 protein sequence (Accession #AAK53823). The arbitrary unit is the unit of hits made by the detectable peptide's mass/ion charge ratio to the mass spectrometer. The searches were performed with maximum false discovery rate of 1% for peptides. Trypsin was set as digestion enzyme with relaxed specificity at the N-termini and a maximum of three missed tryptic peptides. Precursor mass tolerance was set at 15 ppm with a fragment mass tolerance of 0.8 Da, and precursor charge range of +2 to +5. Carbamidomethylation (C) was added as fixed modification, and acetylation at the N-term of the peptide, deamidation (N,Q), and oxidation (M) were set as variable modifications. A maximum number of modifications was set to 3. Manual inspection of each peptide assignment was further performed to confirm each assignment with N-terminal acetylation.

2.8 | Statistics

Results are presented as mean $\pm$ SEMs. To show differences between multiple groups, one-way and two-way ANOVAs with multiple comparison analysis were used on GraphPad Prism; significance is indicated on

the figures as follows: * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$, **** $p \leq .0001$.

3 | RESULTS

3.1 | CE inhibited IL-13-induced CCL26 mRNA upregulation and CCL26 protein release in human airway epithelial cells

Stimulation of BEAS-2B cells with IL-13 (20 ng/mL) for 24 h induced, as expected,⁵² CCL26 mRNA upregulation (Figure 1A). Co-stimulation of BEAS-2B cells with IL-13 and CE (5 $\mu\text{g}/\text{mL}$), however, decreased IL-13-induced CCL26 upregulation (Figure 1A) in a dose (Figure 1B) and time (Figure 1C) dependent manner. Stimulation of BEAS-2B cells with IL-13, CE, or both for 24 h did not affect cell viability (Figure 1D). CE (5 $\mu\text{g}/\text{mL}$) also inhibited IL-13-induced CCL26 protein release from BEAS-2B (Figure 1E). The inhibitory effect of CE was not specific to CCL26 induction by IL-13; CE also inhibited IL-13-induced IL-13R α 2 (Figure 1F) and periostin (Figure 1G) mRNA upregulation.

IL-13 also induced CCL26 mRNA expression and protein release from immersed NHBE cells, and CE prevented this induction at the mRNA and protein levels (Figure 1H,I). We then tested the effects of CE on IL-13-induced CCL26 synthesis and release from NHBE ALI cultures. IL-13 added to the basolateral surface of the ALI cultures induced CCL26 mRNA and addition of CE on the basolateral side decreased this induction (Figure 1J) by at least 70%, while CE addition to the apical side did not affect this IL-13-mediated CCL26 induction. IL-13 added to the apical surface of these cultures induced a small CCL26 mRNA induction, approximately 10-fold increase versus >4000-fold increase when added basolaterally, and this induction decreased when CE was added to the apical surface (data not shown).

IL-13-induced CCL26 expression in epithelial cells is STAT6 dependent.⁵³ Ligation of IL-13R complex with IL-13 mediates pSTAT6-Tyr⁶⁴¹ that peaks at 30 min.^{53–55} IL-13-induced pSTAT6 in BEAS-2B cells after 30 min of activation was decreased in the presence of CE (Figure 2A,B).

3.2 | The CE effect on IL-13-induced CCL26 upregulation is trypsin-like protease dependent

Multiple proteins of cockroach origin were identified to possess proteolytic activity.^{33,34} We therefore tested whether the inhibition of IL-13-induced CCL26 mRNA upregulation by

