German Cockroach Extract Proteolytic Activity Down-regulates Interleukin-13 Dependent Eotaxin-3 (CCL26) Expression in Airway Epithelial Cells

by

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Abstract

Introduction:

Allergy to inhaled allergens is an important factor to develop airway inflammation. The airway epithelium is the main barrier between the host and inhaled allergens, and its activation by allergens is a key step towards the activation of innate and adaptive immunity. German cockroach (*Blattella germanica)* allergens can activate airway epithelial cells through multiple receptors, including protease activated receptor 2 (PAR-2), and induce primarily pro-inflammatory effects. Th2 cytokine, (interleukin-13) IL-13, is a key mediator in allergic inflammation. It activates epithelial cells and induces the production of eotaxin-3 (CCL26), a potent eosinophil chemoattractant. The interactions between the effects of IL-13 and cockroach allergens on airway epithelium have not been studied.

Methods:

A bronchial epithelial cell line (BEAS-2B) and normal human bronchial epithelial cells (NHBE) were cultured in pre-coated multi-well plates and stimulated with IL-13, German cockroach extract (CE) or both. CCL26 mRNA was measured by qRT-PCR and the release of CCL26 protein by ELISA. To test the role of CE proteases,

heat inactivated CE (HICE), boiled CE or CE pre-incubated with protease inhibitors were used. PAR-2 activated peptides were used to examine the role of PAR-2 activation in the inhibition of IL-13 induced CCL26. Western blotting was used to assess STAT-6 phosphorylation and IL-13 protein degradation.

Results:

CE prevented IL-13-mediated up-regulation of CCL26 mRNA and protein from BEAS-2B and NHBE cells in a time and dose dependent manner. HICE and CE pre-incubated with aprotinin, an inhibitor of serine proteases, or soy bean trypsin inhibitor (SBTI) did not inhibit IL-13 mediated CCL26 mRNA induction. PAR-2 activation did not show inhibition of IL-13 induced CCL26 mRNA. STAT-6 activation by IL-13 was not prevented by CE. Western blot detected early onset degradation of IL-13 protein by CE even in the presence of aprotinin or with HICE but not with boiled CE.

Conclusions:

CE inhibited IL-13 induced CCL26 in a time and dose dependant manner. This inhibitory effect is dependent on CE proteases, particularly proteases with trypsin-like activity. Serine protease inhibitor did not inhibit IL-13 protein degradation by

CE, which indicates that CE trypsin like activity prevents IL-13-induced CCL26 expression in a degradation independent manner. PAR-2 activation did not mimic the inhibitory effect of CE on IL-13 induced CCL26. This may be a mechanism for CE to decrease the detrimental effects of Th2 cytokines in allergic asthma.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AHR: Airway Hyper-responsiveness

APC: Antigen Presenting Cell

AP: Activating Peptide

BAL: Broncho-alveolar Lavage

BEGM: Bronchial Epithelial Growth Media

BEBM: Bronchial Epithelial Basal Media

BSMC: Bronchial Smooth Muscle Cell

CE: Cockroach Extract

CFU-GEMM: Colony Forming Unit- Granulocyte, Erythrocyte, Monocyte, Megakaryocyte

CFU-Eo: Colony Forming Unit- Eosinophil

COPD: Chronic Obstructive Pulmonary Disease

CP: Control Peptide

DAMPs: Damage Associated Molecular Patterns

DCs: Dendritic Cells

ECM: Extracellular Matrix

ECP: Eosinophil Cationic Protein

EGF: Epidermal Growth Factor

ELISA: Enzyme Linked Immunosorbent Assay

EPO: Eosinophil Peroxidase

ESR-1, -2: Estrogen Receptor

FEV1: Forced Expiratory Volume 1

GCs: Glucocorticoids

HDM: House Dust Mite

HICE: Heat Inactivated Cockroach Extract

HLPS: Heated Lipopolysaccharide

IgE: Immunoglobulin E

IL-4Rα1: IL-4 receptor alpha 1

IL-13Rα1: IL-13 receptor alpha 1

IL-13Rα2: IL-13 receptor alpha 2

IL-4, -5, -13: Interleukin -4, -5, -13...

ILC2: Innate Lymphoid Cell type 2

IRF-3, -7: Interferon Regulatory Factor -3, -7

IRS-2: Insulin Receptor Substrate 2

JAK1, 3: Janus tyrosine Kinase 1, 3

LPS: Lipopolysaccharide

MBP: Major Basic Protein

MMP-2: Matrix Metalloproteinase 2

NF-kB: Nuclear Factor Kappa-B

NHBE: Normal Human Bronchial Epithelial

NKs: Natural Killer cells

PAMPs: Pathogen Associated Molecular Patterns

PARs: Protease-Activated Receptors

PAR-2: Protease-Activated Receptor-2

PCR: Polymerase Chain Reaction

PI3K: Phosphoinositide 3-Kinase

PKB: Protein Kinase B, known as AKT

PNEC: Pulmonary Neuroendocrine Cell

PRRs: Pattern Recognition Receptors

SBTI: Soy Bean Trypsin Inhibitor

STAT-6: Signal Transducer and Activator of Transcription 6

TI: Type 1 alveolar cell

TII: Type 2 alveolar cell

TAD: Transactivation Domain

TGF-α, -β: Transforming Growth Factor -alpha, -beta

Th2: T-helper 2

TLRs: Toll Like Receptors

TNFα: Tumor Necrosis Factor alpha

TYK2: Tyrosine Kinase 2

VCAM: Vascular Cell Adhesion Molecule

Chapter 1: Introduction

1.1. Airway epithelium

1.1.1. Structure and function

The airway epithelium is the continuous epithelial cell layer that covers the internal surface of the airways (1). This epithelial layer has a central role in gas exchange, regulates lung fluid balance, functions as a physical barrier, and clears inhaled irritants, to maintain normal airways function (2). The lung epithelium responds to pathogens and environmental irritants such as those in air pollution by producing pro-inflammatory mediators which recruit immune cells to the site of inflammation (2). In inflammatory sites immune cells produce oxidative radicals and proteolytic enzymes (3) to clear the pathogenic and environmental irritants which could cause epithelial damage. Airway epithelial components are also capable of inducing epithelial repair events as self-renewal and proliferation to overcome the damaged sites (2). In chronic pulmonary diseases such as asthma and chronic obstructive pulmonary disease (COPD) there is evidence of chronic activation of immune cells and tissue damage, and these are found to be correlated with disease severity (2).

Airway epithelium is composed of different cell types: basal cells, ciliated epithelial cells, goblet cells and Clara cells in the bronchial epithelium beside Type I (TI) and Type II (TII) alveolar cells in the alveoli (Figure 1.1.1A) (2). Those cell types originate mainly from the pulmonary neuroendocrine cell (PNEC) which is the first pulmonary cell type that develops during the prenatal period (4). PNEC represents the pulmonary stem cells in postnatal life that can regenerate the airway epithelial cells (5).

The extracellular matrix (ECM) of the bronchial epithelial basement membrane is underlying the epithelial cell layer and functions to maintain the airway epithelium integrity, as an anchor for epithelial cells adhesion. It also maintains cellular polarity by maintaining the orientation of the apical (upper) and basolateral (bottom) cell surface and forms a barrier between the epithelium layer and the underlying mesenchymal compartment (6-8). The epithelial cells secrete type IV collagen and laminin which form the upper layer of the basement membrane, lamina densa (3). The lower layer, lamina reticularis, of the basement membrane consists mainly of type III and V collagen and fibronectin, which are made by the sub-epithelial fibroblasts (Figure 1.1.1B) (9). Recent studies found immune cells able to migrate through the airway basement membrane through pores of mean diameter 1.76 mu without disturbing the ECM (10, 11).

Basal cells are present in high percentage in large airways and in considerably lower percentage in smaller airways (12). Basal cells are attached to the airway basement membrane via integrins and other adhesion molecules (13) which are transmembrane receptors that mediate cell adhesion to ECM proteins in the basement membrane. Basal cells also facilitate the attachment of columnar cells (columnar ciliated epithelial cells and Clara cells) to the basement membrane (13). Basal cells also possess self-renewal and stem cell-like properties and can regenerate secretory ciliated epithelial cells (14) after airway epithelial injury.

Clara cells are present in large and small airways (3). they have a role in maintaining the airway integrity by producing bronchiolar surfactants which have hydrophilic and hydrophobic regions that regulated air-water interface in the alveoli, reduce surface tension and prevent alveolar collapse by regulating alveolar expansion (15). Clara cells also produce protease inhibitors such as secretory leukocyte protease inhibitor (15) which regulate the action of the harmful proteases that are produced by immune cells during inflammation (16). Some findings also indicate that

Clara cells possess stem cell-like properties and act as progenitor cells for ciliated and mucus producing cells (17).

Columnar ciliated epithelial cells represent more than 50% of the epithelial cells in the airways (18). They are fully differentiated and believed to be generated from basal cells (19). They are also characterized by projecting cilia, to clear up mucus from airways, and the presence of abundant mitochondria beneath the apical surface, which provide the needed energy for cilia movement (20).

Goblet cells or mucus producing cells are characterized by membrane-bound electron-lucent acidic–mucin granules that secrete mucus in the airway lumen (21). The airway mucus layer is present from the level of the trachea down to the bronchioles and consists mainly of glycosylated mucin proteins (2). In normal airways there is an equilibrium between the produced and cleared mucus (22). However, in chronic inflammatory situations, such as in allergic asthma and chronic bronchitis, excessive mucus production takes place due to goblet cell hyperplasia and metaplasia (23). Goblet cells are also capable of self-renewal and may also differentiate into ciliated epithelial cells (13).

Alveolar epithelium has a primary role in gas exchange. Its surface is covered with a thin layer of alveolar fluid that consists mainly of surfactant proteins and phospholipids (24) which is responsible to regulate alveolar surface tension and gas exchange during breathing (2). Alveolar epithelium is composed mainly of TI and TII cells. TI cells are characterized by their thin cytoplasmic layer which provides efficient contact area between air and blood for gas exchange (25). The presence of caveolaes (26) suggest a role for TI cells in metabolic and endocytic activities (27). TII cells are cuboidal cells that have essential secretory role as they pack surfactant granules which called lamellar bodies (25). Findings also showed that TII cells function as progenitor cells in the alveolar epithelium (28).

1.1.1.1. Airway epithelium barrier function

Intact airway epithelium and cell-cell communication plays an essential role to maintain lung function (2). Adhesion junctions play a vital role in forming airway structural integrity, while hemidesmosomes functions to keep the attachment of the epithelial cells to the basement membrane (2). Cell-cell communication is promoted by tight junctions and gap junctions. Tight junctions which connect the apices of adjacent cells regulate the electrolytes diffusion across the epithelium (3). Gap junction channels facilitate the transport of signals and cytoplasmic metabolites from cell to cell (29). Both tight and gap junctions promote the airway epithelium integrity by maintaining cell-cell adhesion.

1.1.1.2. Airway epithelium mucociliary and secretory function

The airway epithelium has a primary function in mucus secretion and mucociliary transport. Mucus cells produce mucus to trap inhaled foreign materials and ciliated cells cooperate to remove trapped materials out of the airways (30). Mucus cells produce mucin, which comes from two protein coding genes MUC5AC and MUC5B (31, 32). MUC5AC is produced mainly from goblet cells at the airway epithelium surface (31, 32) upon acute direct contact with inhaled irritants (33). MUC5B is produced from mucus cells of the sub-mucosal glands (31, 32) and its production may be associated with chronic infections and inflammation (33). Mucin production is shown to be regulated by multiple inflammatory mediators such as lipopolysaccharide (LPS) (34), tumour necrosis factor (TNF-α) (35), interleukin-1 (IL-1) (35), IL-13 (36), IL-17 (37), beside growth factors such as transforming growth factor (TGF-α), and epidermal growth factor (EGF) (38).

1.1.1.3. Airway epithelium immune function

Airway epithelium is the first barrier between the host and the environment, and it represents the first line of defence against inhaled microorganisms and allergens (39). To maintain host immunity against environmental insults, airway epithelial cells express many pattern recognition receptors (PRRs) including toll-like receptors (TLRs), which can detect and respond to pathogen-associated molecular patterns (PAMPs) and also to damage-associated molecular patterns (DAMPs) released in damaged tissue or upon cellular stress (40). Airway epithelial cells express both cell surface and endosomal TLRs which are activated upon agonist ligation to induce series of signals through adaptor proteins resulting in the activation of nuclear factor kappa-B (NF-kB) or the interferon regulatory factors 3 or 7 (IRF-3 or IRF-7) (40). This series of signals results in the production of several pro-inflammatory cytokines, chemokines, interferons, and defensins (41). Another PRR that are expressed by airway epithelial cells are protease-activated receptors (PARs) which are G-protein coupled receptors that are activated by cleavage of their extracellular domains. Activation of PAR-2 leads to activation of signal transduction pathways, transcriptional regulations, and expression of inflammatory mediators (42). Allergens which possess proteolytic activity, such as some pollens, HDM, and cockroach allergens are able to activate PAR receptors, particularly protease-activated receptor 2 (PAR-2) (42). Mucosal administration of protease allergens result in PAR-2 activation which is required for T-helper 2 cells (Th2) induced immunity, suggesting a primary role of PAR-2 activation in epithelial programming of the adaptive immune response by proteolytic allergens (43).

In general, activation of airway epithelial cells through PRRs result in release of cytokines, chemokines, and antimicrobial peptides that attract and activate innate and adaptive immune cells (40). Recent studies have demonstrated that activation of airway epithelial cells is a key trigger in activation of lung resident dendritic cells (DCs) (44), which are lung professional antigen-

presenting cells that also express PRRs. The MHCII+CD11c+ subset of DCs have been described as a central control point to determine the induction of pulmonary immunity or tolerance (45-47). Airway epithelium and the subsequent DC activation result in the migration of DCs from the epithelium to the draining lymph nodes where DCs present antigens to T cells (48). Isolation and transfer of DCs from allergen exposed mice to naïve mice result in the induction of allergen specific Th2 cells (49). Depletion of DCs from the airways of naïve or sensitized mice result in the absence of Th2 specific airway inflammation development (49, 50). It was assumed that activation of DCs via PRRs is sufficient to recognise inhaled foreign materials and to induce immune responses. However, studies in which structural cells deficient to TLR-4, which recognises endotoxin, demonstrated that activation, recruitment, and migration of DCs in response to inhaled endotoxin requires only epithelial TLRs triggering (51). These findings revealed the primary role of airway epithelium activation in maintaining pulmonary immunity.

1.2. Asthma

Asthma is a chronic inflammatory disease of the airways in which innate and adaptive immune cells cooperate with airway epithelial and other tissue resident cells to cause airway hyper-responsiveness (AHR), airway smooth muscle hypertrophy and hyperplasia, excess mucus production, thickness of the basement membrane, and other diagnoses. Asthmatic patients are vulnerable to recurrent episodes of shortness of breath, chest tightness, and wheezing that often require urgent treatment. Asthma treatment is based mainly on two categories of drugs, controller medications that include mainly corticosteroids and relievers that include various forms of bronchodilators (52). Inhaled corticosteroids are used to control and reduce asthma symptoms and future exacerbations by reducing the number and activation status of airway inflammatory cells. Inhaled steroids have been proven to decrease the number of activated Th2, reduce eosinophil

counts in asthmatics BAL fluid, and reduce the thickness of basement membrane (53). Bronchodilators are used to relieve asthma symptoms and prevent exercise-induced bronchoconstriction (52).

Asthma could be categorized as allergic and non-allergic asthma. Allergic asthma is mainly induced by allergen exposure and usually identified by the presence of serum antigen specific immunoglobulin E (IgE) to inhaled allergens such as plant pollens, animal dander, or fungi as well as the presence of Th2 cytokines in patient's sputum and broncho-alveolar lavage (BAL) fluid.

Allergic asthma begins with allergic sensitization due to recurrent exposure to allergens that trigger the immune system and induce the production of antigen specific IgE. Repeat exposure to those allergens in sensitized individuals trigger allergic asthma exacerbations. Simpson, A. et al. found that children with persistent wheezing are more likely to develop asthma (54). In a population-based birth cohort study, children with recurrent wheezing after age 1 and positive methacholine challenge are more vulnerable to childhood asthma (54) .

Non-allergic asthma usually develops later in life and is associated with chronic rhinosinusitis, nasal polyps, or obesity. Non-allergic asthma can be triggered by a non-allergic causative agent like exercise; there is less likely IgE reactivity associated with lower chance of Th2 immune responses (55).

1.2.1. Allergic asthma

Allergic asthma has been associated with Th2 cytokines patterns in patient's serum and BAL fluid. Th2 cells are $CD4^+$ T lymphocytes able to produce IL-5, IL-9, IL-4, and IL-13 (56). Elevated gene expression levels of IL-4, IL-5, and IL-13 were detected in BAL fluid from allergic asthmatics, indicating the principal role of Th2 lymphocytes in asthma pathogenesis (57, 58). There is also increasing evidence for the involvement of innate lymphoid cells type 2 (ILC2) in allergic asthma

because of their production of less amounts of Th2 cytokines (59). ILC2 cells reside mainly in the mucosa and function as part of the innate immunity (59). ILC2s are known to produce IL-5 and IL-13 in response to epithelial IL-25 and IL-33 stimulation (59, 60). A study has shown that airway exposure of naïve mice to fungal allergens induces high levels of IL-33 in BAL fluid followed by IL-13 and IL-5 expression and airway eosinophilia (61). IL-13, IL-5 production and airway eosinophilia were inhibited in mice deficient for the IL-33 receptor in comparison to naïve mice (61). Lung ILC2 were sufficient to induce allergic asthma features upon exposure to fungal allergens. Abundant ILC2 immune cells are found in allergic asthma patients peripheral blood, and isolation of peripheral blood cells from those asthmatics showed significant induction of IL-13 and IL-5, but not IL-4, in the presence of IL-25 and IL-33 (62). Depletion of peripheral blood T-cell before IL-25 stimulation did not decrease IL-13 and IL-5 expression (62) which suggest that IL-13 and IL-5 production is mediated by ILC2 innate immune cells.

