Lipopolysaccharide Inhibits Interleukin–13-induced Chemokine (C-C motif) Ligand 26 in Human Airway Epithelial Cells: Possible Role in Eosinophil Chemotaxis in Allergic Asthma

By

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Abstract

Background

Allergic asthma is characterized by increased level of Interleukin-13 (IL-13) in the lungs. IL-13 promotes eosinophilic infiltration in the airways by stimulating airway epithelial cells to release eotaxin-3 (CCL26) through the Janus activated kinase-2 (JAK-2)/signal transducer and activator of transcription 6 (STAT6) pathway. Eosinophil accumulation in the airways is a hallmark of allergic asthma. There is also evidence that bacterial products, such as LPS, affect the release of eosinophil chemotactic factors and may alter eosinophil accumulation in peripheral tissues. However, the effects of LPS on airway eosinophilia are incompletely understood. Thus, our aim was to study the effects of LPS on IL-13 -induced CCL26 induction in airway epithelial cells and the mechanisms of these effects.

Methods

We used LPS to mimic the bacterial insults on the airway epithelium. The human bronchial epithelial cell line BEAS-2B was stimulated with IL-13 (20 ng/ml) alone or in combination with LPS (10 μg/ml) for 24 hr. CCL26 mRNA levels were measured using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and CCL26 protein was measured in the supernatants of these cells using ELISA. STAT6 phosphorylation was measured by western blot. For NF-kB inhibition, BEAS-2B cells were pre-treated with 3 different NF-kB inhibitors, curcumin (10 μM), arctigenin (1 μM), and bengamide B (1 μM), for 2 hr before activation with IL-13 and/or LPS.

Results

BEAS-2B cell activation with IL-13 for 24 hr strongly induced CCL26 mRNA expression (78.3 ± 3.9 fold over unstimulated cells, n=13, $p < 0.001$) and the release of CCL26 protein (1462 ± 55.1 pg/ml , while there was no detection of CCL26 protein release in unstimulated cells, n=13, $p < 0.001$). Simultaneous treatment of BEAS-2B cells with LPS inhibited IL-13 -induced CCL26 expression (n=13, $p < 0.001$) and CCL26 protein release (n=13, $p < 0.001$). IL-13 also induced STAT6 phosphorylation in BEAS-2B cells, which peaked at 30 min. STAT6 phosphorylation, was attenuated when cells were activated by IL-13 in the presence of LPS (n=3, $p < 0.05$). Pre-incubation of the cells with NF-kB inhibitors prevented the LPS effect on IL-13 -induced CCL26 upregulation and STAT6 phosphorylation.

Conclusions

LPS, a TLR-4 ligand, inhibits the effects of IL-13 on CCL26 expression in airways epithelial cells. This effect may be dependent on LPS interfering with JAK2/STAT6 signaling through NF-kB activation.

Dedicated

To

My father, Saud Dhaifallah Alotaibi

Who prays each day and night to wish me success and honor

My mother, Sha'a Bijad Alotaibi

Who loves me and for being my first teacher and the reason what I become today

My stepmother, Fatimah Abdullah Alotaibi

Who loves me, taught me to trust in Allah, believe in hard work and that so much could be done with little

My soulmates, My Brothers and Sisters

Who are I wish, I had more space to write their names down, but their names are written on my heart

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List of Abbreviations

AHR	Airway hyperresponsiveness
ANOVA	One-way analysis of variance
APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
BCRs	B cell receptors
BEAS-2B cells	Bronchial Epithelial Cell Line
BSA	Bovine serum albumin
CCL	CC chemokine ligand
CCL11	Eotaxin or Eotaxin-1
CCL17/TARC	thymus and activation-regulated chemokine; TARC
CCL2/MCP-1	Monocyte chemotactic protein 1; MCP-1
CCL22/MDC	MDC
CCL24	Eotaxin-2
CCL26	Eotaxin-3
CCL3/MIP-1α	Macrophage inflammatory protein- 1 alpha; MIP-1α
CCL4/MIP-1β	Macrophage inflammatory protein- 1 beta; MIP-1β
CCR3	CC chemokine receptor 3
CD14	Cluster of differentiation 14
DAMP	Pathogen-associated molecular pattern
DMSO	Dimethyl sulfoxide
DCs	Dendritic cells
DTT	Dithiothreitol

dNTPS	Deoxynucleotide triphosphate
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
FEV1	The first second of Forced expiratory volume
FBS	Fetal bovine serum
GAWS	a genome-wide association study
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IgE	Immunoglobulin E
IgE receptor	FcεRI
IKK	IκB kinase
IL-13	Interleukin -13
IL-13Rα1	IL-13 receptor alpha 1
IL-13Rα2	IL-13 receptor alpha 2
IL-4Rα	IL-4 receptor α -chain
IL2R γc	IL-2 receptor common γ -chain
ILC-2	Innate lymphoid cells -2
IRAK4	Interleukin-1 receptor-associated kinase 4
JAK-2	Janus Kinase-2
LTC4	Leukotriene C4
MBP	Major basic protein
MyD88	Myeloid differentiation primary response gene 88
NETs	Neutrophil extracellular traps
PBS	Phosphate-Buffered Saline