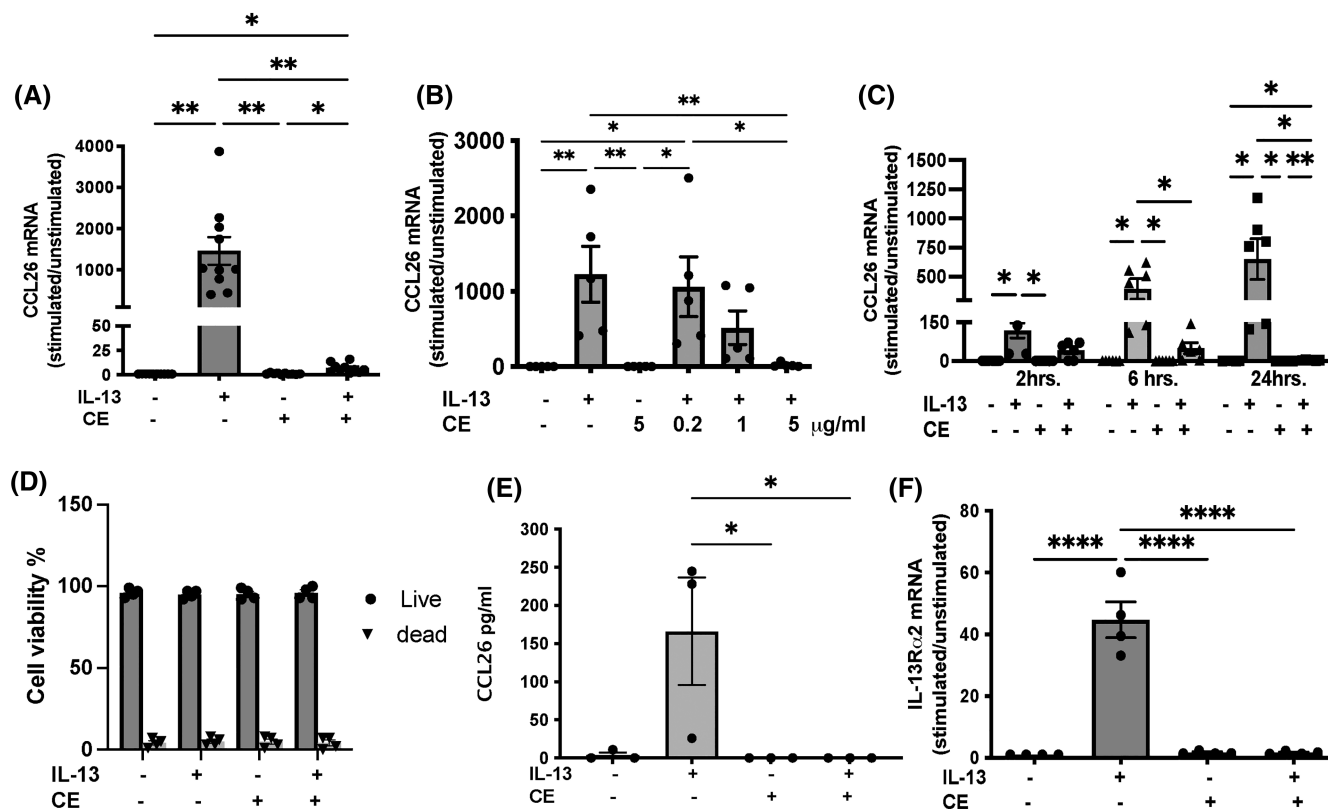


FIGURE 1 CE inhibited IL-13-induced CCL26 mRNA expression and protein release in human airway epithelial cells. (A) Fold induction of CCL26 mRNA following activation of BEAS-2B cells with IL-13 (20 ng/mL), CE (5 µg/mL), or both for 24 h; $n = 9$. IL-13-induced CCL26 expression in BEAS-2B cells incubated with IL-13 (20 ng/mL) and various concentrations of CE; $n = 4$ (B) or CE (5 µg/mL) for different time points (C); $n = 3$. (D) BEAS-2B cell viability 24 h after activation with IL-13 (20 ng/mL) and/or CE (5 µg/mL); $n = 4$. (E) IL-13-induced CCL26 protein release after 24 h. activation with IL-13 (20 ng/mL), CE (5 µg/mL), or both; $n = 3$. (F) Inhibition of IL-13-induced IL-13Ra2 mRNA after 24 h. activation with IL-13 (20 ng/mL), CE (5 µg/mL), or both; $n = 4$. (G) Inhibition of IL-13-induced periostin mRNA after 24 h. activation with IL-13 (20 ng/mL), CE (5 µg/mL), or both; $n = 6$. IL-13-induced CCL26 mRNA levels (H) and CCL26 protein release (I) 24 h. after activation of NHBE cells with IL-13 (20 ng/mL), CE (5 µg/mL), or both; $n = 3$ each. (J) IL-13-induced CCL26 mRNA in stimulated ALI with IL-13 (2 and 20 ng/mL), CE (5 µg/mL) or both in apical or basolateral side as indicated; $n = 3$. Results shown are mean $\pm$ SEM. Results were analyzed with one-way ANOVA, multiple comparisons except in (C) two-way ANOVA multiple comparison was used, ns = non-significant, $*p \leq .05$, $**p \leq .01$, $***p \leq .001$, $****p \leq .0001$.

CE is protease dependent. Heat inactivation decreases CE protease activity³⁶ and HICE did not inhibit IL-13-induced CCL26 mRNA upregulation (Figure 3A). To identify the specific CE protease(s) responsible for inhibiting IL-13-induced CCL26 upregulation, CE was pre-incubated with E-64, aprotinin, or pepstatin A. In contrast with CE pre-incubated with E-64 or pepstatin A, CE pre-incubated with aprotinin did not inhibit IL-13-induced CCL26 mRNA upregulation and protein release (Figure 3B,C), suggesting that the inhibitory effect of CE is mediated through serine proteases. A trypsin-like protease inhibitor, Soybean Trypsin Inhibitor (SBTI), also prevented the effect of CE on IL-13-induced CCL26 mRNA upregulation (Figure 3D), indicating that the CE effect is mediated by a trypsin-like serine protease. In support of these observations, heat-inactivation, SBTI, and aprotinin all decreased trypsin-like protease activity of CE in our experiments (Figure 3E).

CE and certain purified CE serine proteases activate PAR-2.³³ To test whether CE inhibition of IL-13-induced CCL26 mRNA upregulation might be mediated through PAR-2 activation, we treated BEAS-2B cells for 24 h with IL-13 and either the PAR-2-activating peptide (AP) SLIGRL-NH₂ or the control peptide (CP) LRGILS-NH₂. These peptides have been used previously to study PAR-2 activation.⁵⁶ PAR-2 activation did not induce CCL26 mRNA upregulation on its own and had no effect on IL-13-induced CCL26 mRNA upregulation (Figure 3F), suggesting that CE inhibition of IL-13-induced CCL26 upregulation is most likely PAR-2 independent. We validated that PAR-2AP was able to activate BEAS-2B cells, as it induced IL-8 protein release (Figure 3G), as has been shown.⁵⁷

Since CE trypsin-like activity appeared to mediate the inhibition of IL-13-induced CCL26 mRNA upregulation in airway epithelial cells, we then tested whether