1.2.1.1. Th2 cytokines

IL-5 promotes eosinophil maturation and their release from the bone marrow into the circulation (63). Eosinophils and basophils are the primary cells expressing IL-5 receptors (63), so IL-5 has quite restrictive reactivity. High levels of serum IL-5 was found in asthmatic patients during an asthma exacerbation, and the levels were higher in patients with moderate or severe asthma compared to those with mild disease (63). One-week administration of oral corticosteroids in severe asthmatics reduced serum IL-5 levels to under detectable limit (64) which suggests that high serum IL-5 level is associated with more eosinophil infiltration and more asthma severity.

IL-9 boosts the survival of primary mast cells, and it was described as a mast cell growth factor (65). It also stimulates the expression of IgE receptors on mast cells, which suggests that IL-9 may also enhance mast cells reactivity to allergens (65). IL-9 showed in vitro synergistic effect with

IL-4 and IL-5 as it enhances the production of IL-4 mediated IgE in human and murine B cells and promotes eosinophil maturation in response to IL-5 (65).

IL-4 is associated with the development of IgE isotype switching and IgE synthesis by B lymphocytes (66). In allergic asthma IL-4 enhances mucin gene expression and therefore mucus hyper-production (67). IL-4 induces vascular cell adhesion molecule (VCAM)-1 on vascular endothelium which enable T lymphocytes, monocytes, basophils, and eosinophils to migrate to inflammatory sites (67). IL-4 also promotes eosinophilic inflammation by suppressing eosinophil apoptosis and enhancing eosinophil chemotaxis (67). In addition, an essential and special action of IL-4 is its ability to induce naïve lymphocytes differentiation to Th2 cells (67), because T cells express IL-4 receptors which make T cells respond to IL-4. IgE production and VCAM-1 expression induction are shared actions between IL-4 and IL-13 (67). The functional redundancy of IL-13 and IL-4 is due to the sharing of one receptor chain of each interleukin in a receptor they activate (68). All the previous actions of IL-4 reveal it has an essential role in pro-inflammatory cell infiltration and Th2 differentiation in allergic asthma.

IL-13 is a 17-kDa glycoprotein cytokine that is secreted from activated $CD4^+$ cells and to a lesser extent from $CD8^+$ cells, Th1 cells, mast cells, basophils, eosinophils, and natural killer (NK) cells (69). IL-13 has a central role in asthma pathogenesis through multiple effects at all levels of the immune system. IL-13 regulates B cell isotype class switching to IgE (66), induces the expression of the adhesion molecule VCAM-1 on endothelial cells (67), stimulates chemokine (eotaxins) production from epithelial cells which activate and promote the migration and infiltration of eosinophils into the lungs (70), promotes goblet cells proliferation and mucus production (71), promotes the transformation of airway fibroblasts to myo-fibroblasts which increase collagen

deposition (72), cause proliferation of airway smooth muscle (73), and stimulates airways hyper-responsiveness (74).

Functional IL-13 receptor is a heterodimer of IL-4 receptor α subunit (IL-4Rα) chain and IL-13 receptor α1 subunit (IL-13-Rα1) chain. There in an additional IL-13 receptor, the IL-13 receptor α2 subunit (IL-13Rα2) chain, which is composed of a single chain and is a decoy receptor with no known signaling pathway but regulates IL-13 responses (75). IL-13Rα2 found in soluble form in mouse and human serum (76). IL-13Rα2 has also been found in the intracellular environment of multiple cell types such as primary respiratory epithelial cells and monocytes (68, 75). IL-13 functional and decoy receptors are expressed by human B cells, eosinophils, basophils, mast cells, endothelial cells, fibroblasts, monocytes, macrophages, airway epithelial cells, and smooth muscle cells (75). Ligation of IL-13 or IL-4 protein to IL-13Rα1 receptor leads to the recruitment of IL-4Rα chain. Activation of the IL-13Rα1 IL-4Rα dimer by IL-13 or IL-4 protein binding result in activation of JAK1/TYK2 and phosphorylation of the signal transducer and activator of transcription 6 (STAT-6). Phosphorylation of STAT-6 upon IL-13 and IL-4 stimulation results in STAT-6 dimerization and nuclear translocation where it results in CCL26 mRNA transcription (75, 77).

IL-4 can also signal following binding to a second receptor for IL-4. This receptor complex consists of the IL-4Rα chain and the common γc chain and its activation leads to phosphorylation of JAK 1/3 and tyrosines in the IL-4Rα cytoplasmic domain, which in turn activate STAT-6 or/and insulin receptor substrate-2 (IRS-2). Phosphorylation of STAT-6 triggers phospho-STAT-6 dimerization, nuclear translocation and transcription. IRS-2 phosphorylation activates PI3-K, AKT, and NF-kB gene transcription, and cell proliferation (77).

Targeting Th2 cytokines has been used as an approach to treat asthma, especially in patients with uncontrolled and severe asthma. This subset of patients have shown increased concentrations of sputum and bronchial biopsy IL-13 despite the use of corticosteroids (78). Based on that, targeting IL-13 is assume to be a plausible approach to asthma treatment. An anti-IL-13 humanized monoclonal antibody, lebrikizumab (Genentech/Chugai Pharmaceutical), leads to significantly improved lung function in a subset of patients with uncontrolled asthma who have high serum levels of periostin (79). Periostin is an ECM protein that is produced by airway epithelial cells in response to IL-13 stimulation (80). At week 12 of using lebrikizumab, the recorded FEV_1 for the whole lebrikizumab treated group compared to the baseline was significantly increased by 5.5%, it also significantly increased by 8.2% in the high-periostin subset group, but there was no significant increase (1.6%) in the low-periostin subset group (79). FEV is the forced expiratory value in one second and it is used to measure the amount of exhaled air in one second (81).

Another approach to target IL-13 has used dupilumab (SAR231893/REGN668), which is a fully humanized monoclonal antibody against the IL-4Rα chain. This approach is designed to target IL-4Rα chain which is a shared receptor subunit for IL-4 and IL-13 to suppress protein ligation of both IL-13 and IL-4 (82). Dupilumb (300 mg subcutaneously) significantly increased FEV_1 two weeks after administration which was maintained through the 12 weeks of the trial (82). These findings supporting the significant pathogenic role of IL-13 and IL-4 and suggesting that targeting asthma promoting cytokines could be an effective approach to treat asthma.

1.2.2 Role of eosinophils in asthma

Eosinophils are leukocytes with bilobed nuclei that originate from pluripotent haemopoietic stem cells. Stem cells mature to Colony Forming Unit- Granulocyte, Erythrocyte, Monocyte,

Megakaryocyte (CFU-GEMM) progenitor cells then to Colony Forming Unit- Eosinophil (CFU-Eo) progenitor cells in the bone marrow before giving rise to eosinophils. Eosinophils represent less than 5% of leukocytes in the blood stream, and they can be found in higher concentration in bone marrow and certain tissues where they can regulate immune and inflammatory responses (83). Eosinophils contain a number of different specialized compartments in their cytoplasm, such as crystalloid granules, primary granules, small granules, lipid bodies, and secretory vesicles contain multiple proteins that have the ability to kill helminths in worm infections and cause tissue damage in hypersensitivity responses (84). Crystalloid granules are mainly composed of cationic proteins including major basic protein (MBP) (86). MBP is cytotoxic which may cause tissue damage and lung dysfunction in asthma, deposition of MBP is detected in asthmatics sputum and damaged epithelium, which was correlated with smooth muscle hyper-reactivity (85). Eosinophils are also known to secrete mediators such as the preformed granule-derived cationic proteins which include MBP, eosinophil peroxidase (EPO), and eosinophil cationic protein (ECP); oxidative metabolites such as reactive oxygen species; cytokines, chemokines and growth factors such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-16, TGF-α, TGF-β, eotaxins and TNF-α (84).

Eosinophil accumulation in the blood and tissue is found to be related to multiple inflammatory diseases (83). Tissue deposition of eosinophil-associated proteins contributes to disease pathogenesis (85). Multiple studies have established a causative link between eosinophils and allergic asthma pathogenesis (87-89). Sensitized and challenged eosinophil deficient mice demonstrate significantly lower airway epithelial hypertrophy, reduced goblet cell metaplasia and mucus accumulation, lower airway hyper-responsiveness (87, 88), as well as significant decrease in Th2 cytokines in BAL fluid, compared to sensitized and challenged wild type mice (88). The

significant decrease in Th2 cytokines in eosinophil deficient mice is also suggesting a critical role of eosinophils in lung immune regulation. Another study has defined a connection between eosinophils and allergic airways remodeling as they revealed that eosinophil deficient mice have demonstrated significant reduction of airways collagen deposition and smooth muscle hypertrophy (89). All of these data highlight the critical role of eosinophils in the development of allergic airways hallmark features.

1.2.2.1 Eosinophil chemotactic factors

1.2.2.1.1 Eotaxins

Eotaxins are a family of CC chemokines, chemokine proteins that have two adjacent cysteines at their amino terminus, that include CCL11/Eotaxin-1, CCL24/Eotaxin-2, and CCL26/Eotaxin-3. These proteins are typically 8-10 kDa in size and produced by epithelial cells, smooth muscle cells, endothelial cells, macrophages, eosinophils, T-cells, and fibroblasts (90). Eotaxins are known to induce a directional migration of leukocytes during allergic inflammation through activation of the CCR3 receptor (90). CCR3 is a G-protein coupled receptor and its expression on the cell surface is restricted to eosinophils, basophils, Th2 cells, and mast cells (90).

BAL fluid, sputum, and biopsies from asthmatic patients contain high numbers of eosinophils (55). In animal models of lung allergic inflammation, eosinophilic infiltration is correlated positively with eotaxins production (91). Eosinophilia in lung tissue is promoted by IL-5 and eotaxins, and reduction of airway eosinophil count by corticosteroids or by IL-5 neutralization are used therapies to reduce asthma exacerbation (92). IL-5 promotes eosinophils maturation from the bone marrow and release into the circulation whereas, eotaxins 1, 2, and 3 (CCL11, CCL24, and CCL26

respectively) serve as eosinophil chemoattractants by activating CCR3 receptors, which are highly expressed on eosinophils (93).

A study aimed to examine the involvement of CCR3 receptor in eosinophil recruitment by using eotaxin specific migration. Complete CCR3 blockade completely inhibits eotaxin2/CCL24-induced eosinophil migration for up to 18 hours (93). However, ~10% of eotaxin1/CCL11-induced migration and ~30% of eotaxin3/CCL26-induced migration was not inhibited under the same conditions (93). The eosinophil migration assay for each eotaxin was assessed for up to 18 hours. Eotaxin1/CCL11 and eotaxin2/CCL24 have a quick time course and reach maximum migration effect at 6 hours, while eotaxin3/CCL26 induced migration is still active at 12 hours and shows a second or late phase of migration at 18 hours. These data revealed that CCR3-induced migration is not the only signaling pathway used by eotaxins to recruit eosinophils. Interestingly, this study also found that CCL26-induced migration is the least inhibited by CCR3 blockade beside its ability to induce early and late phases of migration, which suggest that CCL26 may be the most effective target for treating eosinophil migration in asthma.

Moreover, eosinophil migration assays using increasing concentrations of each eotaxin found that CCL11 and CCL24 reached their maximum effect at concentrations around 10nM, but 100nM of CCL26 was needed for maximum effect (93). The same assay assessed the mean cell migration for CCL11, CCL24, and CCL26. Highest eosinophil migration (59%) was induced by CCL26. That means that CCL11 and CCL24 are more potent than CCL26 in terms of maximum concentration for cells migration, but the results also indicate that CCL26 is more effective than CCL11 and CCL24 because it caused the maximum percentage of eosinophils migration. CCL26 mRNA also found to be the highest expressed eotaxin in cultured airway epithelial cells obtained from healthy donors upon stimulation with IL-13 and IL-4 (94).

1.3 Airway remodeling in asthma

Airway remodeling is characterized by structural changes including fibrosis, increased airway wall thickness, and epithelial cell hypertrophy. Chronic asthma patients may show progressive decrease of lung function that is thought to be caused by airway remodeling and increased thickness of airways basement membrane, which includes smooth muscle hyperplasia and sub-epithelial fibrosis.

Eosinophils also contribute to airway remodeling. It induces fibrosis by releasing the potent fibrogenic factor transforming growth factor (TGF)-β (95). Adding TGF-β to fibroblasts promote fibroblasts proliferation and collagen synthesis (95). TGF-β was also detected to stimulate myo-fibroblast differentiation and deposition of ECM in vivo and in vitro (95). A study supported the connection between eosinophils and ECM production by finding a specific decrease in bronchial mucosa eosinophils, in mild asthmatics, by using anti-IL-5 which was associated with significant reduction in ECM proteins production in the airway basement membrane (96).

Other immune cells like macrophages may contribute to airway remodeling by secreting pro-fibrotic mediators, such as basic fibroblast growth factor and TGF-β (97). Some data showed an increase T cell count was associated with airway fibrosis (98).

Mast cells may have important role in airways fibrosis (97). Mast cells produce fibroblast-migration and proliferation factors besides its ability to produce collagen which is a component of airway basement membrane (97). These findings suggest a strong connection between airways immune cell activation and remodeling in asthma.

Another feature of airways remodeling is excessive mucus production due to goblet cells and submucosal glands hyperplasia. Some findings showed a roll for IL-13 in the induction of mucus production (99) by using human STAT-6 transgenic and deficient mice. STAT-6 is a critical

signaling molecule activated by IL-13 and essential for allergen-induced asthma in experimental models; and its expression in airway epithelial cells is usually increased in asthma. STAT-6 deficient mice were protected from IL-13 induced AHR and mucus production. However, reconstitution of STAT-6 in airway epithelial cells was sufficient to show IL-13 induced AHR and mucus production in the absence of inflammation or other lung pathology (99). Another study has also shown that IL-13 induces collagen type-1 mRNA and protein expression in airway fibroblasts in mild asthmatics compared to healthy subjects (100). IL-13 induced collagen expression was significantly attenuated in the case of using matrix metalloproteinase 2 (MMP-2) inhibitor (100) which indicated that IL-13 induced collagen production is probably through activation of MMP-2. MMP-2 is a proteolytic enzyme that is known to degrade ECM components especially collagen more efficiently (101). However, it was demonstrated that collagen type-1 production is dependent on IL-13 activated MMP-2 and consequently the activation of TGF-β which contributed to the production of collagen from airway fibroblasts in asthma (100).

1.4 Role of common allergens in allergic airway inflammation

Exposure to aeroallergens is considered a key trigger to allergic airway inflammation. Though the molecular basis by which aeroallergens induce allergic inflammation is still not fully understood, it is suggested in multiple studies that airborne allergens are inducing allergic reactions in proteolytic dependent mechanisms (102). Cysteine, serine, and aspartic proteases were defined to be present in multiple airborne allergens such as house dust mite (HDM), cockroach, fungi, as well as pollens (102). Different effects of proteolytic activity have been identified to contribute to airway allergic inflammation. Proteases disruption of airway anti-protease defences in mucosa lead to enhanced tissue damage and activated immune reaction (102). Some of the HDM identified

cysteine proteases and German cockroach frass proteases can degrade airway residue α1-antitrypsin inhibitor (103) beside the ability of some HDM proteases to degrade airway epithelium surfactant proteins (104). Epithelium integrity breakdown, which increase the epithelium permeability, is another mechanism that HDM cysteine proteases and other allergens use to gain access to submucosal tissues where they induce pro-inflammatory reactions (105). Direct cell activation and induction of inflammatory mediators is a well-established mechanism that molds, cockroach, pollens, and HDM proteases use to induce allergic reactions. Direct cell activation was shown to be at least mediated by PAR-2 activation pathways (102). Airway remodeling is another effect of allergic reaction to protease allergens that contributed to airway tissue damage (102). Though the exact mechanism of the remodeling process is not well elucidated, it is known that inflammatory cell mediators, proteases as well as growth factors all have a role in remodeling (106).

1.4.1 Role of cockroach allergens

Exposure to cockroach allergens is a major risk factor for asthma development (107). There are two species of cockroaches that have been studied extensively in association with asthma, German cockroach (*Blattella germanica*) and American cockroach (*Periplaneta Americana*). *B. germanica* is mostly distributed in warm and humid residential areas, whereas *P. americana* reside outside in sewers and draining systems (108). Inner city asthma studies found exposure to household cockroach to be one of the principal allergens associated with asthma severity (107). This is also supported by data showing that early-life exposure to cockroach allergens Bla g1 and Bla g2 were also associated with higher risk of allergen specific Th2 lymphocyte proliferative responses, which skew the immune system toward allergic inflammation (109).

Our laboratory showed that mucosal sensitization of mice using a whole-body *B. germanica* extract followed by challenge with the same antigen led to AHR, serum cockroach-specific IgG, and airway increased levels of Th2 cytokines and high inflammatory cell count, primarily eosinophils (110). This allergic sensitization was found to be dependent on *B. germanica* proteolytic activity and its activation of PAR-2 (111). PAR-2 receptor expression was detected in airway epithelial cells, fibroblasts, and smooth muscle and its activation by serine proteases found connected with the development of airway inflammatory reactions (112). Using anti-PAR-2 antibody during the sensitization phase has completely inhibited AHR and airway inflammation (111). These findings highlight the role of cockroach extract (CE) proteolytic activity in the airway inflammation and allergic asthma.

Three distinct serine proteases (E1, E2, and E3) have been identified and isolated from *B. germanica* cockroach extract which are used in clinical skin test (113). Serine proteases are enzymes that able to cleave proteins peptide bonds. In these proteases serine amino acid at the enzyme active site serves as nucleophilic (amino acid donates electron pair to form a chemical bond). To determine the proteases enzymatic biochemical activity, three different serine protease substrates were used. Results showed highest cleavage to trypsin substrate, which was completely blocked by soy bean trypsin inhibitor (SBTI) (113). These CE proteases have different primary amino acid sequences, with high homology, and all three were able to activate PAR-2 leading to Ca^{++} and MAPK signaling (113). No other *B. germanica* allergnes, other than E1, E2, and E3, are known to have proteolytic activity (114). From that we could not claim all the proteolytic activity-related pathogenesis to those allergens since the effect could be a result of collaborated trigger of each or with other unidentified allergens.