PIC	protease inhibitor cocktail
PGD2	Prostaglandin D2
PRRs	Pattern recognition receptors
RIPA	Radioimmunoprecipitation assay
siRNA	Small interfering RNA
STAT6	Signal transducer and activator of transcription 6
TAK1	TGF-β activated kinase 1
TBS	Tris-buffered saline
TGF-β	Transforming growth factor beta
T_H2	T helper 2
TLR-4	Toll-like receptor-4
TNF	tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
Bc	Common beta-chain

Chapter 1

Introduction

1.1 Asthma Overview

Asthma is a chronic inflammatory disease of the airways characterized by airway hyperresponsiveness (AHR) to a variety of stimuli such as histamine and methacholine and airway inflammation leading to remodeling. Asthma is characterized clinically by episodic reversible airway obstruction, shortness of breath, wheezing, chest tightness and airway remodeling (1,2). Asthma has become an epidemic, increasing in prevalence in urbanized populations worldwide, affecting 330 million in the world (3). There are almost 2.4 million Canadians aged 12 years and over that suffer from asthma (8.4 % of the general population, 9.8% of females and 7.0% of males) (4,5). Asthma can severely compromise quality of life and even though not very common can even lead to death. According to asthma society report, asthma is the main reason of absenteeism from school and the third cause of work loss (6). In 2015, there were more than 70,000 emergency room visits due to asthma in Canada (7).

1.2 Asthma Pathogenesis: Genetic and Environmental Factors

A complex interplay between genetic and environmental factors contributes to asthma pathogenesis. Asthma is a familial disease and has significant genetic contributions, with heritability estimates varying between 35% and 95% for asthma (8–18). Several studies showed that more than 118 genes are associated with asthma. Some of these genes affect the development of asthma and others change asthma severity or the patient's response to therapy. In related to this thesis, *IL-13, IL-4, FcεRI-β, CCL11, CCL24 and CCL26* are some examples of genes that are associated with asthma (19–25).

In addition to genetic factors, environmental factors also play a role in asthma pathogenesis.

Exposure to environmental factors such as allergens, air pollutants, diet, viruses, and bacteria can trigger asthma in genetically susceptible individuals (26). In related to bacteria, some studies showed bacterial infection is involved in asthma pathogenesis and highly associated with asthma exacerbations and AHR in asthmatic patients (27,28). However, bacterial infection and it is associated with asthma is matter of debate, increasing evidences suggesting exposure to normal microflora bacteria is beneficial for the development of the human immune system (20). These evidence support 'the Hygiene hypothesis' that state an environment rich in microbes provides protection against diseases.

Altogether susceptibility and development of asthma are affected by internal as well as external factors.

1.3 Asthma Phenotypes

Asthma is a clinical disorder with many phenotypes and each phenotype can have biologic markers that reflect the underlying disease such as eosinophilic or neutrophilic asthma and some phenotypes represent trigger- induced asthma such as allergic asthma and aspirin-exacerbated respiratory disease and exercise- induced asthma (31). The diversity of asthma phenotypes continues to make asthma a challenge to classify, and to treat. Here we will review primarily allergic asthma, the most common phenotype of asthma.

1.3.1 Allergic Asthma

Allergic asthma usually starts in childhood and pathophysiology involves the inhalation of an allergen such as house dust mite or cockroach. These allergens trigger the immune response and activate different immune cells that they participate in allergic asthma.

Cells Contributing to Allergic Asthma Pathogenesis

When an allergen enters the body for the first time, an individual with an inherited predisposition to this allergen will begin to develop sensitization (specific IgE production). Allergen will interact with pattern recognition receptors (PRRs) and protease activating receptors on epithelial cells. This recognition can cause the activation of dendritic cells (DCs) which uptakes the allergen and present it to naïve $CD4^+$ T cells in the lymph nodes. $CD4^+$ T cells will proliferate and differentiate to T helper cells including T helper 2 (T_H2) cells (32). T_H2 cells produce IL-4, which stimulates B cells to begin production of immunoglobulin E (IgE) antibodies. Secreted IgE circulates in the blood and binds to mast cells and basophils through an IgE receptor (FcεRI) (33,34).