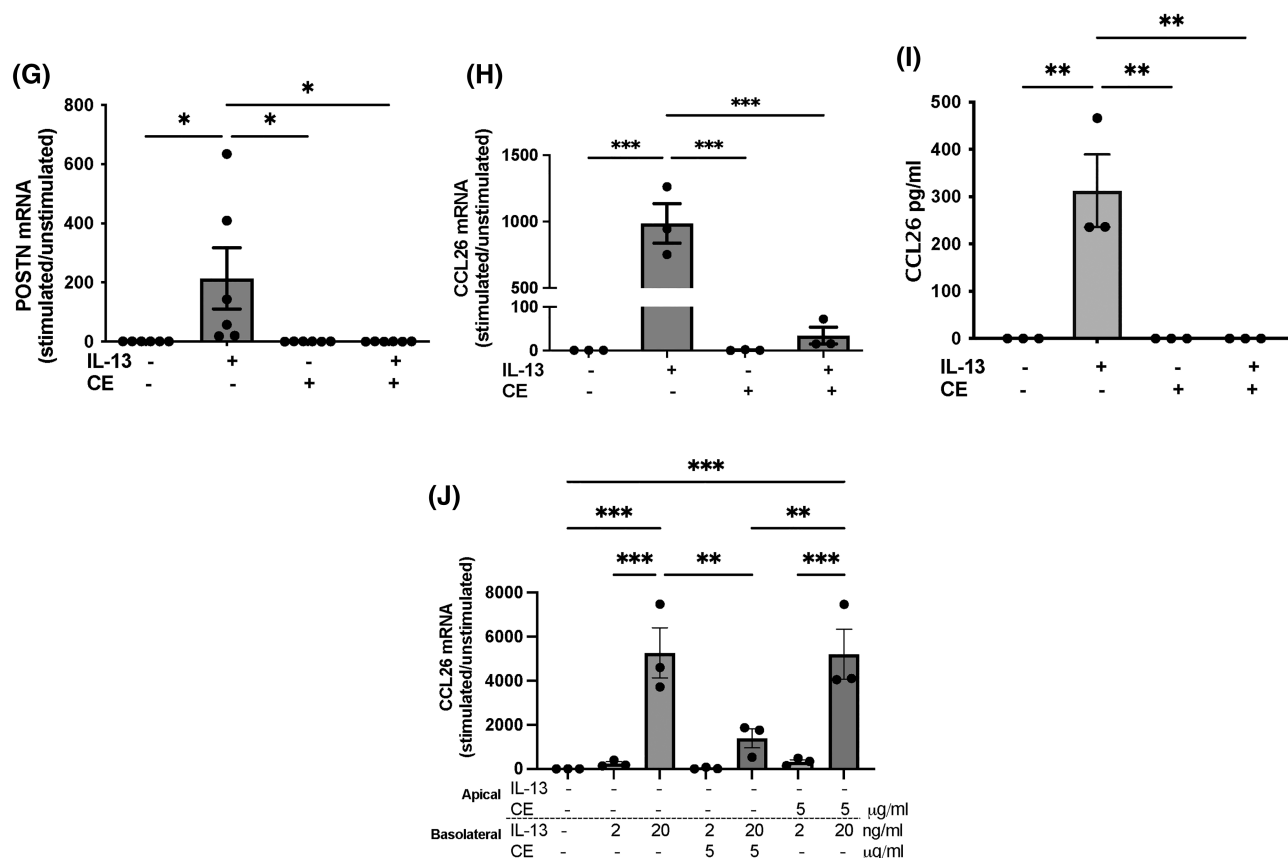


FIGURE 1 (Continued)

this is a general effect of trypsin-like proteases. Bovine pancreatic trypsin, in concentrations that had similar trypsin-like activity to the concentrations of CE used in our experiments (Figure 3E), was not able to inhibit IL-13-induced CCL26 mRNA upregulation (Figure 3H). Similarly, HDM extract, which contains serine protease activity,^{44,58} did not inhibit IL-13-induced CCL26 upregulation (Figure 3I).

3.3 | CE did not decrease IL-13R α 1 expression on airway epithelial cells

IL-13 signaling in epithelial cells is mediated through the IL-13R α 1, that is a heterodimer of the IL-4R α and IL-13R α 1 chains.⁵⁹ Alteration of IL-13R expression by CE, could potentially mediate the inhibitory effects of CE on IL-13-induced CCL26 mRNA upregulation. Incubation of BEAS-2B cells with CE for 24 h did not alter IL-13R α 1 expression on the cell surface (Figure 4A,B). In addition, as we discussed above (Figure 1F), CE did not alter IL-13R α 2 mRNA expression, a decoy receptor that binds strongly to free IL-13 and could limit its activity, on its own, but in contrast, decreased IL-13-induced IL-13R α 2 mRNA upregulation.

3.4 | CE proteolytic activity cleaves IL-13

We next examined whether CE may cause proteolysis of IL-13 and therefore inhibit its induced effects. We detected IL-13 cleavage after incubation of recombinant IL-13 with CE for 15 min at 37°C as evidenced by decreased intensity of the full-length IL-13 band and the generation of short forms of IL-13 in the 7.85–9.21 MW range (Figure 5A,B). HICE and BCE did not induce IL-13 cleavage. Inhibition of serine proteases with aprotinin did not affect cleavage in general but preserved more of degradation products, which were not as evident in the case of IL-13 incubated with fully active CE. It is interesting that inhibition of cysteine proteases with E64 prevented to some extent IL-13 degradation by CE and generated cleavage products of slightly different MW compared to products generated by CE incubated with aprotinin (Figure 5A,B). Quantification of the cleavage products is shown in Figure 5C.

3.5 | Identification of cleavage sites on IL-13 by mass spectrometry

We then performed chemical modification and mass spectrometry analysis of the fragments generated upon

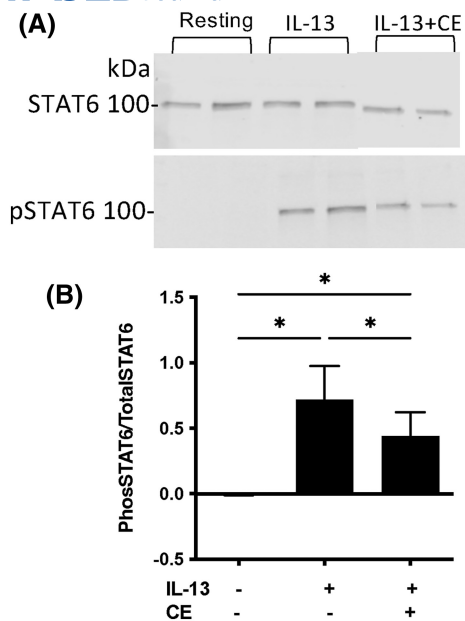


FIGURE 2 CE decreased IL-13-induced STAT6 phosphorylation. BEAS-2B cells were treated with IL-13 (20 ng/mL), CE (5 μ g/mL), or both for 30 min. (A) Western blot of total STAT6 (top) and pSTAT6-Tyr⁶⁴¹ (bottom); a representative of three experiments is shown. (B) Densitometric analysis of protein abundance determined as ratio of optical density signal of pSTAT6-Tyr⁶⁴¹/STAT6. Values are shown as mean $\pm$ SEM, $n=3$. Results were analyzed with one-way ANOVA, multiple comparisons, $*p \leq .05$.