1.4.2 Role of house dust mite allergens

HDM are microscopic organisms found in household dust in warm and humid areas feeding on people and pets skin flakes. HDM is known to induce allergy. General dusting and vacuuming process, which disturb HDM to become airborn allergen, are associated with allergy symptoms such as watery eyes, runny nose, and sneezing.

HDM is one of the most common triggers of allergic airway diseases. More than 20 structural proteins and enzymes in HDM have been identified as allergens (115) which can activate various receptors. For example, HDM allergens Der p1 and Der p5 enhanced IL-6 and IL-8 cytokines production in a dose dependent fashion in cultured human alveolar epithelial cell line (116). In terms of PAR-2 activation by Der p1 or Der p5, Der p1 induced IL-6 and IL-8 release were found to be independent of PAR-2 activation, while Der p5 showed PAR-2 dependent release of IL-6 and IL-8 (116). The same study found the Der p1 protease activity inhibited by cysteine-protease inhibitors, but this was not true for Der p5, which was not inhibited by either serine or cysteine protease inhibitors. Heat treatment (65°C, 30 minutes) for HDM allergens result in complete block of IL-6 and IL-8 cytokines production upon treatment with heated Der p1, and partial reduction of IL-6 and IL-8 induction with heated Der p 5 (116) that would give insight that some effects of HDM allergens on airway cells are protease dependent.

Release of CCL2 and CCL20 in response to HDM in airway epithelial cell highlights the HDM role in the recruitment of monocytes, T cells, mast cells, and dendritic cells (115). Other data suggest that HDM induces the upregulation of adhesion molecules on epithelial and inflammatory cells which promotes the pro-inflammatory cells recruitment and infiltration (115).

The proteolytic activity of HDM extract has been shown to degrade CXCL-5 in vivo, a chemokine mediating chemotaxis of neutrophils, which is released from bronchial smooth muscle cells (117).

Cultured bronchial smooth muscle cells (BSMC) were treated with HDM extract which resulted in reduction of CXCL-5 protein release but not CXCL5 mRNA levels (117). This observation suggested that HDM proteolytic activity degraded CXCL-5 protein, and that was confirmed by showing recombinant CXCL-5 degradation upon incubation with HDM extract. These results showed the ability of HDM extract to degrade chemotactic factor CXCL-5, which may limit the ability of certain inflammatory cells to accumulate in areas of inflammation and may bias the inflammatory reaction towards non-affected cells.

1.5 Rationale

Sensitization to cockroach allergens are a predictor of morbidity in the US Inner City asthma studies, and up to 36.8% of the recruited children in these studies were positive to cockroach skin prick test (107).

Airway epithelial cells recognize and interact with inhaled allergens, which result in release of airway epithelial pro-inflammatory mediators such as IL-33 and IL-1α (118). Further recognition and presentation of those allergens by antigen presenting cells (APC) build the bridge to activate the adaptive immune response such as that mediated by Th2 cells (118).

A central mediator and remodeling cytokine in asthma, IL-13 was found to induce allergic asthma features such as AHR, and production of antigen specific immunoglobulin IgE (119). Furthermore, IL-13 and IL-4 are known to induce the expression of airway epithelium CCL26 (94), the most effective eosinophil chemotactic factor (93). CCL26 is the only eotaxin that induces two phases of eosinophil migration and is the least affected by CCR3 blockade (93) which emphasizes its role in asthma pathogenesis and makes it a potential therapeutic target in allergic asthma.

In view of the above and to reveal the interactions of cockroach allergens with IL-13 and CCL26 induction in airway epithelium, which is considered a core interaction in asthma triggered by

cockroach allergens (Figure 1.5), we sought to understand the mechanism by which cockroach interact with the airway epithelial cells and how that affects the expression of IL-13 induced CCL26.

1.6 Hypothesis

Based on previous studies that found CE induces sensitization and airway eosinophilic inflammation (110, 120), we hypothesized that CE augments the production of IL-13 induced CCL26 in airway epithelial cells.

1.7 Objectives

1) Examine the expression of IL-13 mediated CCL26 in BEAS-2B cells.

2) Study the effect of CE on the IL-13 induced CCL26 mRNA and protein.

3) Find the role of CE proteases.

4) Compare the effect of CE with the effect of HDM on IL-13 mediated CCL26 expression.

5) Examine the effect of CE on IL-4 induced CCL26.

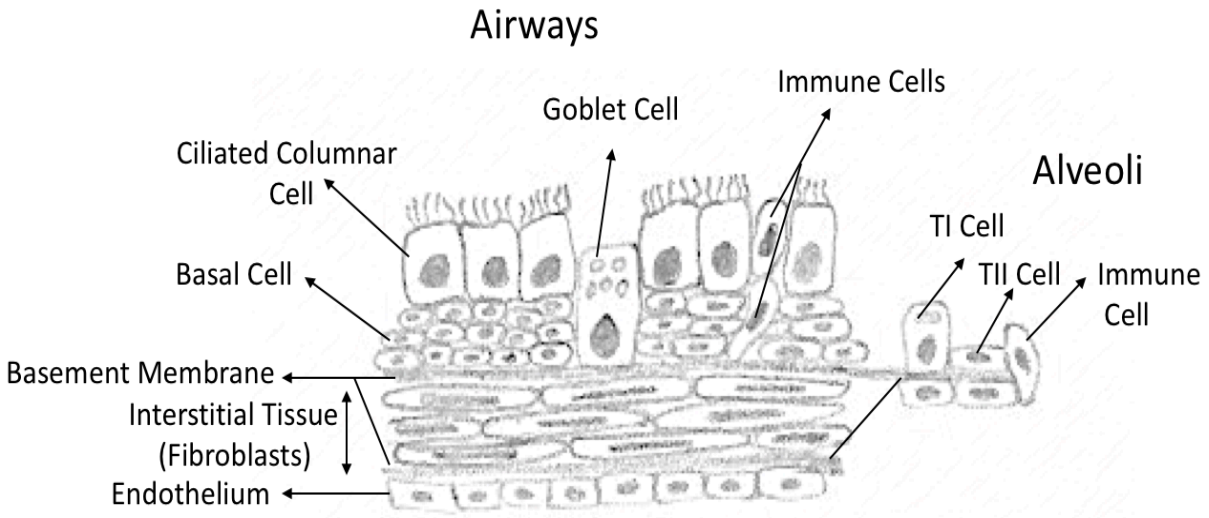


Figure 1.1.1A: Major cell types of the airway epithelium. Large airways major cell types are: ciliates columnar cell, goblet cell, and basal cell. The small airways major cell types are similar to the large airways with more Clara cell. The alveolar major cell types are the TI and TII. Basement membrane is separating the epithelial cell layer from the interstitial tissue that contain the fibroblasts as major cell type. Another layer of the basement membrane is separating the interstitial tissue from the endothelium and blood stream. This figure is a simplified hand drawing based on (Figure 1) at Tam A et al, The airway epithelium: More than just a structural barrier. Ther Adv Respir Dis 2011;5(4):255-273

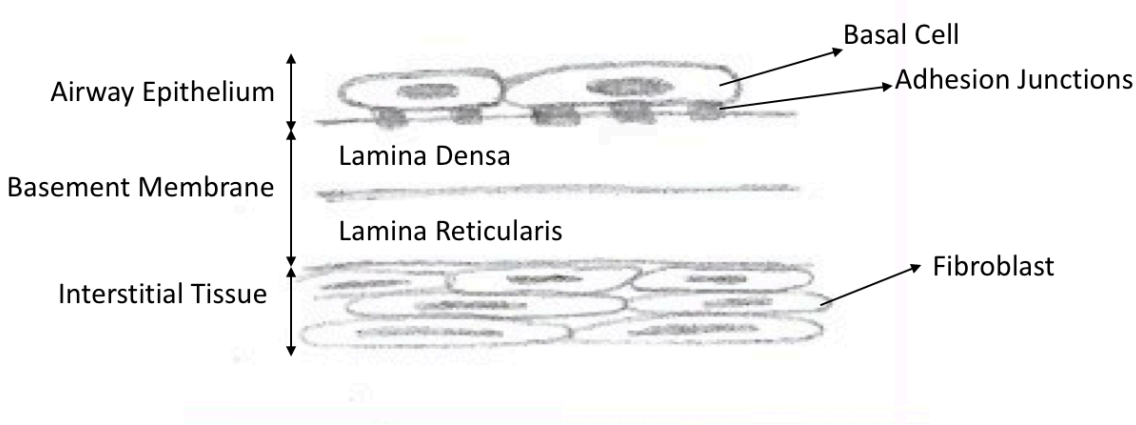


Figure 1.1.1B: Airway epithelium basement membrane composed of two layers. Lamina Densa is made mainly of type IV collagen and Laminin. This layer separates the basal epithelial cells from the second layer of the basement membrane Lamina Reticularis, which is made mainly of type III collagen and fibronectin. Lamina Reticularis separates Lamina Densa from the interstitial tissue. This figure is a simplified hand drawing based on (Figure 2) at Tam A et al, The airway epithelium: More than just a structural barrier. Ther Adv Respir Dis 2011;5(4):255-273

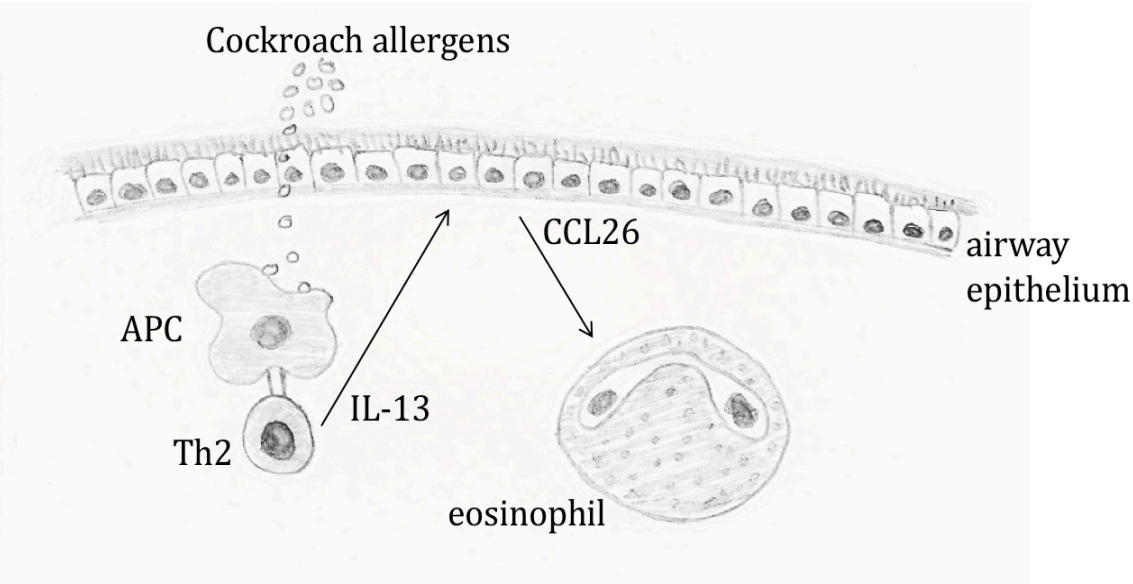


Figure 1.5: Model of cockroach allergens and airway epithelial interaction

Chapter 2. Materials and Methods

2.1. Cell culture

The virus transformed human bronchial epithelial cell line BEAS-2B was purchased from American Type Cell Collection (ATCC®, Manassas, VA) (cat # CRL-9609). Cells were cultured in multi-well pre-coated plates using Bronchial Epithelial cell Growth Media (BEGM) consisting of Bronchial Epithelial cell Basal Media (BEBM) (Lonza; Walkersville, MD) (cat # CC-3171) with the addition of SingleQuots (cat # CC-4175), except Gentamycin, Amphotericin and Retinoic Acid, and in addition to Penicillin/Streptomycin (100U/ml & 100ug/ml respectively). Cell culture flasks and multi-well plates were pre-coated for 8 h with 0.03 mg/ml bovine Collagen Type I (Life Technologies, Grand Island, NY, USA) (cat # A10644-01), 0.01mg/ml bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO, USA) (cat # A3059-50G), and 0.002 mg/ml Human Fibronectin (Gibco/Life Technologies; Grand Island, NY) (cat # PHE0023) in BEBM. Cell growth media was replaced with fresh media every 2-3 days according to the ATCC cell culture protocol. Primary Normal Human Bronchial Epithelial cells (NHBE), from two different donors, were purchased from Lonza (Walkersville, MD) (cat # CC-2540). NHBE cells were cultured in pre-coated multi-well plates with 0.03 mg/ml bovine collagen type I (life technologies) in BEBM. Cells were grown in BEGM media from Lonza (Walkersville, MD) with all the SingleQuots growth factors to culture the cells. NHBE cell experiments were done on passage 5. All cells were grown in incubators at 37^{o}C with 5% CO2.

2.2 Cell activation

BEAS-2B and NHBE cells were cultured in pre-coated multi-well plates till 80-90% confluent. BEGM was then changed to starvation media (BEBM plus 1% Penicillin/Streptomycin) and cells

were activated 24 hours later. Cells were activated with different concentrations of recombinant human interleukin-13 (rhIL-13) (R&D Systems, Minneapolis, MN, USA) (cat # 213-ILB/CF), CE (Greer Laboratories, Lenoir, NC) (cat # XPB46D3A4) or both.

To examine the effect of CE proteases, BEAS-2B cells were activated with heat inactivated CE (HICE) heated at 65^{o}C for 30 minutes and reconstituted to final concentration 5ug/ml in BEBM or CE incubated with specific protease inhibitors. E-64 from SIGMA (cat # E3132) in final concentration 10uM, aprotinin from (Roche Applied Science, Mannheim, Germany) (cat # 70512326) 2ug/ml final concentration, or 1ug/ml pepstatin A from SIGMA (cat # P5318) was used separately. 5ug/ml of CE was incubated with each inhibitor for 30 minutes at room temperature prior to cell activation. CE protease activity was also examined by using trypsin inhibitor from Glycine max (soybean) (Sigma-Aldrich) (cat # T6522) by incubating 5ug/ml of CE with (.5-1 ug/ml) of soybean trypsin inhibitor (SBTI) for 30 minutes at 37^{o}C prior to cell activation.

To examine the role of PAR-2 receptor activation in the observation we are examining, BEAS-2B cell culture was activated with stimulated with either IL-13, IL-13 plus PAR-2 activated peptide (AP) (SLIGKV-NH2) (250uM), or IL-13 plus PAR-2 control peptide (CP) (VKGILS-NH2) (250uM). Due to results inconsistency, we have not further pursued using those peptides. The experiment repeated later by using other PAR-2 peptides, AP (SLIGRL-NH2) (50uM) and CP (LRGILS-NH2) (50uM) (121).

In some experiments recombinant human interleukin-4 (rhIL-4) (R&D Systems) (cat # 204-IL) was used to stimulate BEAS-2B instead of IL-13. In addition, different concentrations of HDM (Greer) (cat # XPB82D3A2.5) or Escherichia Coli (E. Coli) lipopolysaccharide (LPS) (cat # L4391-1MG) was used with IL-13 instead of CE for comparative studies.

For IL-13 and CE sequential treatment experiment BEAS-2B cells activated with 20ng/ml of IL-13 for 90 minutes. IL-13 then washed and the cells incubated to complete 24 hours in absence of IL-13. In another condition stimulation with IL-13 was for 90 minutes then CE added, and cells continued incubation for 24 hours total. In the last condition, cells washed after 90 minutes stimulation with IL-13 then CE added and cells incubated for 24 hours total.

To examine whether CE alters the stability of IL-13 induced CCL26 mRNA, BEAS-2B cell cultures were stimulated with 20ng/ml of IL-13 for 12 hours then 5ug/ml of CE added for another 2 hours. After the indicated time IL-13 and CE was washed and 5ug/ml of Actinomycin D (Sigma-Aldrich) (cat # A9415) added. Cells then incubated for 1,3, or 6 hours before harvesting. To validate this methodology, cells were also stimulated with IL-13 for 12 hours then IL-13 was washed. Actinomycin D added for 5 minutes before CE stimulation. Cells then incubated for 1 ,3, or 6 hours before harvesting.

2.3. Reverse transcription and polymerase chain reaction

Total RNA was isolated from cell lysates by using RNeasy Mini Kit from (QIAGEN, Valencia, CA) (cat # 74104) then the RNA concentration was measured by Nanodrop 2000c (Thermo Scientific). For reverse transcription reaction, 1ul of 100mM of dNTPs (2'-deoxynucleoside 5'-triphosphate) from (Invitrogen, Carlsbad, CA) (cat # 10297-117) and 1ul of 0.5ug of Oligo(dT) 12-18 Primer from Invitrogen (cat # 18418012) was added to 0.5ug of RNA in 20ul system. The mixture was then heated at 65^{o}C for 5 minutes in PTC-100 machine (Programmable Thermal Controller, MJ Research, Inc) to hybridize the poly(A) tail of mRNA. A mixture of 4ul of 5X First-Strand Buffer, 2ul of 0.1 M DTT (dithiothreitol), which is a reducing agent that reduces disulfide bonds and inhibits RNases activity, both from Invitrogen (cat # 28025-013) and 1ul of RNase OUT, which is a recombinant ribonucleases inhibitor from Invitrogen (cat # 10777-019) was added

to each sample prior to heating at 37°C for 2 minutes. The samples were then heated at 37°C for 50 minutes after adding 200U per sample of MMLV (Moloney Murine Leukemia Virus Reverse Transcriptase) from Invitrogen (cat # 28025013) followed by 15 minutes at 70°C to form cDNA.

The product cDNA was used in Taqman polymerase chain reaction (PCR). CCL26 gene expression assay (cat # Hs00171146_m1) and the housekeeping gene GAPDH gene expression assay (cat # Hs99999905_m1) (both from Thermo Scientific) were used to study CCL26 mRNA expression. For interleukin13-receptor subunit alpha 2 (IL-13Rα2) PCR, IL-13Rα2 gene expression assay from Thermo Scientific (cat # Hs00152924_m1) was used with GAPDH housekeeping gene.

In general, PCR program was done in 40 cycles of 95°C for 15 seconds for denaturing and 60°C for 1 minute for annealing. The Ct value for each sample is captured when the amplified product hit the threshold. The Ct value is the number of the cycles required to amplify the product and give fluorescence exceeds the threshold level.

2.4. Enzyme linked immunosorbent assay (ELISA)

DuoSet ELISA kit for human CCL26/Eotaxin-3 was purchased from R&D systems (DY346) and the assay was performed according to the manufacturer's instructions. 96 well flat-bottom microplates were coated overnight at room temperature with 1ug/ml anti-human CCL26 capture antibody in PBS Sigma (cat # P5368), and then blocked with 1% BSA (Sigma) in PBS Sigma for 1 hour at room temperature. A seven point 2-fold standard curve of recombinant human CCL26 was used with highest concentration of 4000pg/ml. BEAS-2B cell culture supernatants and standards were then added to the pre-coated microplates and incubated for 2 hours at room temperature. Detection antibody was added later to the plate and incubated for another 2 hours at

room temperature. The plate was then incubated for 20 minutes in the dark with Streptavidin-HRP before adding the substrate solution and incubate for another 20 minutes in the dark. The plate was washed three times with washing buffer made of 0.05% Tween 20 in PBS between each step. Stop solution 2N H2SO4 was used to stop the reaction. Plate reader Power Wave XS (BIO-TEK) measured the optical density immediately, then a four-parameter logistic (4-PL) standard curve was generated by Graph Prism.

2.5. Flow cytometry

Cultured BEAS-2B cells were stimulated with or without 5ug/ml of CE for 24 hours before harvesting with Trypsin. For each analysis, 1×10^6 cells were counted and placed in a test tube on ice. Cells were washed with PBS:FACS buffer (0.5% BSA, 0.1% NaN3 and 3% FBS in 1x PBS) and centrifuged at 200 g for 5 minutes at 4°C. Supernatants were then disposed and the cell pellets were suspended in fresh PBS:FACS solution. A mixture of mouse IgG Isotype Control antibody from Invitrogen (Cat # 10400C) and human FcR Blocking Reagent from Miltenyi Biotec (cat #1 30-059-901) was used for 15 minutes blocking to minimize nonspecific binding of the target antibody. The cells were then washed with PBS:FACS buffer as previously described. The test cells were incubated with monoclonal Human IL-13Rα1 APC-conjugated antibody (R&D systems) (cat # FAB1462A) for 30 minutes on ice. Control cells were incubated with Mouse IgG2B APC-conjugated control antibody (R&D systems) (cat # IC0041A) for 30 minutes on ice. Then both were washed as previously described. Finally, the cells were fixed with 2% paraformaldehyde PFA before analysis.

2.6. Protein extraction and western blot

2.6.1 Protein extraction and western blotting for STAT6

BEAS-2B cells following activation with IL-13 (20ng/ml), CE (5ug/ml), or IL-13 in combination with CE, was incubated for 30 minutes before protein extraction. The plate was then placed on ice and the supernatants were vacuumed from each well. 200ul/well of a mixture of RIPA (lysis buffer) from Santa Cruz (#sc-24948) and protease inhibitor cocktail (PIC) from Sigma (#P-8340) (40ul of PIC/1ml of RIPA) was used to lysate the cells for 5 minutes. Each well was scrapped with a plastic scrapper then the content was transferred to Eppendorf tubes. The lysates were then vortexed for 10 minutes then placed on shaker for 15 minutes in cold room to complete the extraction of intracellular proteins. The tubes were then centrifuged on full speed (3000g) for 10 minutes at 4^{o}C to get rid of cell debris. 90:10 units of Red loading buffer from BioLabs (cat # B7709S) and 30x Reducing agent (DTT) from BioLabs (#B7705S) were respectively added for each lysate, before placing on hot plate 100^{o}C for 5 minutes. The samples were then ready to transform to 10% PAGE (polyacrylamide gel electrophoresis) or stored in -80^{o}C till the running time.

To do western blot, 10% PAGE gels Mini-PROTEAN® TGX™ Gels (cat # 456-1034) were loaded in Bio-Rad mini Protean Cell that was connected to Bio-Rad PowerPac200 power supply. Two similar gels were loaded in every experiment. One was used to detect total STAT6 with a monoclonal mouse anti-human anti-STAT6 antibody (BD Bioscience; cat # 611290) and the other to detect phosphorylated STAT6 by using a polyclonal rabbit anti-human anti-pSTAT6 (Tyr641) from Cell Signaling (cat # 9361S). The membranes were imaged using Odyssey Infrared Imager (LI-COR) and analyzed by Image Studio software (5.2.5) from LI-COR. The results were calculated as a ratio of phosphorylated STAT6/total STAT6 has been calculated.

2.6.2 Western blotting for IL-13

Samples of IL-13 final concentration (10ug/ml), CE final concentration (2.5mg/ml) or a combination of both were prepared in BEBM and incubated for various time points (15minutes, 45minutes, 2hours, 6hours, and 24hours). Red loading buffer (BioLabs) and 30x Reducing agent (DTT) (BioLabs) were then added for each sample, 90:10 units respectively, before placing on hot plate 100^{o}C for 5 minutes. The samples were then ready to be transformed to 16% PAGE (polyacrylamide gel electrophoresis) or stored in -80^{o}C till the running time.

To do western blot, 16% PAGE gels loaded in Bio-Rad mini Protean Cell and connected to Bio-Rad PowerPac200 power supply used. Polyclonal goat anti-human IL-13 Antibody (R&D cat # AF-213-SP) was used to detect IL-13 protein. The membranes were imaged using Odyssey Infrared Imager (LI-COR) and analyzed by Image Studio software (5.2.5) from LI-COR

2.7. Quantification of trypsin-like activity

CE trypsin-like activity was measured by using fluorogenic peptide substrate butoxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin (AMC)·HCl (QAR-AMC) from BACHEM (cat # I-1550.0005). A standard of Trypsin from bovine pancreas from Sigma (cat # T1426), with known BAEE unit/mg, in PBS was used. On Ice, in a 96 well flat bottom plate reconstituted CE and Trypsin were placed then QAR-AMC substrate was added. The plate was then incubated for 10 and 20 minutes at room temperature before using Microplate Fluorescence Reader (BIO-TEK) to measure QAR-AMC substrate fluoresce. A standard curve was then generated by GraphPad Prism 5 to calculate CE trypsin-like activity. Similar assay was used to measure CE trypsin like activity in other studies (111).

Chapter 3. Introduction

Airway epithelial cells are known to respond to Th2 cytokines with number of effects. IL-13 and IL-4 are known to induce eotaxin production from airway epithelial cells (122), which suggest that those Th2 cytokines may mediate eosinophil recruitment in the airways by epithelial cell activation. Common allergens also have effects on epithelial cells through activation of multiple epithelial cell receptors. For example, development of allergy to HDM allergens is mediated by the activation of epithelial TLR-4 (51).

In our lab we have been interested in understanding the effects of cockroach allergens on airway epithelial cells through PAR-2 activation. Previous work in our lab showed that mucosal exposure to *B. germanica* cockroach extract induces AHR and airways high inflammatory cell count, primarily eosinophils, were completely inhibited by using anti-PAR-2 blocking antibody (111). Another student in our lab who was studying cytokine/chemokine production from airway epithelial cells by allergens in vitro noticed that *B. germanica* CE interfered with IL-13 induced CCL26 expression, and in this project I have followed this observation.

To investigate the role of CE in the interference of IL-13 induced CCL26 expression, BEAS-2B and NHBE cells were used. Results showed time and dose dependent inhibition of IL-13 mediated CCL26 mRNA and protein by CE (explanatory figure 3A). This inhibitory effect by CE found to be mediated by CE proteolytic activity. HICE and CE incubated with trypsin protease, type of serine proteases, inhibitors abolished the inhibitory effect of CE on IL-13 induced CCL26 mRNA. Consistence with the protease inhibitor results, there was below detection level trypsin like activity in HICE and CE pre-incubated with trypsin inhibitors by trypsin-like activity quantification method. CE proteolytic activity did not change the expression of IL-13Rα1 on BEAS-2B cell

surface and inhibited the upregulate IL-13Rα2 mRNA in BEAS-2B cells. Results also revealed that PAR-2 activation did not mimic the CE inhibitory effect on IL-13 induced CCL26 expression. Data also showed that longer time window between adding CE to IL-13 resulted in less effect of CE on IL-13 induced CCL26 mRNA expression. The inhibitory effect of CE on IL-13 induced CCL26 did not result in significant reduction of CCL26 mRNA stability nor with significant inhibition of IL-13 induced STAT-6 phosphorylation.

CE proteolytic activity was shown to be responsible for the early onset of IL-13 protein degradation, which increased with longer incubation of IL-13 with CE. Surprisingly, IL-13 protein degradation was also detected in case of IL-13 incubation with HICE or CE perincubated with aprotinin.

Results:

3.1 IL-13 induced CCL26 expression in airway epithelial cells

To study the ability of IL-13 to induce CCL26 mRNA in BEAS-2B airway epithelial cells, we cultured BEAS-2B cells with various concentrations of IL-13 (4, 20, or 100ng/ml) in starvation media, BEBM plus 1% Penicillin/Streptomycin, for 2, 6 and 24 h (Figure 3.1A). A pilot experiment was performed to identify the optimal time and concentration of IL-13 to induce CCL26 mRNA expression. This experiment (n=1) showed an over 50-fold induction of CCL26 after at least 6 hours and 20 ng/ml of IL-13. Another experiment was done using (4, 20, 100ng/ml) of IL-13 for 24 hours (n=2) (Figure 3.1B). Since 20 ng/ml of IL-13 was giving over 50-fold induction of CCL26 mRNA in 24 hours, we continued all our experiments using 20 ng/ml IL-13. There was high range of results variability and that might be due to cell confluency variability at stimulation time, temperature, or time variabilities in PCR process . However, 20 ng/ml of IL-13

induced 57.71 ± 20.19-fold increase in CCL26 mRNA expression (n=6). To show significant results and overcome the indicated variabilities in some data, the percentage of IL-13 stimulated expression was calculated and explained where it is applied.

3.2. CE inhibited the expression of IL-13-induced CCL26 mRNA and protein in BEAS-2B cells

BEAS-2B cells were cultured with CE (5ug/ml) for 24 hours. CE had minor effect in CCL26 mRNA induction. CE-induced CCL26 mRNA expression was detected in all experiments, but the level of induction compared to resting cells was very low (Figure 3.2A). However, CE induced CCL26 mRNA level was not significant in case of analysing results with one-way ANOVA and multiple comparisons. Besides, CE did not induce detectable levels of CCL26 protein as it is shown in the CCL26 protein release results.

To study the interactions between CE and IL-13 for CCL26 induction we activated BEAS-2B cells with IL-13 (20ng/ml) or CE (0.2, 1, 5ug/ml) alone or together for 24hrs before harvesting. Higher levels of CE caused more inhibition of IL-13-induced CCL26 mRNA (expression of IL-13 induced CCL26 mRNA with 0.2ug/ml of CE > the expression of IL-13 induced CCL26 mRNA with 5ug/ml of CE) (Figure 3.2B). However, the difference between expression of IL-13 induced CCL26 mRNA and inhibition caused by the three different doses of CE was not statistically significant over three experiments, possibly because of the observed high variability between experiments. To overcome the variability, the effect of CE on IL-13 induced CCL26 expression was calculated in percentage of IL-13 stimulated expression (Figure 3.2C). To do this, the absolute number of IL-13 induced CCL26 expression (fold expression of stimulated over unstimulated cells) of each experiment was considered to be 100%, and the absolute number of IL-13 induced CCL26

expression (fold) in the presence of CE was calculated in percentage over the IL-13 induced CCL26 expression.

In light of the previous results, 5ug/ml of CE was the dose causing complete inhibition of IL-13 induced CCL26 mRNA in 24 hours in response to 20ng/ml stimulation of IL-13. To examine the inhibition of IL-13 induced CCL26 mRNA by CE in different time points, BEAS-2B cells activated with either IL-13 (20ng/ml), CE (5ug/ml) or both for 2, 6, or 24 hours. Higher IL-13 induced CCL26 mRNA was detected with longer time of incubation (induction in 2 hours < 24 hours). Moreover, CE inhibited IL-13 induced CCL26 mRNA expression with longer time of incubation (inhibition in 2 hours < 24 hours) (Figure 3.2D). Inhibition of IL-13 induced CCL26 in 2, 6, or 24 hours is shown in percentage of IL-13 stimulated expression in figure 3.2E.

To understand whether CE also decreases IL-13 induced CCL26 protein release, we measured IL-13 induced CCL26 protein release in the supernatants of IL-13 and/or CE activated cells using ELISA. BEAS-2B cell activation with IL-13 (20ng/ml) for 24 hours led to significant release of CCL26 protein. However, cells incubated with CE, and IL-13 plus CE had no detectable CCL26 protein level (Figure 3.2F).

3.3 CE inhibited IL-13-induced CCL26 mRNA expression and protein release from Normal Human Bronchial Epithelial cells (NHBE)

To validate the inhibition of IL-13 induced CCL26 by CE in normal human cells, NHBE cells were cultured and stimulated with either IL-13 (20ng/ml), CE (5ug/ml), or both. Cells and supernatants were harvested 24 hours later for IL-13 induced CCL26 mRNA and protein measurements respectively. IL-13 also induced CCL26 mRNA expression and protein release in

NHBE. Results showed CE inhibited IL-13 induced CCL26 mRNA (Figure 3.3A), and protein (Figure 3.3B) in NHBE cells.

3.4. The effect of CE on IL-13 induced CCL26 mRNA expression is serine, specifically trypsin-like protease dependent

It was shown that airway allergic reactions to CE is proteases dependent (109, 111). To examine the effect of CE proteolytic activity on IL-13 induced CCL26, CE was heated at 65°C for 30 min to inactivate CE proteases (111). Cells were incubated with IL-13 (20ng/ml), IL-13 plus CE (5ug/ml), or IL-13 plus HICE (5ug/ml). In contrast to CE, HICE did not prevent CCL26 induction by IL-13 (Figure 3.4A). Heating CE abolished the inhibitory activity of CE on IL-13 mediated CCL26 mRNA induction. In percentage of IL-13 stimulated expression, there was 99.79-97.45% inhibition of IL-13 mediated CCL26 mRNA induction with CE, and 0-48.98% of inhibition with HICE (Figure 3.4B).

To identify the CE protease(s) responsible for the inhibition of the IL-13 dependant CCL26 induction, CE was pre-incubated with three different protease inhibitors E-64 (10uM), a cysteine protease inhibitor, aprotinin (2ug/ml), serine protease inhibitor mainly for trypsin-like activity, and pepstatin A (1ug/ml), an acid proteases inhibitor. In contrast with CE incubated with cysteine and acid protease inhibitors, CE incubated with the serine protease inhibitor aprotinin, did not inhibit IL-13 induced CCL26 mRNA expression (Figure 3.4C). The three inhibitors E-64, aprotinin, and pepstatin A were diluted in DMSO, DDH2O, and methanol and acetic acid respectively. To confirm that those diluents did not affect IL-13 induced CCL26 induction, a control experiment was performed where the same concentrations of DMSO, DDH2O, and methanol acetic acid used in the inhibitors experiments were added (Figure 3.4D). The control experiment was done one time and showed no effect on the IL-13 induced CCL26 or CE proteolytic activity beside that the used

diluents to aliquot each proteases inhibitor did not exceed the toxicity level (less than 0.1 solvents in 1 ml of media).

To verify that IL-13 induced CCL26 inhibition is CE trypsin-like activity dependent, a trypsin inhibitor from Glycine max (soybean), Soybean Trypsin Inhibitor (SBTI), was used. CE incubated with SBTI did not inhibit IL-13 induced CCL26 expression (Figure 3.4E).

HICE and CE incubated with SBTI have also failed to induce CE allergic sensitization and airway inflammation in mice model of allergic airway inflammation (111).

3.4.1 CE trypsin-like activity by using QAR-AMC substrate assay

To validate and assess the trypsin-like activity of CE, we measured CE trypsin-like activity by using a QAR-AMC substrate-based assay. In constant with the cell culture results the trypsin-like activity of HICE or CE pre-incubated with aprotinin or SBTI was below the detection limit. However, 5ug/ml of CE contains 0.4-0.5 BAEE (Nα-Benzoyl-L-arginine ethyl ester) units, which is equivalent to 80-100 BAEE units in 1mg/ml of CE (Figure 3.4F). Trypsin-like activity measurements for three experiments are shown in (Table 3.4).

3.4.2 Trypsin did not mimic the CE effect on IL-13 induced CCL26 mRNA expression

Based on the CE trypsin-like activity results, we hypothesised that trypsin with equivalent BAEE activity as in 5ug/ml of CE will mimic the CE effect on IL-13 induced CCL26 expression. To test this BEAS-2B cell were stimulated with (20ng/ml) of IL-13 plus 30ng/ml or 40ng/ml of trypsin to cover the maximum range of trypsin activity. Trypsin did not inhibit IL-13 induced CCL26 mRNA expression (Figure 3.4G). This suggests that CE has a trypsin-like activity that differs from the activity of the pancreatic trypsin (Trypsin from bovine pancreas) used in this experiment.

From the previous results we know that the inhibitory effect of CE on IL-13 induced CCL26 is trypsin-like activity dependent. We hypothesized that this protease activity could modify or cleave the IL-13 receptor on the BEAS-2B cell surface, activate PAR-2, decrease CCL26 mRNA stability, or even degrade the IL-13 protein. So, by using one or more of the stated mechanisms CE inhibits IL-13 induced CCL26 expression. The experiments below examined the proposed hypotheses.

3.5 CE had no effect on IL-13Rα1 expression

IL-13 receptor is a heterodimer of IL-4Rα chain and IL-13Rα1 chain. There is an additional IL-13 receptor that consists of the IL-13Rα2 chain. This α2 receptor is a decoy receptor that has no known signalling pathway but regulates the IL-13 response (75). It was also found to be present in intracellular pools in multiple cell types and to be soluble in extracellular fluid (75, 77). We hypothesized that CE proteolytic activity could cleave the IL-13Rα1 and by that mechanism CE would inhibit the IL-13Rα1 activation on the surface of BEAS-2B cells and consequently inhibit IL-13 induced CCL26 expression. Flow cytometry analysis showed that CE did not change IL-13Rα1 expression on the BEAS-2B cell surface. Median fluorescent intensity and the percentage of BEAS-2B cell expression of IL-13Rα1 on the cell surface were similar in CE treated and untreated cells (Figure 3.5 A, B, C).