incubation of IL-13 with CE or CE pretreated with aprotinin to identify the potential cleavage sites that may affect IL-13 activity in our assays.^{60,61} Our chemoselective labeling approach allowed the identification of several N-terminally modified peptides that might correspond to cleavage events on the IL-13 sequence by the proteases in the CE. A total of 5, 4, and 10 acetylated peptides were detected for IL-13, IL-13 + CE, or IL-13 + CE pretreated with aprotinin, respectively (Figure 6A).

Out of those cleavage candidates, only a few acetylated peptides showed the trypsin-like specificity observed in the CE proteases (cleavage after K and R amino acids). A trypsin-like specific was observed for the cleavage at position 95 in IL-13 + CE and IL-13 + CE preincubated with aprotinin samples. Another cleavage event at position 119 was only observed in the sample treated with aprotinin. The distribution of all the acetylated peptides in the IL-13 sequence is shown in (Figure 6B), cleavages at position 95 and 119 are highlighted.

3.6 | CE decreased IL-4-induced CCL26 upregulation and cleaved IL-4

IL-13R α 1/IL-4R α complex can be activated by either IL-13 or IL-4.⁵⁹ CE also inhibited IL-4-induced CCL26 mRNA

upregulation from BEAS-2B cells (Figure 7A) and mediated IL-4 cleavage that was evident within 5 min and led to almost complete degradation of IL-4 by 2 h (Figure 7B,C).

4 | DISCUSSION

In this study, we showed that CE inhibited IL-13-induced CCL26 mRNA upregulation and protein release from bronchial epithelial cells in a time- and dose-dependent manner. CE antagonized all IL-13 effects on airway epithelial cells, since we showed that it also prevented upregulation of other IL-13-regulated genes, such as periostin and IL-13R α 2, and decreased IL-13-induced STAT6 phosphorylation. We also showed that this CE effect was trypsin-like protease dependent as it was prevented by heat inactivation of CE or pre-treatment of CE with serine and trypsin-like protease inhibitors. We present evidence that suggest this inhibition of the IL-13-induced effects is mediated, to a large degree, by degradation of IL-13 by CE serine proteases. The data we present from experiments that used ALI cultures of airway epithelial cells support the degradation scenario, since inhibition of IL-13-induced CCL26 was evident only when IL-13 and CE were added to the same side of the ALI cultures, and no inhibition was detected when IL-13 and CE were added in opposite sides of ALI. We finally showed that CE can also degrade IL-4 and inhibits IL-4-induced CCL26 upregulation in airway epithelial cells.

Since bovine trypsin, in concentrations with similar serine protease activity to that in CE, and HDM extract, another extract that contains allergens with serine protease activity,^{44,58} did not have the same effect with CE on IL-13-induced CCL26 expression, we conclude that the inhibitory effect of CE is not a general effect of serine proteases, but a specific effect of one or more serine proteases present in CE. Recently, our group described three distinct serine proteases from *Blattella Germanica* extract,³³ the same extract we used for the experiments presented here. It is likely that one or more of these proteases is/are responsible for the effects we describe in this manuscript. Further work will be needed to identify the specific cockroach protease capable of inducing IL-13 degradation.

We showed here for the first time that CE can cleave IL-13 protein. IL-13 is also susceptible to degradation by mast cells proteases.^{62,63} In one of these studies⁶³ mast cell degranulation was shown to decrease the biological effects of IL-13 through IL-13 degradation. If cockroach proteases can also cleave IL-13, as well as IL-4, in vivo, then inhaled cockroach proteases may be able to alter the biological activity of IL-13 in allergic diseases. A recent observation indicates that CCL26 may also facilitate clearance of eosinophilic inflammation through activation of CX₃CR1 expressing macrophages,²⁸ indicating that degradation of IL-13 by

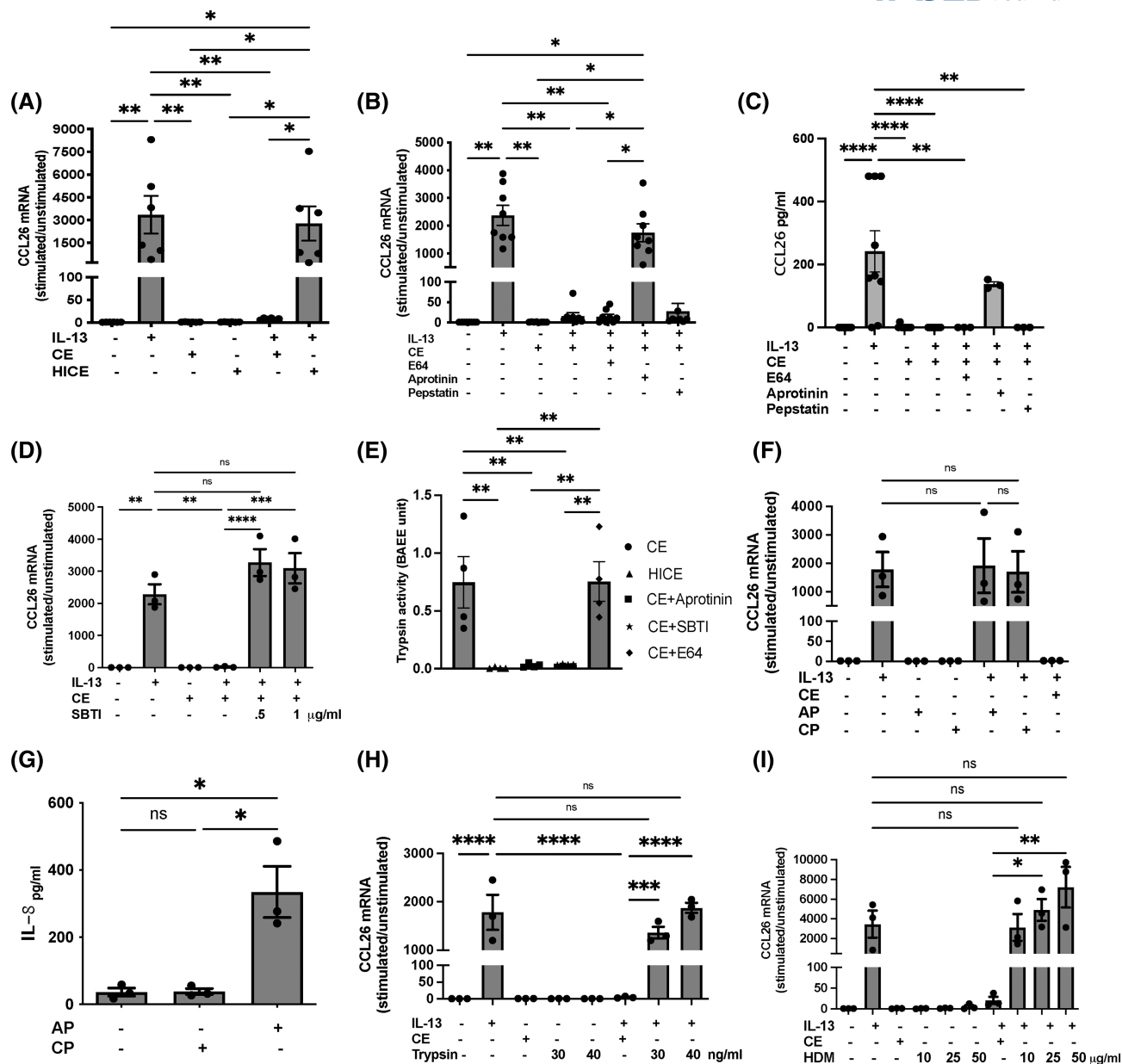


FIGURE 3 The inhibitory effect of CE on IL-13-induced CCL26 upregulation is trypsin-like protease dependent. (A) IL-13-induced CCL26 mRNA in BEAS-2B cells stimulated with IL-13 in the presence of CE or HICE for 24 h; $n = 3$. IL-13-induced CCL26 mRNA; $n = 4$ (B) and protein release; $n = 3$ (C) in cells treated with IL-13, CE or CE pre-incubated with protease inhibitors. (D) IL-13-induced CCL26 mRNA in cells treated with IL-13, or IL-13 with either CE or CE pre-incubated with SBTI; $n = 3$. (E) Trypsin activity in BAEE unit in solutions of CE (5 $\mu\text{g/mL}$), HICE (5 $\mu\text{g/mL}$), or CE preincubated with aprotinin, SBTI, or E64; $n = 4$. IL-13-induced CCL26 mRNA upregulation (F) and IL-8 protein release (G) in cells treated with IL-13, or IL-13 with either CE, PAR-2AP or PAR-2CP; $n = 3$ each. (H) IL-13-induced CCL26 mRNA in cells treated with IL-13, or IL-13 with either CE or bovine trypsin; $n = 3$. (I) IL-13-induced CCL26 mRNA in cells treated with IL-13, or IL-13 with HDM for 24 h; $n = 3$. Results shown are mean $\pm$ SEM. Results were analyzed with one-way ANOVA, multiple comparisons, ns = non-significant, $* \leq .05$, $** p \leq .01$, $*** p \leq .001$, $**** p \leq .0001$.

CE may further aggravate airway inflammation by delaying eosinophil clearance from the airways. Alternatively, we cannot exclude the possibility that exposure to high levels of cockroach proteases may limit the pro-inflammatory and pro-fibrotic effects of excessive IL-13 production during an allergic response, and possibly reduce tissue remodeling. Detailed in vivo studies in mice will be required to fully

understand the biological significance of our results. Our ALI data also indicate that CE should have access to the basolateral epithelial surface to degrade IL-13 and prevent CCL26 induction; this is possible since it is known that epithelial barrier is damaged in allergic asthma⁶⁴ and allergen proteases can directly digest epithelial tight junction proteins and increase epithelial permeability.^{65,66}

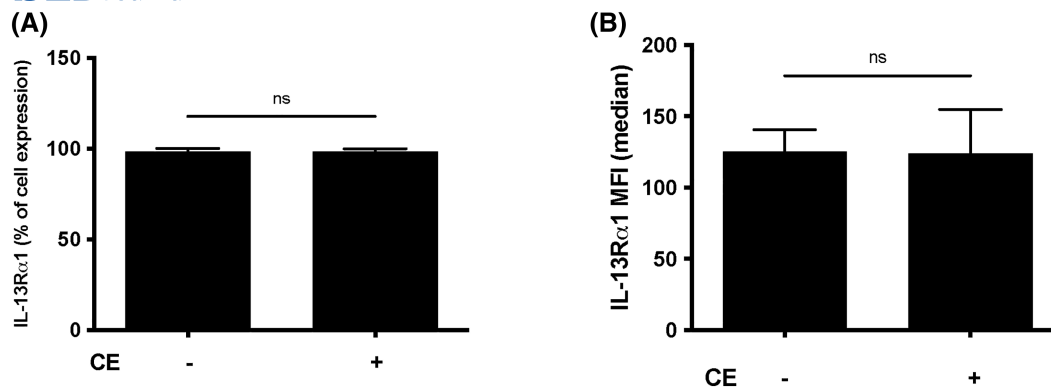


FIGURE 4 CE did not decrease IL-13Rα1 expression on the surface of airway epithelial cells. BEAS-2B cells were untreated or treated with CE (5 µg/mL) for 24 h. (A) % of BEAS-2B cells expressing IL-13Rα1 on cell surface. (B) Median fluorescent intensity (MFI) of IL-13Rα1 expression on BEAS-2B cells. Results shown are mean ± SEM; $n = 3$, ns = non-significant.