Then we examined whether CE up-regulates IL-13Rα2 expression, since this mechanism could prevent IL-13 protein from activating IL-13 Rα1 and would decrease CCL26 induction. Results showed up-regulation of the decoy receptor mRNA in IL-13 activated cells, which has been shown before and considered as feedback regulation to IL-13 stimulation response (75). CE blocked the upregulation of IL-13Ra2 by IL-13 in cells stimulated with IL-13 plus CE (Figure 3.5D).

3.6 PAR-2 activation did not prevent CCL26 induction

CE is known to activate PAR-2 (111). To study whether this ability of CE is important for its ability to inhibit IL-13 mediated CCL26 induction we activated epithelial cells using PAR-2 activating peptides. BEAS-2B cells were cultured and stimulated with either IL-13, IL-13 plus PAR-2 activated peptide (AP) (SLIGKV-NH2) (250uM), or IL-13 plus PAR-2 control peptide (CP) (VKGILS-NH2) (250uM) (Figure 3.6A). Due to results inconsistency, we have not further pursued using those peptides. The experiment repeated later by using other PAR-2 peptides (121), AP (SLIGRL-NH2) (50uM) and CP (LRGILS-NH2) (50uM). Results showed IL-13 plus PAR-2 AP induced IL-13 dependent CCL26, PAR-2 activation with AP did not mimic CE inhibitory effect, which reveal that the inhibition of IL-13 induced CCL26 mRNA expression by CE is more likely PAR-2 independent (Figure 3.6B).

3.7. LPS did not mimic the CE effect on IL-13 induced CCL26 mRNA expression

It has been shown that CE contains LPS contamination (123), so we aimed to examine whether LPS may be responsible for the observed inhibition by CE. 10ug/ml of LPS or heated LPS (HLPS) were used in cells activation, HLPS was used to examine whether heat will abolish the inhibitory effect of LPS in comparison with HICE. Data showed no significant inhibition of IL-13 induced CCL26 by LPS (Figure 3.7A) in contrast of complete inhibition of IL-13 induced CCL26 by CE. Percentage of IL-13 stimulated expression results of LPS are shown (Figure 3.7B). To validate LPS activity, IL-8 mRNA expression after LPS stimulation was measured (Figure 3.7C). It is known that LPS induces IL-8 release in epithelial cells (124). Measuring LPS induced IL-8 (n=2) showed that LPS induced IL-8 mRNA in BEAS-2B cells which is expected and constant with the what was shown in previous study (124).

3.8 Longer time window between adding CE to cells already activated with IL-13 resulted in less inhibitory effect of CE on IL-13 induced CCL26 mRNA expression

To better understand the CE-mediated inhibition of IL-13 induced CCL26 expression, BEAS-2B cells were stimulated with IL-13 and CE sequentially. The cells were stimulated with IL-13 for 24 hours and CE was added either at the same time with IL-13 or 90 minutes, 6 h, or 12 h later. The results showed that the inhibitory effect of CE on IL-13 induced CCL26 was less evicent with increasing the time interval between stimulation with IL-13 and addition of CE (Figure 3.8A). In terms of percentage of IL-13 stimulated expression, adding CE to IL-13 90 minutes, 6 hours, and 12 hours later inhibited 98.44%±0.69, 85.43%±6.31, and 73.33%±9.20 of the CCL26 induction respectively (Figure 3.8B).

3.9 IL-13 removal resulted in loss of CCL26 mRNA induction

To examine the effect of sequential stimulation of IL-13 and CE on IL-13 induced CCL26 mRNA, 90 minutes time window and stimulation removal were used in different conditions as described in the methodology section. Removal of IL-13 after 90 minutes stimulation resulted in very low induction of CCL26 mRNA (3.3-fold) 24 hours after addition of IL-13. Adding CE 90 minutes later to IL-13 was enough to cause complete inhibition of IL-13 induced CCL26 mRNA (Figure 3.9). These observations from one experiment was not followed since the removal of IL-13 was not the best way to do the sequential stimulation. Removal of IL-13 showed similar effect with what has been shown previously for the removal of IL-4 (125).

3.10 CE effect on IL-13 induced CCL26 mRNA stability

The time window between adding CE later to IL-13 resulted in less effect of CE on IL-13 induced CCL26 mRNA expression, suggested that CE could be affecting IL-13 induced CCL26 mRNA

expression by decreasing its stability or in other words increasing the rate of CCL26 mRNA degradation. To examine this hypothesis IL-13 induced CCL26 mRNA rate of degradation was examined in the presence of actinomycin D. Actinomycin D is known to inhibit cellular transcription by intercalating with DNA and inhibiting mRNA synthesis (126, 127). Cells were stimulated with IL-13 for 12 hours then CE was added for 2 hours followed by actinomycin D for (1, 3, or 6 hours). There was no significant effect on IL-13 induced CCL26 mRNA rate of degradation (Figure 3.10A). Neither adding CE for (1, 3, or 6 hours) after IL-13 stimulation and actinomycin D treatment did not significantly increase CCL26 mRNA rate of degradation (Figure 3.10B).

3.11 IL-13 protein degradation by CE

As the above results showed that IL-13 induced CCL26 inhibition is mediated by CE proteases activity, we sought to know whether CE proteolytic activity digested IL-13 protein. To test this hypothesis, IL-13 was incubated with either CE, HICE, or CE pre-incubated with aprotinin. We also examined IL-13 protein auto-degradation by incubating IL-13 alone. The samples were then analyzed by western blot for the presence of full length IL-13 or degradation products. Results showed early onset of IL-13 degradation by CE within 15 min, which increased with longer incubation times. Surprisingly, IL-13 protein degradation was also detected with visible and detactable degradation products in case of IL-13 incubation with HICE or CE preincubated with aprotinin (Figure 3.11A). To examine if IL-13 protein degradation is occurring because of CE proteases we aimed to use boiled CE (100°C for 30 minutes). Most proteins become unfunctional upon boiling at 100°C for 30 minutes, which cause protein unfolding. In contrast to CE, boiled CE did not cause degradation of IL-13 protein even with 24 hours of incubation (Figure 3.11B). Boiled CE was used in one experiment (western blot) and showed expected effect as boiling causes protein

unfolding and therfore lose of functional proteins in CE. This experiment will be followed by using boiled CE for cell stimulation, but results are not available yet.

3.12 CE had no significant effect on IL-13 induced STAT-6 phosphorylation

It is known that IL-13 induces expression of CCL26 mRNA by STAT-6 activation dependent mechanism (128). To examine the effect of CE on the IL-13 induced STAT-6 phosphorylation, BEAS-2 cells were activated with IL-13 and CE prior to harvesting, protein extraction and detection of total and phosphorylated STAT (pSTAT-6) done using western blotting. CE did not significantly inhibit IL-13 induced STAT-6 phosphorylation (Figure 3.12A, 3.12B). In percentage of inhibition, figure 3.11C shows the inhibition of STAT-6 phosphorylation by CE in percentage.

3.13 HDM did not inhibit IL-13 mediated CCL26 expression

HDM is known to have proteases activity (116). To study the effect of HDM and compare it with the CE inhibitory effect on IL-13 induced CCL26 mRNA, we used HDM instead of CE to stimulate BEAS-2B cells. BEAS-2B cells were stimulated with IL-13 (20ng/ml), HDM (10, 25, or 50ug/ml) or both for 24 hours before harvesting. Results showed that HDM did not inhibit IL-13-induced CCL26 expression (Figure 3.13). Using the trypsin like activity assay we found that the HDM extract at the concentrations used had below detection limit trypsin like activity.

3.14 No significant inhibition of IL-4 induced CCL26 mRNA expression by CE

IL-13 receptor is a heterodimer of IL-4Rα subunit and IL-13Rα1 subunit and this receptor can be activated by IL-13 and IL-4 protein ligation (75). We aimed to examine the effect of CE on the expression of IL-4 induced CCL26 mRNA and if that will mimic the CE inhibitory effect on IL-13 induced CCL26. BEAS-2B cells were cultured and activated with IL-4 (20ng/ml), CE (5ug/ml)

or both then harvested 24 hours later. CE did not inhibit IL-4 induced CCL26 mRNA (Figure 3.14).

Figures:

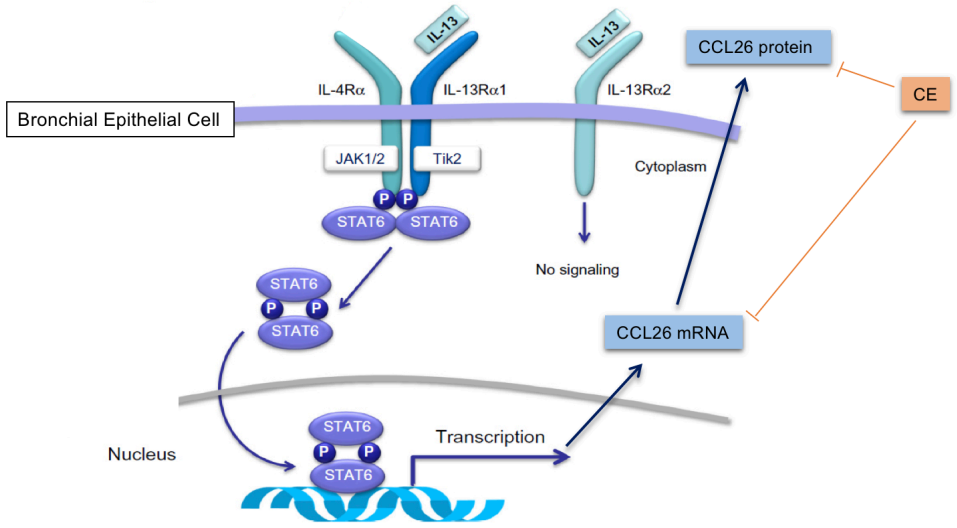
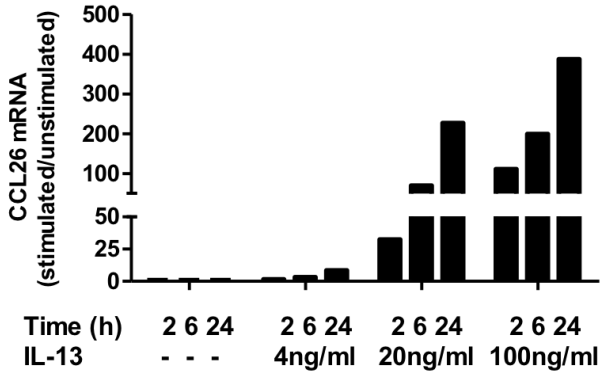


Figure 3.A: Inhibition of IL-13 induced expression of CCL26 mRNA and protein by CE in airway epithelia cells. This figure is modified from Vatrella A, et al, *Dupilumab: A novel treatment for asthma. J Asthma Allerg* 2014;7:123-130, with permission from the publisher: DOVE Medical Press

A)



B)

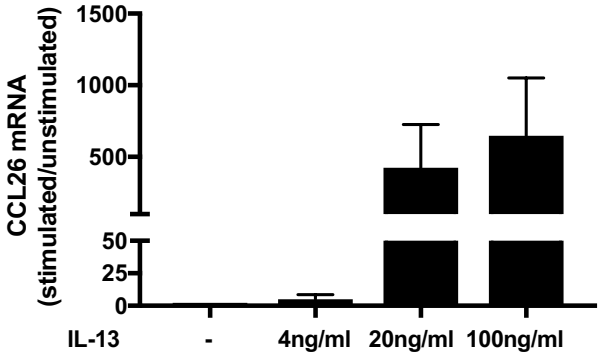
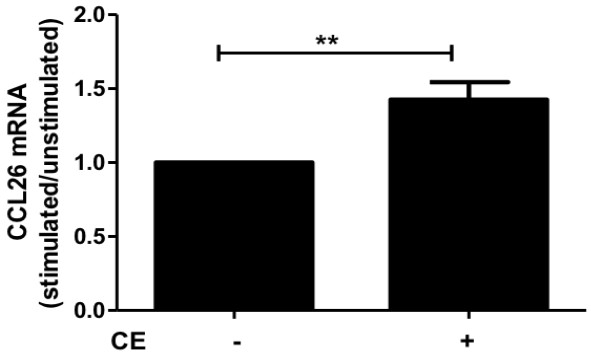
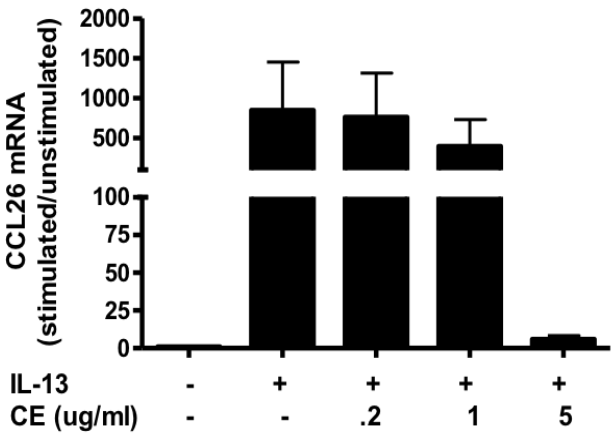


Figure 3.1: A) IL-13 induced CCL26 mRNA expression in response to dose (4, 20, 100ng/ml) and time (2, 6, 24 hours). Results for n=1. **B)** IL-13-induced CCL26 mRNA expression in response to (4, 20, 100ng/ml) of IL-13 for 24 hours. Results for n=2.

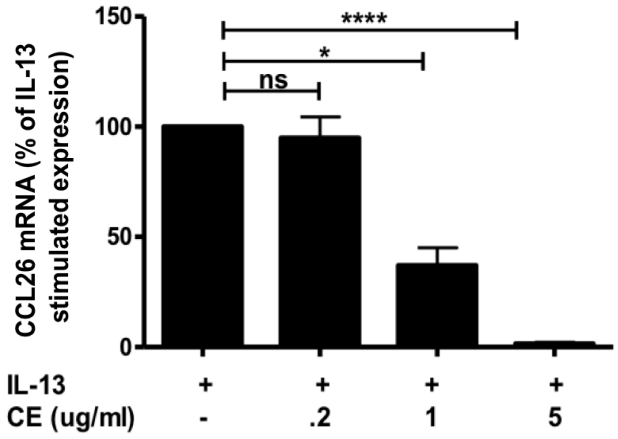
A)


CCL26 mRNA
(stimulated/unstimulated)
2.0
1.5
1.0
0.5
0.0
**
CE
-
+

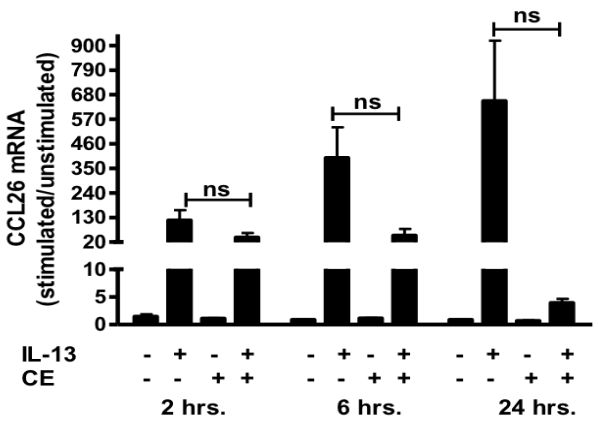
B)


CCL26 mRNA
(stimulated/unstimulated)
2000
1500
1000
500
100
75
50
25
0
IL-13
CE (ug/ml)
- + + + +
- - .2 1 5

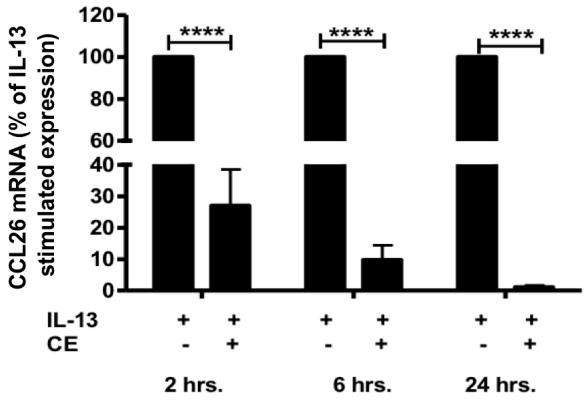
C)


CCL26 mRNA (% of IL-13
stimulated expression)
150
100
50
0
****
*
ns
IL-13
CE (ug/ml)
+ + + +
- .2 1 5

D)



E)



F)

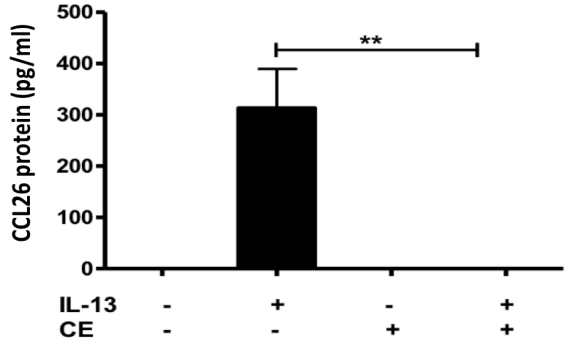


Figure 3.2: CE inhibited the expression of IL-13-induced CCL26 mRNA and protein in BEAS-2B. **A)** CE induced CCL26 mRNA in 24 h, n=9 **B)** CE inhibited IL-13 induced CCL26 mRNA induction, n=3 **C)** CE inhibited IL-13 induced CCL26 mRNA expression presented as percentage of IL-13 stimulated expression **D)** 2, 6, 24 hours inhibition of IL-13 induced CCL26 mRNA by CE, n=3 **E)** Inhibition of IL-13 induced CCL26 in percentage of IL-13 stimulated expression at 2, 6, 24 hours, n=3 **F)** IL-13 induced CCL26 protein inhibition by CE, n=3. Results shown are mean ± SEM of values. P value and statistical analysis have been done by T-test (3.2 A), two-way ANOVA (3.2 D and E) or one-way ANOVA (3.2 B, C, and F).

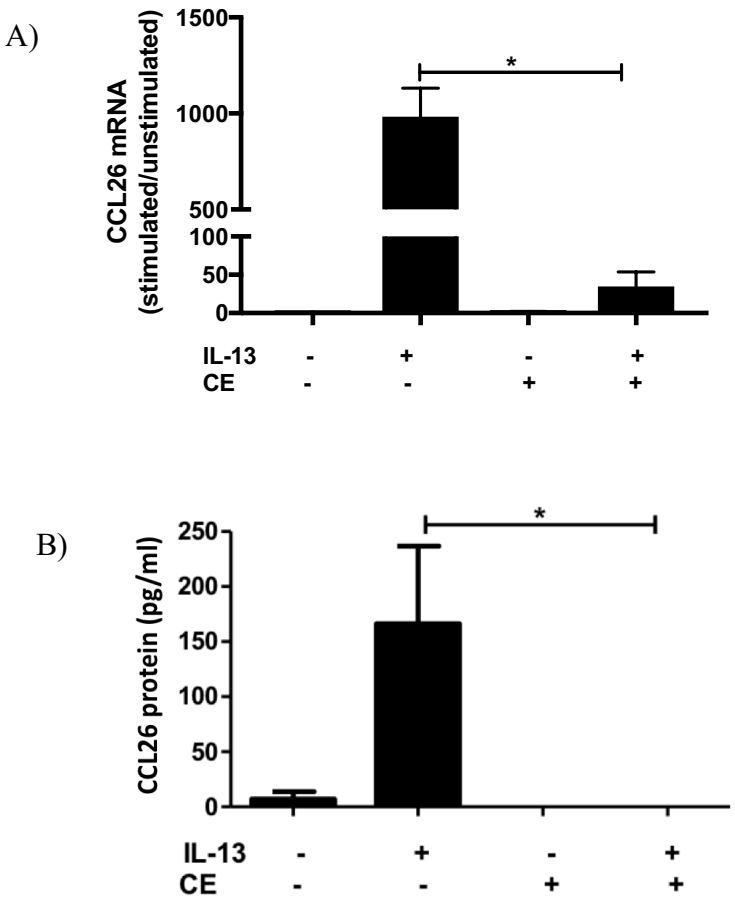
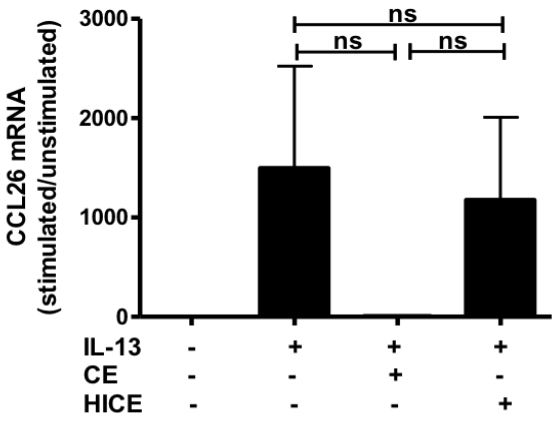
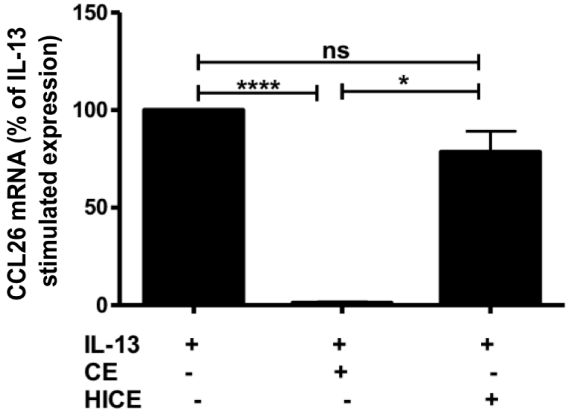


Figure 3.3: CE inhibited IL-13-induced CCL26 mRNA expression and protein release from Normal Human Bronchial Epithelial cells (NHBE) **A)** IL-13 induced CCL26 mRNA, n=3 **B)** IL-13 induced CCL26 protein inhibition by CE, n=3. Results shown are mean ± SEM of values. P value and statistical analysis have been done by one-way ANOVA.

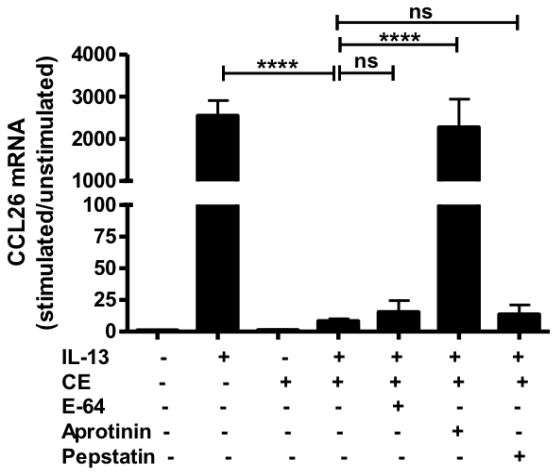
A)


CCL26 mRNA
(stimulated/unstimulated)
3000
2000
1000
0
ns
ns
ns
IL-13 - + + +
CE - - + -
HICE - - - +

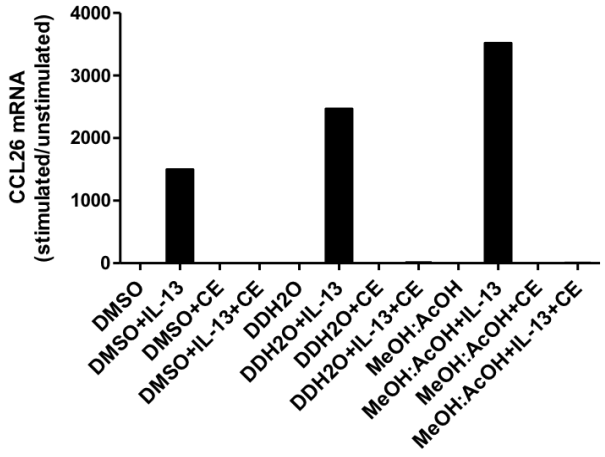
B)


CCL26 mRNA (% of IL-13
stimulated expression)
150
100
50
0
ns
****
*
IL-13 + + +
CE - + -
HICE - - +

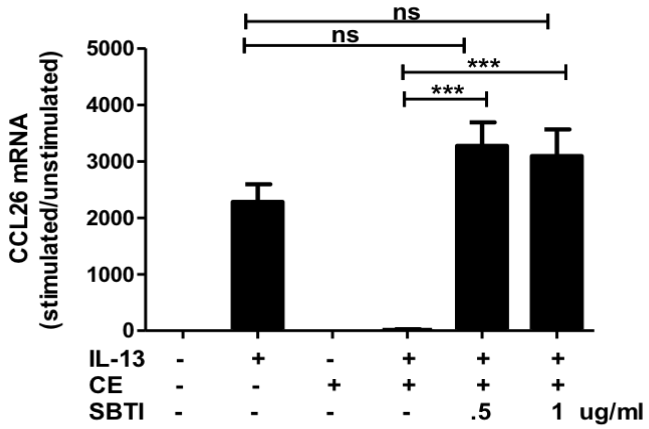
C)


CCL26 mRNA
(stimulated/unstimulated)
4000
3000
2000
1000
100
75
50
25
0
ns
****
****
ns
IL-13 - + - + + + +
CE - - + + + + +
E-64 - - - - + - -
Aprotinin - - - - - + -
Pepstatin - - - - - - +

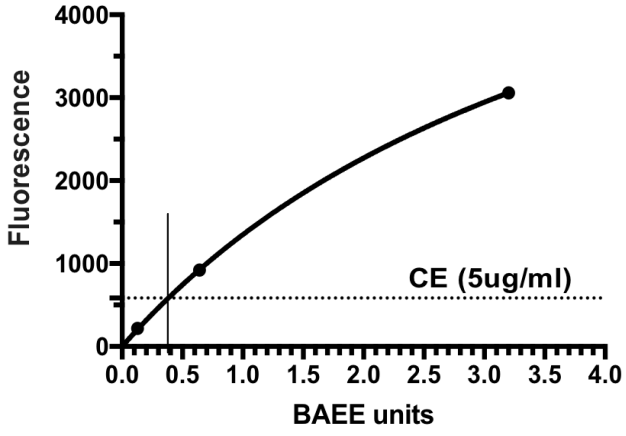
D)


CCL26 mRNA
(stimulated/unstimulated)
4000
3000
2000
1000
0
DMSO
DMSO+IL-13
DMSO+CE
DMSO+IL-13+CE
DDH2O
DDH2O+IL-13
DDH2O+CE
DDH2O+IL-13+CE
MeOH:AcOH
MeOH:AcOH+IL-13
MeOH:AcOH+CE
MeOH:AcOH+IL-13+CE

E)


ns
ns
***
***
CCL26 mRNA
(stimulated/unstimulated)
5000
4000
3000
2000
1000
0
IL-13 - + - + + +
CE - - + + + +
SBTI - - - - .5 1 ug/ml

F)


Fluorescence
4000
3000
2000
1000
0
CE (5ug/ml)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0
BAEE units

G)

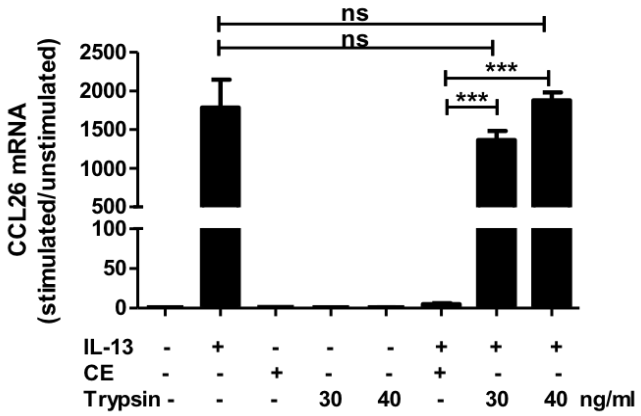
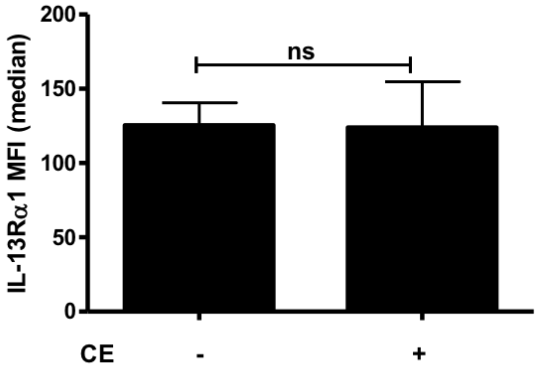
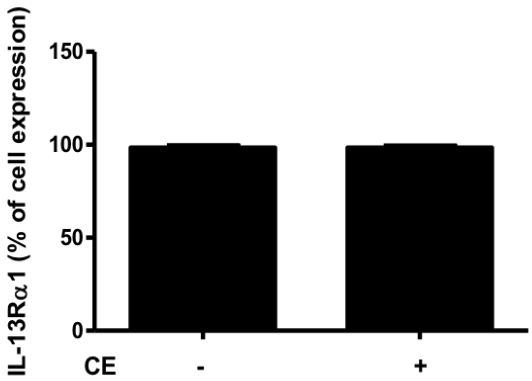


Figure 3.4: CE trypsin-like activity and trypsin effect on IL-13 induced CCL26. **A)** Heating CE abolished the inhibitory activity of CE on IL-13 induced CCL26 mRNA expression, n=3. **B)** Shows the effect of HICE on IL-13 induced CCL26 in percentage of IL-13 stimulated expression, n=3. **C)** CE incubated with serine protease inhibitor, aprotinin, did not inhibit IL-13 induced CCL26 mRNA expression, n=3. **D)** Control experiment for protease inhibitors, n=1 **E)** CE incubated with trypsin-like activity inhibitor, SBTI, did not inhibit IL-13 induced CCL26 mRNA expression, n=3. **F)** CE trypsin-like activity by using QAR-AMC substrate assay. The BAEE unit measurement is for one experiment representative of three **G)** Trypsin did not mimic CE effect on IL-13 induced CCL26 mRNA, n=3. Results shown are mean ± SEM of values. P value and statistical analysis have been done by one-way ANOVA.

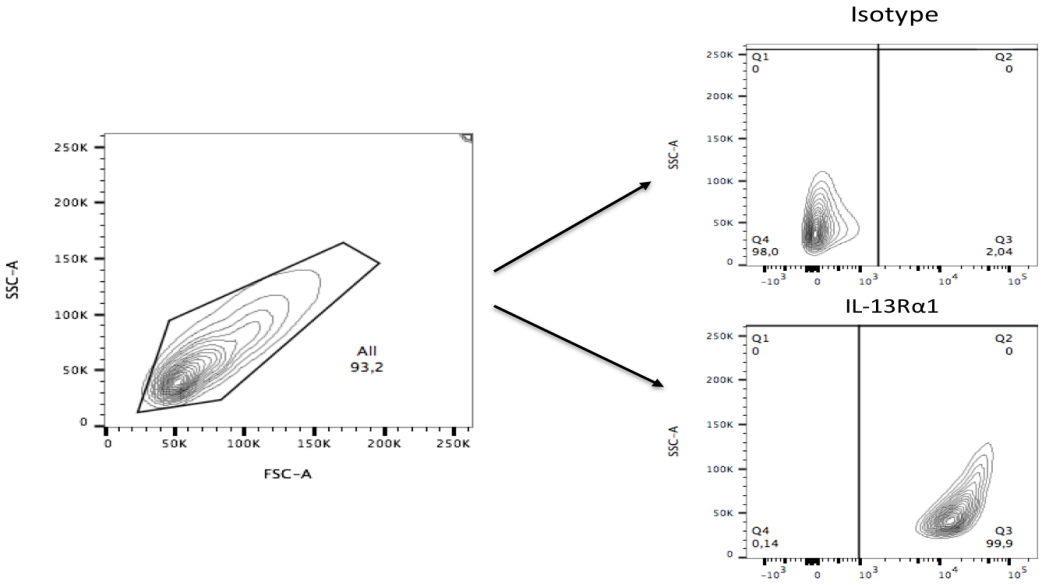
A)


ns
IL-13Rα1 MFI (median)
200
150
100
50
0
CE
-
+

B)


IL-13Rα1 (% of cell expression)
150
100
50
0
CE
-
+

C)


SSC-A
FSC-A
All
93,2
Isotype
Q1
0
Q2
0
Q4
98,0
Q3
2,04
IL-13Rα1
Q1
0
Q2
0
Q4
0,14
Q3
99,9

D)

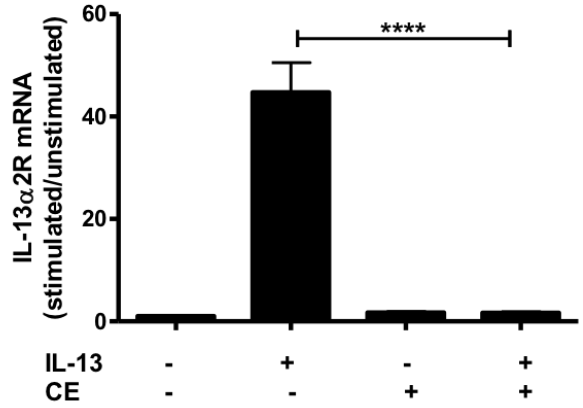


Figure 3.5: CE had no effect on IL-13Rα1 expression. **A)** IL-13Rα1 expression on BEAS-2B cell surface by flow cytometry. Results show median fluorescent intensity (MFI) of IL-13Rα1, n=3. **B)** MFI of IL-13Rα1 by percentage of inhibition, n=3. **C)** IL-13Rα1 expression on BEAS-2B cell surface (contour plot) followed by gating of isotype and IL-13Rα1 labeled cells. Contour plot is for one experiment representative of three. **D)** Induction of IL-13Rα2 mRNA in BEAS-2B, n=3. Results shown are mean ± SEM of values. P value and statistical analysis have been done by one-way ANOVA.