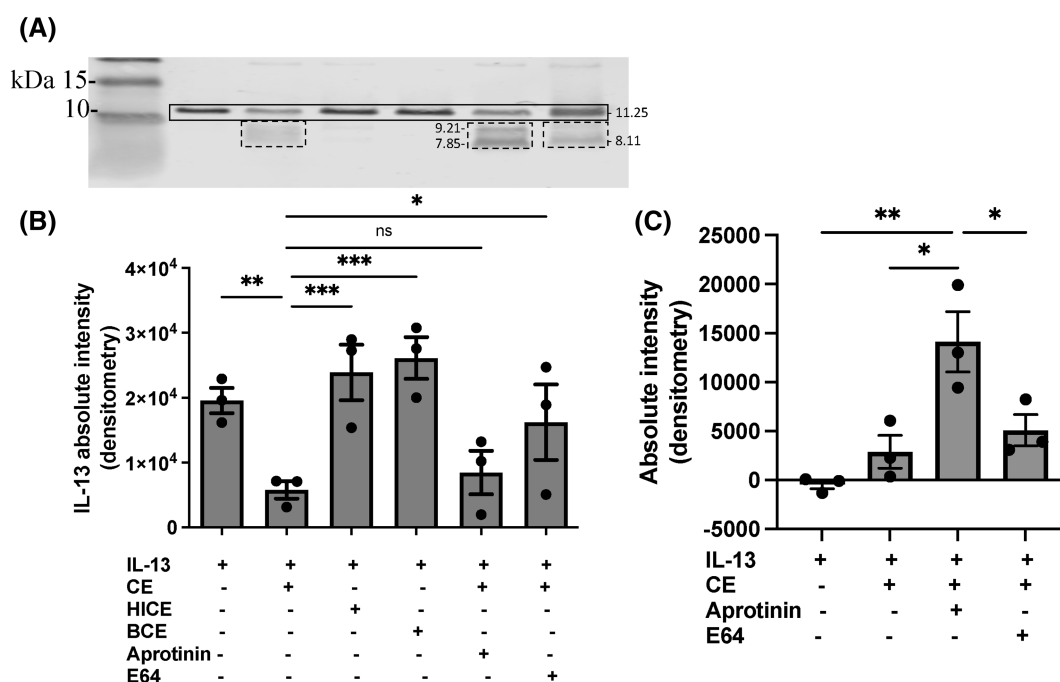


FIGURE 5 CE proteolytic activity cleaved IL-13. (A) Western blot of IL-13 or IL-13 treated with CE, HICE, BCE, or CE preincubated with aprotinin or E64; a representative of three experiments is shown. (B) Quantification of signal intensity of full-length IL-13 at 11.25 kDa (solid box) and (C) Quantification of IL-13 cleavage products signal intensity (dashed boxes); $n = 3$. Results shown are mean ± SEM. Results were analyzed with one-way ANOVA, multiple comparisons, ns = non-significant, * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$.

The exact amount of CE allergens inhaled in everyday life is difficult to estimate. Quantitative measurement of airborne cockroach allergens in apartments with asthmatic children showed that the mean content of the cockroach allergen Bla g2 was 0.38 µg/g of total suspended particulates,⁶⁷ while levels as high as 1.06 µg/g have been seen in settled dust in another study.⁶⁸ The mean of Bla g2 content in 13 *Blattella Germanica* cockroach extracts was 18.01 µg in a total protein content of 4.02 mg.⁶⁹ Based on these observations, a 5 µg/mL solution of CE, as the one we use on our experiments, should contain around 0.022 µg of Bla g2, or the equivalent

amount present in 0.05–0.021 g of dust. Whether it is possible to have exposure to this level of cockroach allergens/proteins in everyday life, or whether cockroach proteases may accumulate in the surface airway liquid and increase an individual's exposure, is something that needs further study. There is some evidence that high levels of inhaled allergens may protect from allergic sensitization.⁷⁰ Whether the same happens with cockroach allergen exposure and whether proteases are important for this effect are not known.

IL-13 cleavage by CE was completely inhibited by heat inactivation or boiling of the extract indicating