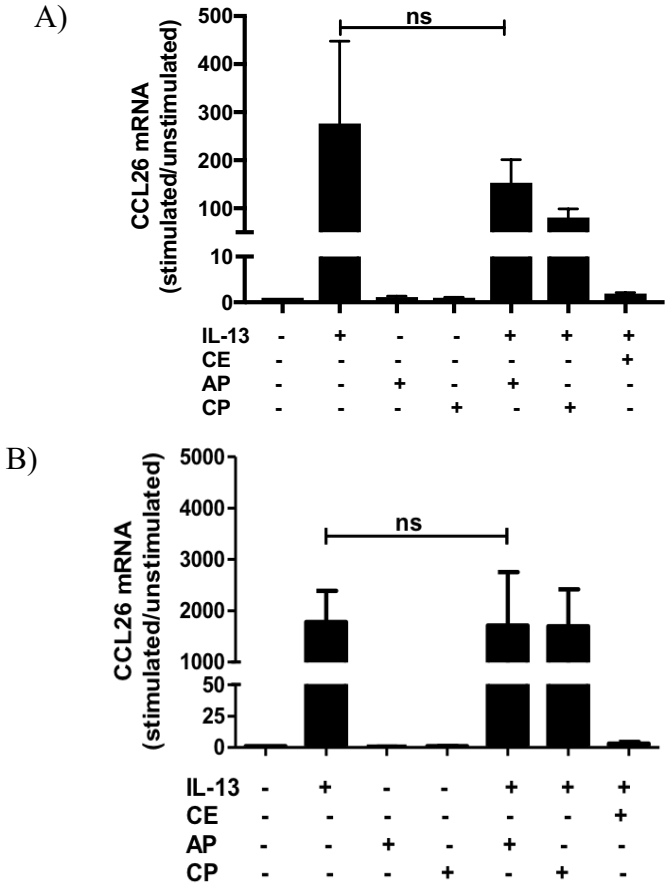
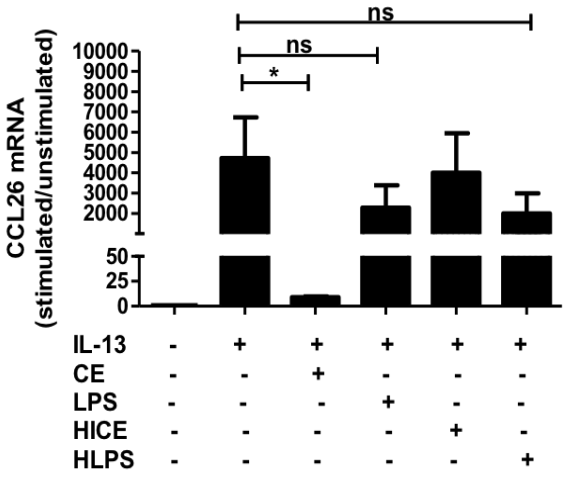
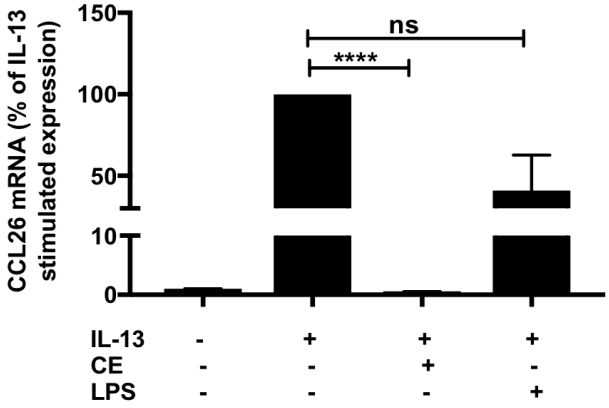


Figure 3.6: PAR-2 activation did not prevent IL-13-induced CCL26 mRNA expression. **A)** IL-13 induced CCL26 mRNA in IL-13, CE, and PAR-2 peptides treated cells (inconsistent results) **B)** IL-13 induced CCL26 mRNA in IL-13, CE, and PAR-2 peptides treated cells. Results shown are mean ± SEM of values from n=3. P value and statistical analysis have been done by one-way ANOVA.

A)



B)



C)

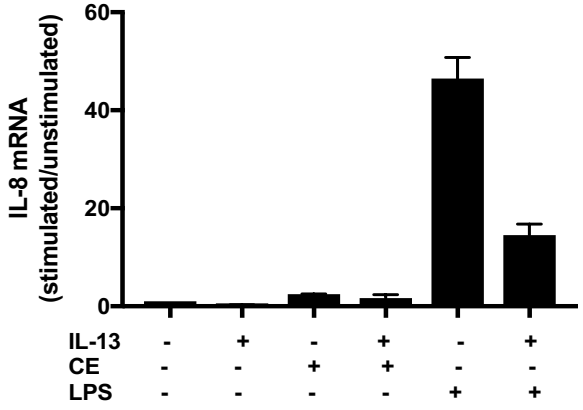


Figure 3.7: A) LPS did not mimic the CE effect on IL-13 induced CCL26 mRNA expression, n=3 B) IL-13 induced CCL26 mRNA in percentage of IL-13 stimulated expression, n=3. **C)** IL-8 mRNA induction by LPS stimulation, n=2. Results shown are mean ± SEM of values. P value and statistical analysis have been done by one-way ANOVA.

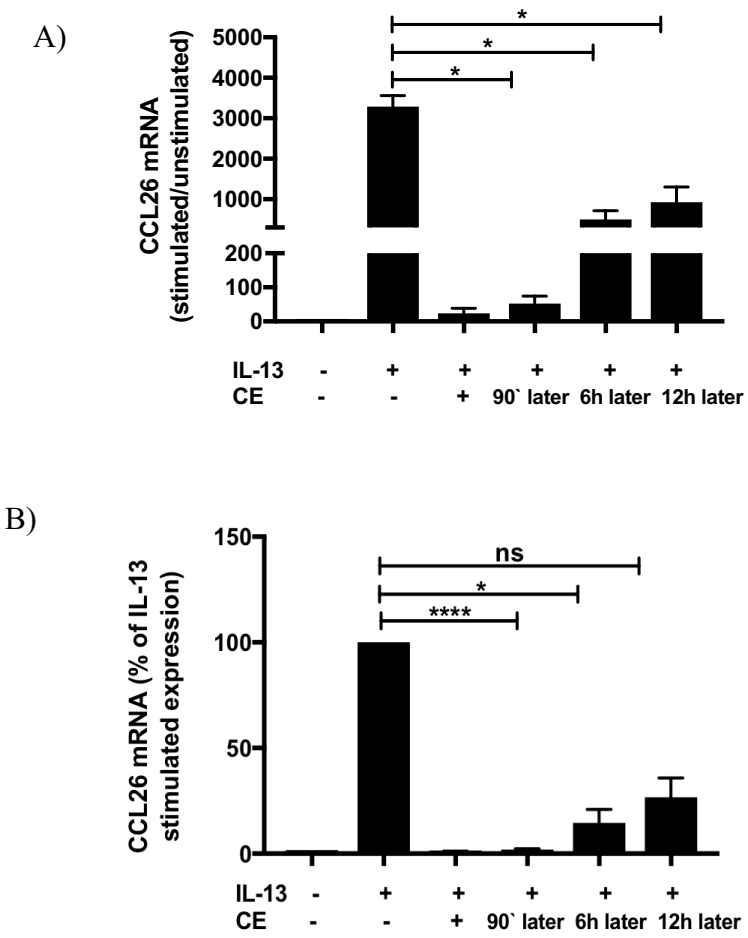


Figure 3.8: Longer time window between adding CE to cells already activated with IL-13 resulted in less inhibitory effect of CE on IL-13-induced CCL26 mRNA expression. **A)** Less inhibitory effect of CE on IL-13 induced CCL26 mRNA associated with longer time window between adding CE to IL-13 (90 minutes, 6 hours, or 12 hours), n=3. **B)** Shows the percentage of IL-13 stimulated expression in association with times between adding CE to IL-13, n=3. Results shown are mean ± SEM of values. P value and statistical analysis have been done by one-way ANOVA.

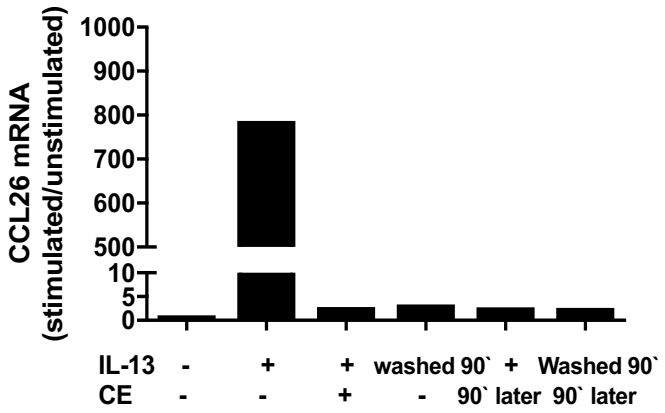
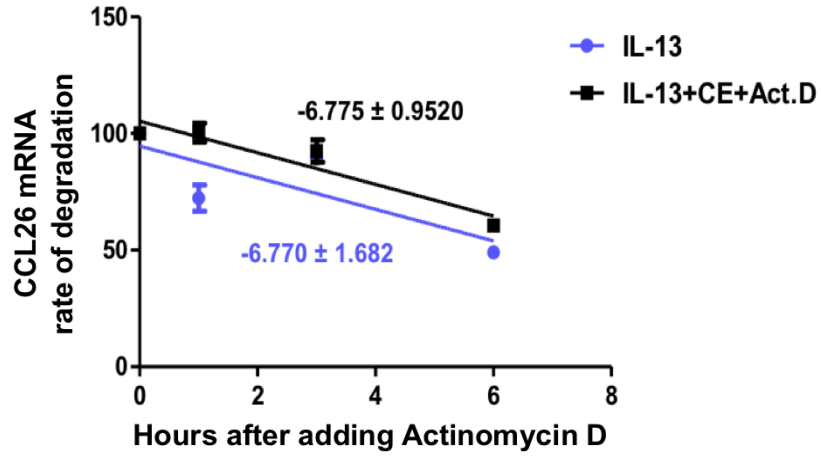


Figure 3.9: Effect of washing IL-13 on the expression of IL-13-induced CCL26 mRNA. Removal of IL-13 resulted in loss of CCL26 mRNA induction, n=1.

A)



B)

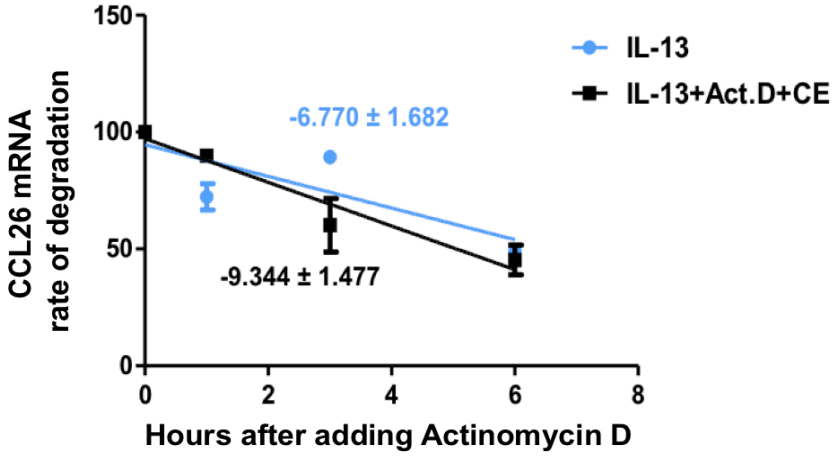
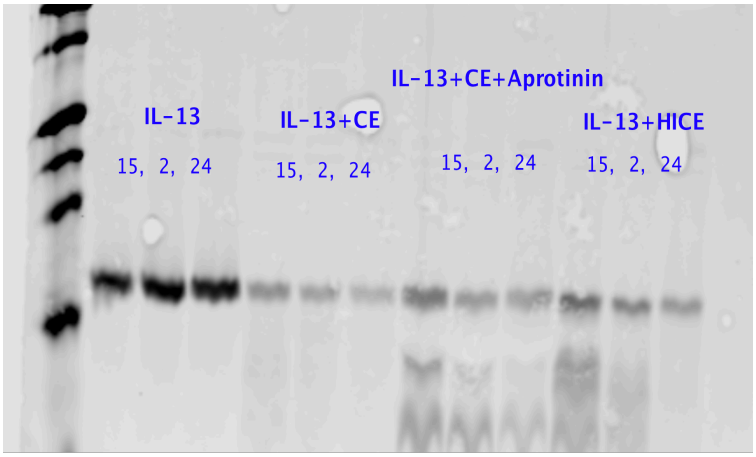


Figure 3.10: CE effect on IL-13 induced CCL26 mRNA stability. **A)** The rate of IL-13 induced CCL26 mRNA degradation for 6 hours in the absence of CE (purple) or presence of CE (black) followed by 1, 3, or 6 hours of adding Actinomycin D, n=3 **B)** The rate of IL-13 induced CCL26 mRNA degradation for 6 hours in the absence of CE (blue) or presence of CE (black) after Actinomycin D treatment followed by 1, 3, or 6 hours of adding CE, n=3.

A)



B)

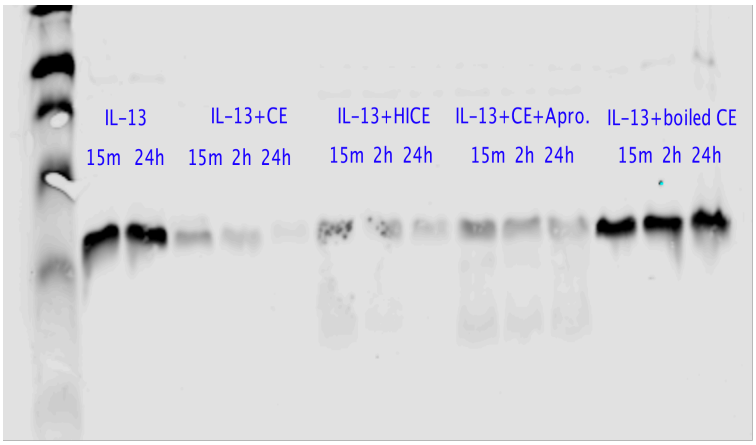


Figure 3.11: IL-13 protein degradation by CE. **A)** Western blot of IL-13 protein degradation in 15 minutes, 2 hours, or 24 hours, n=3. Shown western blot image of one experiment representative of three separate experiments. **B)** IL-13 western blot shows the condition of IL-13 incubation with boiled CE n=1.

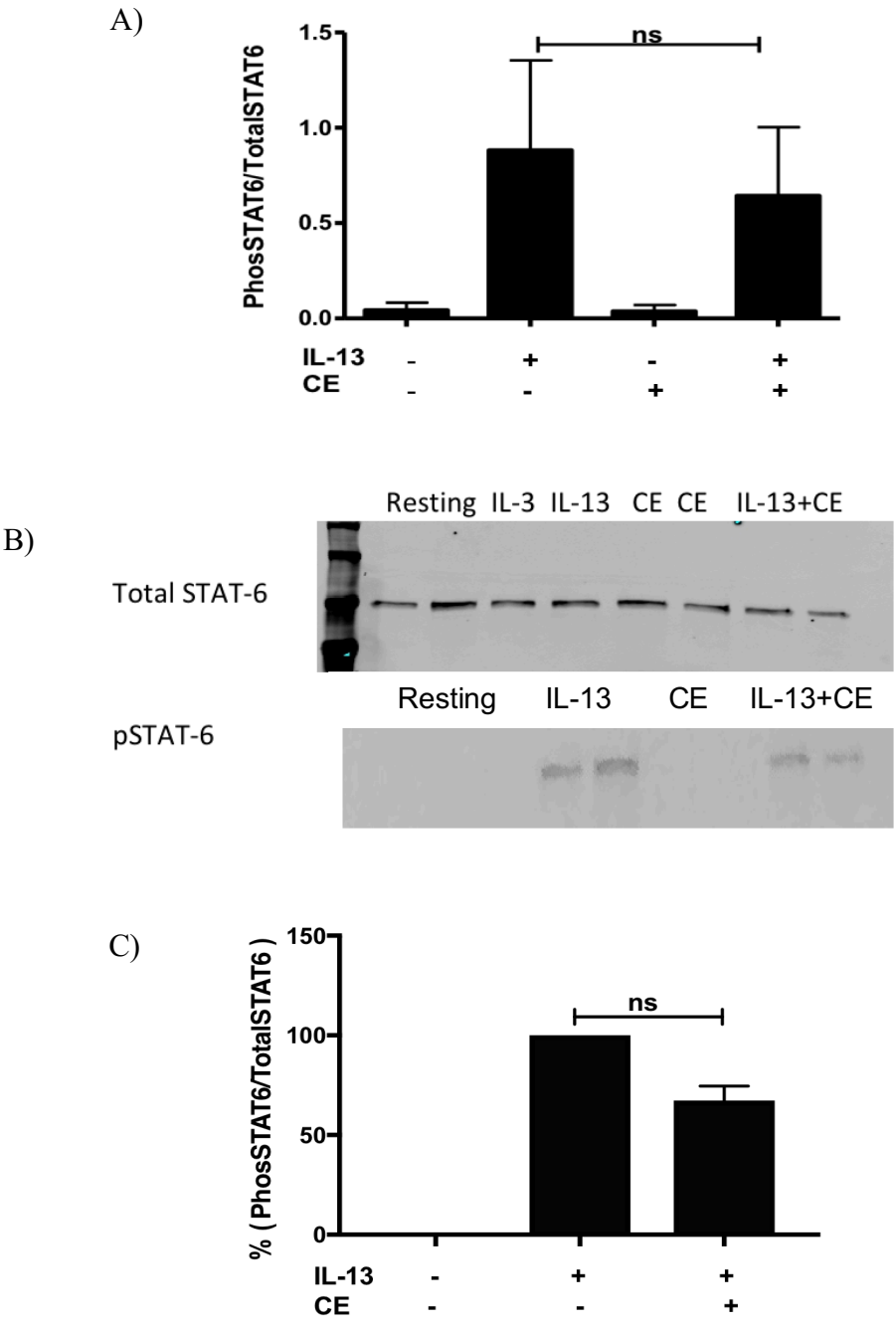


Figure 3.12: CE had no significant effect on IL-13 induced STAT-6 phosphorylation. **A)** Quantification of phosphoSTAT-6/Total STAT-6 western blot, n=3 **B)** Western blot image of Total STAT-6 (Top) and PhosphoSTAT-6 (Bottom). Image is representative of three experiments. **C)** Inhibition of STAT-6 phosphorylation by CE in percentage, n=3. Results shown are for mean ± SEM of values. P value and statistical analysis have been done by one-way ANOVA.

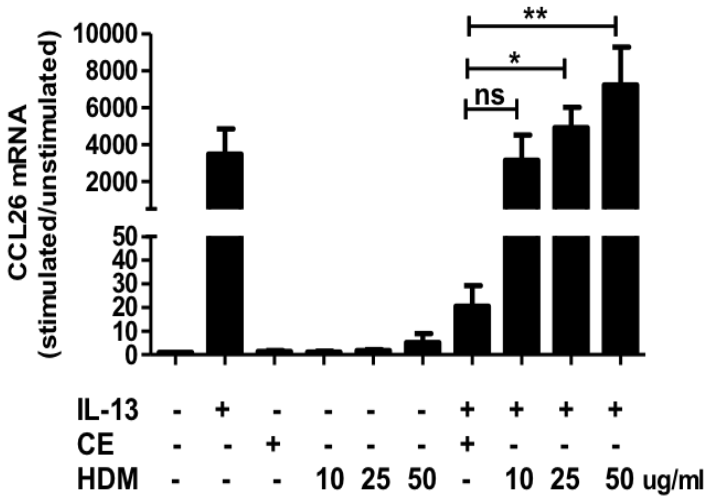


Figure 3.13: HDM did not inhibit IL-13-induced CCL26 mRNA expression. Results shown are mean ± SEM of values from n=3. P value and statistical analysis have been done by one-way ANOVA.