(A) N-terminal acetylation of IL-13 cleavage products

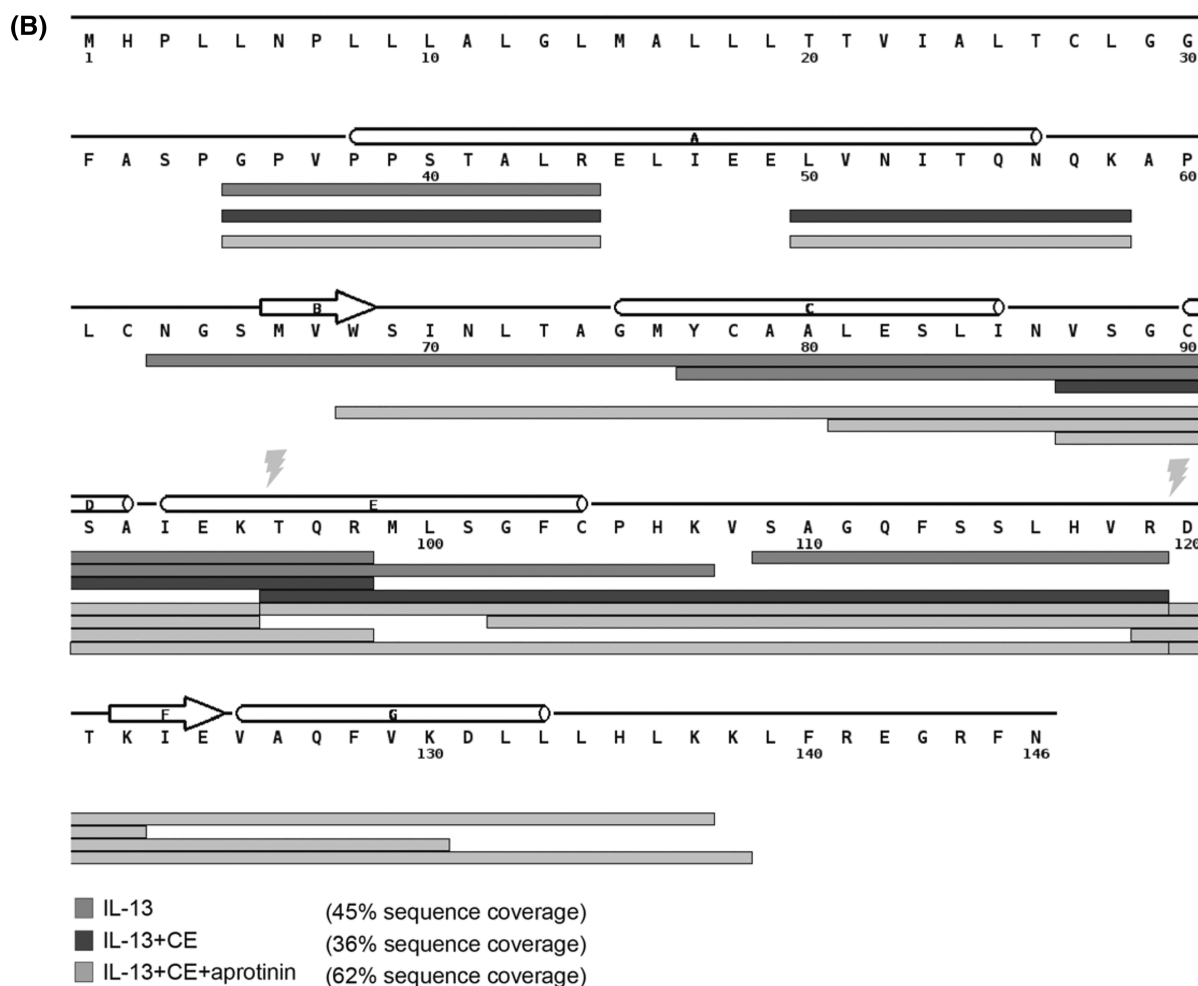
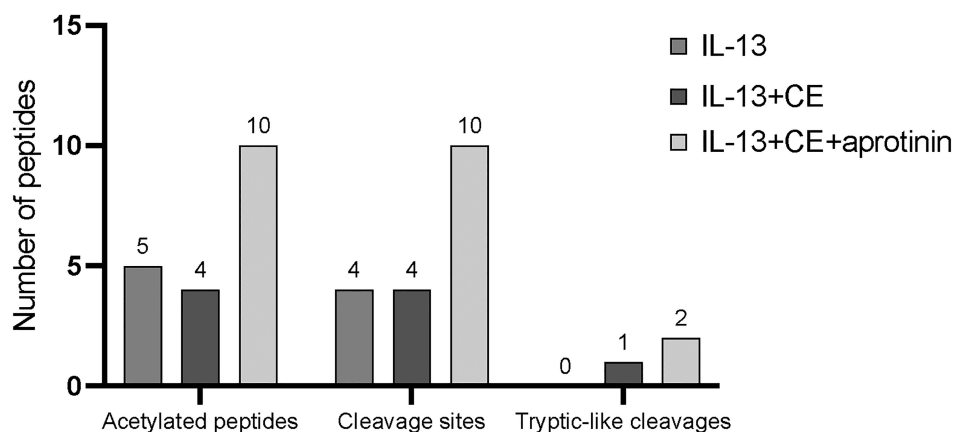


FIGURE 6 Identification of cleavage events in IL-13 by mass spectrometry. (A) Summary of the MS results from in-gel acetylation reaction. (B) Peptide map showing primary sequence of IL-13, secondary structural features, acetylated peptide distribution and sequence coverage. Two possible cleavage sites in the IL13 sequence show the P1 = K/R specificity are highlighted (K95|T96 and R119|D120).

the presence of heat labile proteases. Even though neutralization of serine proteases with aprotinin prevented CE-mediated inhibition of IL-13-induced CCL26

upregulation, it did not prevent cleavage of the full molecule, but large IL-13 fragments are more prominent in this case than when IL-13 is degraded by fully active CE