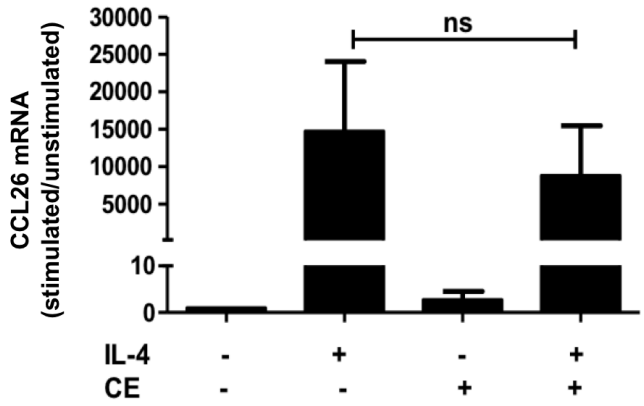


Figure 3.14: CE did not inhibit IL-4-induced CCL26 mRNA expression. Results shown are mean ± SEM of values, n=5. P value and statistical analysis have been done by one-way ANOVA.

BAEE unit/ml	CE (5ug/ml)	HICE (5ug/ml)	CE+Aprotinin	CE+SBTI
	0.495	0.018	0.049	0.041
	0.357	0.029	0.024	0.032
	0.364	0.053	0.039	0.038
Mean	0.405	0.033	0.037	0.037
SEM	0.045	0.010	0.007	0.003

Table 3.4: Trypsin-like activity measurements of CE (5ug/ml), HICE (5ug/ml), CE pre-incubated with aprotinin or SBTI. Results are mean ± SEM; n=3.

Chapter 4. Discussion and future directions

4.1 Discussion

Allergic asthma prevalence is increasing worldwide. According to the World Health Organization White Book on Allergy 2011-2012, there are 300 million patients suffering from asthma worldwide, and this number is expected to increase to 400 million by 2025. Allergic sensitization rates have also increased. Sensitization to one or more common allergens is affecting around 40-50% of school age children worldwide.

In asthma, inhaled allergens activate epithelial cells and PRRs, which triggers innate and adaptive immune responses. In particular, proteolytic allergens prompt epithelial cells to secrete cytokines and chemokines which orchestrate the subsequent immune response (40).

In this project we have shown that IL-13 induces CCL26 mRNA and protein from the airway bronchial epithelial cell line (BEAS-2B) and normal human primary epithelial cells (NHBE), which has also been shown previously (129, 130). CCL26 was the highest induced eotaxin in cultured nasal, bronchial, and small airway epithelial cells upon IL-13 and IL-4 stimulation for 24 hours (94). In primary bronchial epithelial cell cultures from asthmatic individuals, the levels of IL-13-induced CCL26 expression correlates with asthma severity and with sputum eosinophilia (131). IL-13 receptor α-chain knockout mice sensitized to ovalbumin and exposed to aerosolized antigen demonstrate reduced accumulation of eosinophils and other inflammatory cells, as well as reduced mucus cells hyperplasia and fibrosis (132). These data suggest a critical role of IL-13 in the induction of CCL26 and consequently the recruitment of eosinophils, chronic inflammatory cells, and severity of asthma. CCL26 expression was detected in response to IL-13 and IL-4 stimulation in airway epithelial cells, but this was not true in response to IL-1β stimulation (130).

TNF-α enhanced CCL26 production synergistically with either IL-13 or IL-4, in contrast TNF-α alone has minimum effect to stimulate CCL26 production in airway epithelial cells (130).

The inhibitory effect of CE on IL-13-induced CCL26 expression is serine, particularly trypsin-like, activity dependent. There was no inhibition of IL-13-induced CCL26 with HICE neither with CE pre-incubated with serine and trypsin-like activity inhibitors. Depletion of CE protease activity by heating or pre-incubation with SBTI failed to induce eosinophilic airway inflammation in a murine model of mucosal sensitization to CE (111). Another study showed reduced AHR and mucus production upon mucosal sensitization of mice with protease depleted *B. germanica* frass (133). Our observation is also supported by the trypsin-like activity assay results, which showed that trypsin-like activity of HICE and CE pre-incubated with serine protease inhibitors was below the detection limit in comparison to CE trypsin-like activity. Using trypsin with similar trypsin-like activity to 5ug/ml of CE did not show an inhibitory effect on IL-13 induced CCL26 that could mean that CE contains one or multiple trypsin like proteases of a unique type.

There are five basic categories of serine proteases based on their substrate; two of them are: trypsin-like and thrombin-like (134). Thrombin-like activity includes plasmin protease. Heat-stable plasmin from bacterial origin *pseudomonas* was found to be resistance to ultra-high temperature (135-150°C for few seconds) used in pasteurization of milk products; and that contributed to reduced shelf-life of milk products that have residual activity of heat-stable proteases such as plasmin (135). It is possible that CE contains heat-stable proteases from bacterial origin such as plasmin, which is serine protease but not trypsin-like, that is responsible for the degradation of IL-13 protein without affecting its biological activity.

Whole body CE has been found to have three distinct serine, particularly trypsin-like, proteases E1, E2, and E3. By using four fluorogenic substrates, crude CE cleaved all the used four AMC-labelled substrates in variable degrees with maximum cleavage to QAR-AMC trypsin-like substrate. Those three proteases were also found able to activate Ca^{++} and MAPK via PAR-2 in PAR-2 transfected cells (113). As we have a future plan to use and examine these extracted CE proteases on IL-13-induced CCL26 expression, the three CE trypsin like proteases are expected to mimic the CE in not affecting IL-13 receptors expression.

Functional IL-13 receptor is a heterodimer of IL-4Rα chain and IL-13Rα1 chain. We have not examined the CE effect on IL-4 receptor expression. CE effect on IL-4Rα chain is less likely to happen since we found IL-13 receptor is able to induce STAT-6 phosphorylation upon IL-13 plus CE cell stimulation.

PAR-2 activation of BEAS-2B cells did not mimic the CE inhibitory effect on IL-13 induced CCL26 expression. Multiple evidence showed that using the mucosal CE administration root in the sensitization phase in vivo appears to be a determinant for PAR-2 dependant activation. Studies used mucosal sensitization with *B. germanica* CE in wild type mice (43, 111) found increased airway allergic inflammation, which was characterized by eosinophilic airway inflammation, mucus production, AHR, and CE specific IgG. We have not examined whether CE activates PAR-2 in BEAS-2B cell culture. Besides, the methodology we used is not dependent on in vivo mucosal sensitization, which could explain why PAR-2 activation by activated peptides did not contribute to the inhibitory effect on IL-13 induced CCL26 as expected.

Investigating the role of LPS depletion in house dust collected from asthmatics houses in downregulation of airway inflammation found that LPS-reduced house dust challenged mice had

significant reduction in TNFα, IgE, and IgG1 levels (123). In addition, LPS reduction had no inhibitory effect on inflammatory eosinophil and neutrophil cell recruitment but had significantly induced higher levels of IL-13 and IL-5 in BAL fluid in comparison with crude house dust challenged mice (123). Surprisingly, it was also demonstrated in the same lab that used house dust to induce asthma-like inflammation that induction of asthma-like inflammation by house dust extract is cockroach allergens specific (136). Our project results showed using LPS had no inhibitory effect on IL-13 induced CCL26, which goes along with the finding stated that LPS reduced house dust did not reduce inflammatory eosinophil recruitment and more likely had not reduced IL-13 induced CCL26.

Detection of early IL-13 protein degradation by CE was not prevented by using HICE instead of CE nor by pre-incubation of CE with serine protease inhibitor, aprotinin, but was completely prevented by using boiled CE. A similar finding was shown in a study that used skin-derived mast cells to assess the effect of de-granulated mast cell proteases on asthma cytokines (137). IL-13 was the most susceptible cytokine for degradation by mast cells chymase and cathepsin G, which are serine proteases. IL-13 was recovered by pre-incubation of mast cells with aprotinin and SBTI (137). We found the degradation of IL-13 is taking place any way even in the presence of trypsin-like protease inhibitor, aprotinin, or using HICE. Degradation of IL-13 by CE proteases could be a mechanism of CE allergens to attenuate or alter Th2 mediated inflammation in allergic asthma, which suggest that CE allergic asthma may be mediated by other inflammatory immune cells, beside Th2. However, translating our data to in vivo should consider the presence of the immune cells produced protease inhibitors which could augment the CE allergens inflammatory responses.

Investigating the role of CE in the IL-13-induced STAT-6 phosphorylation; it is known that CCL26 induction upon IL-13 or IL-4 stimulation is mediated by STAT-6 (128), particularly STAT-6 Tyr641 phosphorylation (138) dependent. IL-13-mediated CCL26 induction in BEAS-2B cells is STAT-6 dependent (129). Based on that, we examined STAT-6 phosphorylation after IL-13 or IL-13 plus CE cell activation and found no significant inhibition of STAT-6 phosphorylation by CE. Other phosphorylation sites, other than Tyr641, was not examined in this project since it was shown that Tyr641phosphorylation is enough to induce the nuclear translocation of STAT-6 (138).

A study found negative regulation of STAT-6 DNA-binding due to serine phosphorylation (138). Multiple serine residues phosphorylation on transactivation domain (TAD) of STAT6 result in conformational changes and loss of STAT-6 DNA binding (138). Surprisingly, this serine phosphorylation of STAT-6 TAD domain did not affect Tyr641 phosphorylation, STAT-6 dimerization, or nuclear translocation (138). In light of that, one hypothesis would be that CE may induce STAT-6 serine phosphorylation which result in loss of STAT-6 DNA binding and therefore CCL26 mRNA induction. This hypothesis was not investigated.

It is also possible that IL-13 protein degradation by CE decreases or de-phosphorylate STAT-6. This hypothesis is supported in two ways; first we showed that washing IL-13 after 90 minutes of stimulation results in loss of CCL26 induction. This means that IL-13 continued stimulation is required for STAT-6 tyrosine phosphorylation and IL-13 removal causes de-phosphorylation of STAT-6. This was also shown in a study that used IL-4 stimulation and washing to study the effect of IL-4 removal on STAT-6 phosphorylation (125). Human bronchial epithelial cells were cultured in 10ng/ml of recombinant human IL-4 for 15 minutes or 2 hours; IL-4 was washed and the cells

cultured for a longer period of time in the absence of IL-4. The presence of IL-4 was required for STAT-6 tyrosine phosphorylation, while removal of IL-4 resulted in gradual STAT-6 de-phosphorylation (125). Second, we consider the effect of early degradation of IL-13 as the effect of washing, so gradual degradation of IL-13 protein by CE causes gradual de-phosphorylation of STAT-6. The exact mechanism by which CE proteases affect STAT-6 phosphorylation remains unclear.

HDM had no inhibitory effect on IL-13 induced CCL26 and that goes along with our finding that HDM has no detectable trypsin-like activity in our assay. IL-4 induced CCL26 mRNA was not completely inhibited by CE. The IL-4 protein degradation and IL-4 induced STAT-6 phosphorylation was not investigated.

Cockroach allergens were associated with allergic airway inflammation in several studies and some other studies have shown that CE serine protease inhibitors administration to mice had significantly reduced airway inflammation (139). In this study, mice models of asthma were sensitized with CE, then treated with broad specificity serine protease inhibitor 1 hour before or after challenge. CE challenged mice showed high airway resistance and increased level of inflammatory cells in BAL fluid. Serine protease inhibitor treated mice before CE challenge showed significantly lowered airway resistance and reduced number of eosinophil and neutrophil count.

In our project we found dose effect of CE in the inhibition of IL-13 induced CCL26 induction which could go along with the hygiene hypothesis. Our data showed low dose of CE (0.2 ug/ml) has induced significant level of IL-13-induced CCL26 expression. In contrast, high dose of CE (5 ug/ml) has caused complete inhibition of IL-13-induced CCL26 expression. The hygiene

hypothesis states that lack of early childhood exposure to infections, as well as allergens, increases susceptibility to infections and allergic diseases later in life. A study showed evidence of that by finding that oral tolerance inhibits pulmonary eosinophilia in a cockroach allergen induced model of asthma (140). This study investigated and compared pulmonary inflammation in mice that received four cockroach allergens feedings before sensitization and challenge to mice that had received PBS feedings before sensitization and challenge with cockroach allergens. AHR was significantly reduced in cockroach fed mice and that was not due to reduction of Th2 cytokines and inflammatory cell chemokines but mainly due to reduced eosinophil count in BAL fluid. Reduced eosinophilia was not also associated with lower levels of eotaxin 1 and eotaxin 2 (CCL11 and CCL24 respectively) but mostly is related to increased levels of IL-10 in the lungs, high levels of IL-10 is correlated with lower eosinophil recruitment to the lungs (140). In light of our project results, an alternative hypothesis could be that reduced eosinophilia is mostly connected to lower levels of CCL26. However, the authors did not show the role of CCL26 in the reduced eosinophilia. This study provided an evidence that cockroach allergens tolerance can improve respiratory health in mice model of asthma (140). In our data we showed that reduction of IL-13 induced CCL26 by CE is dose dependent, which could be translated into exposure to higher doses of cockroach allergens is protective and result in attenuated eosinophilia and inflammatory responses in allergic asthma.

4.2 Future directions

This project showed inhibitory effect of *B. germanica* CE on IL-13 induced CCL26 in airway epithelial cells, but many questions remained unanswered. The mechanism by which CE inhibits IL-13-induced CCL26 remained unclear. We do not know if IL-13 protein degradation by CE proteolytic activity is connected with low or loss of STAT-6 phosphorylation. Besides, the questionable IL-13 biological activity after degradation by CE and how does it promote CCL26 mRNA induction in case of using CE pre-incubated with aprotinin.

Examining the effect of the CE isolated serine proteases (113) on IL-13 induced CCL26 induction and IL-13 protein degradation will solidify our results and help to identify which protease(s) are responsible for the inhibition of IL-13 induced CCL26 and IL-13 protein degradation.

Our results showed that CE did not significantly inhibit STAT-6 Tyr641phosphorylation. However, it is possible that CE induces STAT-6 TAD serine resides phosphorylation which contribute to STAT-6 conformational changes and cause loss of STAT-6 DNA-binding. STAT-6 TAD serine phosphorylation by CE needs to be studied. This will clarify whether this is the mechanism CE uses to inhibit IL-13 induced CCL26 induction.

We also showed that HDM has below detection limit trypsin-like activity and HDM did not inhibit IL-13-induced CCL26, in contrast to CE. Exploring HDM proteases activity and its ability to degrade IL-13 would provide evidence about the proteases responsible to degrade IL-13 and if that is contributing to inhibit IL-13 mediated CCL26 induction.

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Appendix:

The data presented here were generated to collaborate with Dr. Lisa Cameron from the University of Western Ontario (UWO).

Dr. Cameron is interested in understanding the connection between the expression of estrogen receptors (ESR1 and ESR2) genes in whole blood cells in severe asthmatic women and men and investigate if that is related to the observed high circulating Th2 lymphocytes in severe asthmatics women vs. men. Cameron hypothesized that high levels of circulating Th2 lymphocytes in women with severe asthma could be related to a gender-specific efficacy of the inhaled glucocorticoids (GCs). This work is a part of bigger study that Dr. Cameron is conducting.

A study has shown that females aged 13-18 years old have higher rates of asthma diagnosis, wheezing (141) and AHR (142) compared to males of similar age. This high rate of asthma diagnosis in females has been found to be related to female hormonal changes. 25-40% of asthmatic females in reproductive age showed perimenstrual asthma syndrome associated with perimenstrual estrogen level changes (143). Estrogen has two receptors to act through: ERα and ERβ which are encoded by ESR1, and ESR2 genes respectively (144). ERs are expressed on lung tissue and some inflammatory cells: mast cells, macrophages, lymphocytes, and monocytes (145). Estrogen has been implicated in promoting the mobilization of bone marrow eosinophils as well as eosinophils infiltration in airways, goblet cell hyperplasia, and baseline lung resistance in mouse model of asthma (146). Importantly, estrogen also stimulated Th2 lymphocyte cytokines IL-5 and IL-13 production which was suppressed by estrogen receptor antagonists (146). In asthmatics, inhaled glucocorticoids (GCs) suppress asthma by inhibiting Th2 cytokine production and induction of Th2 apoptosis (147). Other data showed estrogen interfered with GCs signaling in

Th2 lymphocytes (148) which may contribute to GCs inhibitory effects on Th2 lymphocytes in asthma.

The above data suggest that estrogen signaling in Th2 lymphocytes may interact with the Th2 responses to GCs by enhancing the Th2 rate of survival and function. To examine the hypothesized GCs interactions with estrogen signaling in Th2 lymphocytes, Cameron's study aimed to activate ERs by agonist to see if that interferes with the anti-inflammatory effects of GC, which could explain the higher level of Th2 cells in women compared to men with severe asthma.

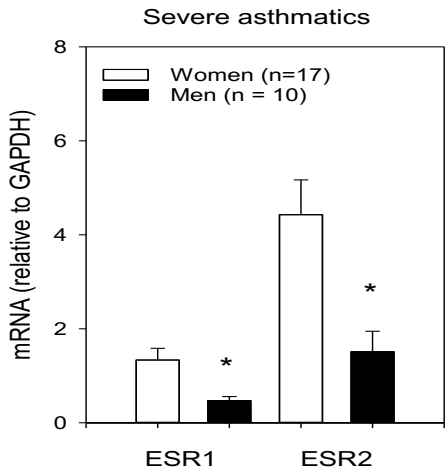
Methodology:

We received 32 RNA samples isolated from whole blood from subjects recruited at UWO. I performed qRT-PCR (same methodology as described in Chapter 2) to screen all the 32 samples for ESR1, ESR2 and CRTh2 (Th2 cell surface receptor) gene expression.

Results:

Results showed severe asthmatic women have higher ESR1 and ESR2 gene expression levels in whole blood cells in comparison to men. Results are shown in figure 5.

Figure 5:



Expression of ESR1 and ESR2 mRNA in whole blood cells of women and men with severe asthma. Significant by Mann Whitney Rank Sum test (and Student's t-test) n=27, ESR1 p = 0.002; ESR2 p = 0.007