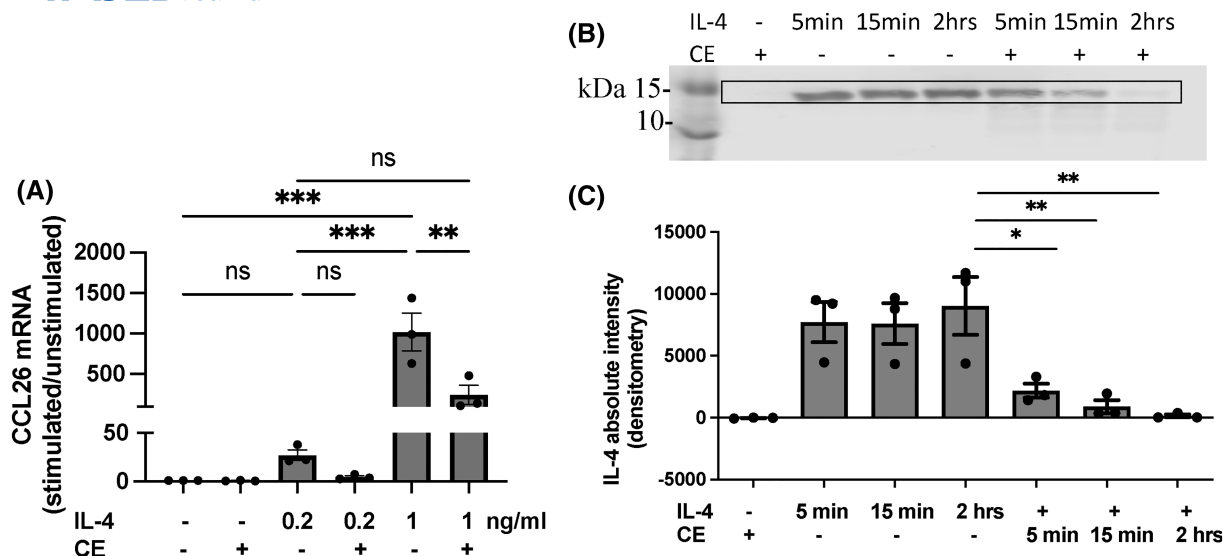


FIGURE 7 CE decreased IL-4-induced CCL26 upregulation and cleaved IL-4. (A) CCL26 mRNA in BEAS-2B cells after activation for 24 h. with IL-4 (0.2 or 1 ng/mL) or IL-4 with CE (5 μ g/mL); $n = 3$. (B) Western blot analysis of IL-4 or IL-4 treated with CE for 5, 15 min, and 2 h; a representative of three experiments is shown. (C) Quantification of signal intensity of full-length IL-4 (solid box); $n = 3$. Results shown are mean $\pm$ SEM. Results were analyzed with one-way ANOVA, multiple comparisons, ns = non-significant, * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$.

proteases. Inhibition of cysteine proteases inhibited IL-13 cleavage but that was not enough to prevent CE-mediated inhibition of IL-13-induced CCL26 upregulation. These observations suggest that there is one or more specific cleavage sites responsible for abolishing IL-13-induced effects.

We have not been able to identify the exact cleavage site abolishing the IL-13 activity, but one site, cleavage at residue 119, which was evident only in samples incubated with IL-13 and CE pretreated with aprotinin, is likely relevant to explain the loss of activity. Theoretically, cleavage of recombinant IL-13 at site 119 would be predicted to generate two fragments of a detectable one at 9.11 and 3.23 kDa that cannot be detected in our gel. In the case of IL-13 cleaved by CE with aprotinin, we see a prominent band at 9.21 kDa that is not seen in other degradation products of IL-13. We believe that this fragment may represent cleavage at position 119 and may appear in this case because aprotinin inhibits the ability of CE to further cleave this peptide. This IL-13 fragment is probably functional since IL-13 in the presence of CE preincubated with aprotinin can still induce CCL26 upregulation. Further identification of cleavage at other sites, prevented by aprotinin, would explain the ability of CE to prevent IL-13-induced CCL26 upregulation. Further work will be needed to identify those sites.

STAT6 phosphorylation at Tyr⁶⁴¹ mediates STAT6 translocation to the nucleus and induces CCL26 mRNA transcription.^{53–55} We have showed that cells activated with IL-13 in the presence of CE exhibit decreased pSTAT6-Tyr⁶⁴¹ compared to cells activated with IL-13 alone. Early degradation of IL-13 by CE and decreased binding to

IL-13R may be the reason for this difference. However, early pSTAT6-Tyr⁶⁴¹ was detected, even though CCL26 expression was completely abolished. It is therefore likely that other signaling events mediated after prolonged activation of the IL-13R are required for sustained CCL26 induction. Phosphorylation of serine residue in transactivation domain (TAD) of STAT6 results in conformational changes and loss of STAT6 DNA binding⁵⁵ without affecting Tyr⁶⁴¹ phosphorylation, STAT6 dimerization, or nuclear translocation. It would be interesting to test whether AEC activation by IL-13 and CE may induce STAT6 TAD domain serine phosphorylation leading to loss of STAT6 DNA binding and therefore complete inhibition of CCL26 mRNA expression. Though IL-13 degradation may explain the CE-mediated effects we describe here, we also tested whether other mechanisms that may be involved. One such mechanism might be PAR-2 activation by CE proteases.³³ However, PAR-2 activation by synthetic activating peptides did not inhibit the IL-13 effects on airway epithelial cells suggesting that the effect of CE on IL-13-induced CCL26 mRNA upregulation was independent of PAR-2 activation.

5 | CONCLUSION

Our study shows that CE serine proteases have the capacity to degrade IL-13 and decrease IL-13-mediated CCL26 induction in vitro, suggesting that CE may also have the ability to modulate allergic airway inflammation in vivo by interfering directly with the pro-inflammatory effects of IL-13^{71,72} or delay eosinophil

clearance in allergic inflammation.²⁸ Further work is required to investigate the effect of the identified cleavage sites on IL-13 biological activity in the context of allergic airway inflammation.

AUTHOR CONTRIBUTIONS

Khadija Rashed Alzahrani, Vivek Gandhi, and Harissios Vliagoftis conceived and designed the research; Vivek Gandhi and Cheryl Laratta set up the experimental methods and performed the first experiments; Khadija Rashed Alzahrani, Nami Shrestha Palikhe, and Erik Gomez-Cardona performed most of the experiments presented in the manuscript, acquired data from those experiments, and analyzed all data; Khadija Rashed Alzahrani, Erik Gomez-Cardona, Olivier Julien, and Harissios Vliagoftis interpreted the data. Khadija Rashed Alzahrani prepared the first draft of the manuscript and revised the manuscript with Harissios Vliagoftis. All authors approved the final manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study will be made available by the authors to any research with a reasonable request.

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