Upon a subsequent exposure to the same allergen, it will bind to IgE on the surface of mast cells and basophils and activate them. They will release inflammatory mediators (e.g. histamine, leukotrienes, and prostaglandins) into the surrounding tissue causing some features of asthma including vasodilation and bronchoconstriction and shortness of breath (35). Furthermore, this reaction attracts many other inflammatory cells to the site such as eosinophils, and neutrophils which are discussed below (36,37).

Epithelial Cells

Epithelial cells form a first line of defense to protect underlying organs, such as the lungs, from potentially harmful external stimuli such as bacteria, viruses, toxins and also allergens (38). The airways, from the nose to the alveoli, are covered by a continuous epithelial sheet and are constantly exposed to the external environment. Epithelial cells are connected to each other by tight junction proteins such as zonula occludens, occludin, and claudin (38). This structure provides the airway with a physical barrier property to control transport of water, gasses, and ions. It also prevents inhaled pathogens and other environmental substances from penetrating or injuring the human airway.

There are several different types of epithelial cells within the airways to provide its complex functionality (38). In the large airways, the epithelium is pseudostratified and contains goblet cells, ciliated, undifferentiated columnar, and basal cells. Goblet cells produce mucus, thick and viscous fluid, which serves as a lubricant and forms a viscoelastic blanket that covers the entire bronchial epithelium and helps to remove bacteria and other foreign particles from the airway (39). Ciliated cells are responsible for driving the tracheobronchial secretions toward the pharynx. Basal cells facilitate attachment of other epithelial cells to the basement membrane and give the epithelium of the larger airways its pseudostratified appearance. These cells gradually disappear from upper airways down to the alveoli (40,41). Basal cells can function as precursors to other airway epithelial cells in sites of injury by differentiating and restoring a healthy epithelial cell layer (42). In the small airways, the airway epithelium changes to columnar and cuboidal. The cells in small airway are similar, with relatively more ciliated cells, and some clara cells (43). In alveoli, there are alveolar type I and type II cells. Alveolar type II cells are

responsible for surfactant production which increases pulmonary compliance and prevents the collapse of the alveoli at the end of exhalation by the decrease in surface pressure (44). Alveolar type II cells are progenitors for both types I and II cells (44).

Airway epithelial cells can detect allergens using proteinase-activated receptors (PARs). PARs are G protein-coupled receptors (GPCRs) that are uniquely activated by proteases from allergens. In turn, these can alter epithelial tight junction proteins and increase epithelial permeability, which facilitate allergen entry and uptake by dendritic cells. The interaction between allergens and airway epithelium can cause the release of epithelial driven cytokines including IL-33 and TSLP that can activate the immune system. IL-33 and TSLP produced by epithelial cells regulate dendritic cell functions to facilitate T_H2 cells differentiation and migration into the airway (45,46).

In addition to allergens, airway epithelial cells are frequently exposed to bacteria and various bacterial products. They recognize conserved structural motifs in bacteria, termed pathogen - associated molecular patterns (PAMPs) through surface receptors, both on the cell surface and in endosomes (47). These surface receptors must be exposed apically to recognize bacterial components present in the lumen of the airways (48). Under unstimulated conditions, these receptors are presented at low density on the cell surface that is likely to prevent excessive immune responses that might affect lung function (49). Airway epithelial cells can use antimicrobial activity (e.g. lysozyme, lactoferrin, secretory phospholipase A2, human beta defense (HBD) … etc.) as apart of pulmonary innate defense to eliminate invading bacteria without an inflammatory response or the activation of adaptive immunity (50). However, upon repeated bacterial stimulation more receptors are upregulated and recruited to the apical surface where they initiate the inflammatory response when this is required to clear the infection

(47,51,52). Airway epithelial cells recognize bacteria by pattern recognition receptors (PRRs) such as Toll like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors (NLRs)) (51,52). For example, Toll like receptor-2 (TLR-2) recognizes a variety of microbial components, including lipoproteins/lipopeptides, lipoteicoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, and phenol soluble modulins from Staphylococci (53,54). TLR-4 recognizes lipopolysaccharide (LPS) found in the outer layer of gram-negative bacteria (55). TLR-5 recognizes flagellin, the principal component of flagella, from both Gram-positive and Gram-negative bacteria (56). In addition to cell surface receptors that recognize microbial components, mammalian cells also have NLRs to recognize PAMPs in the cytosol of infected cells. The NLR family of proteins include many receptors of which nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 are expressed by airway epithelial cells (52). NOD1 recognizes peptidoglycans containing meso-diaminopimelate (meso-DAP) acid found mainly in Gram-negative bacteria (57,58). NOD2 recognizes muramyl dipeptide (MDP) in peptidoglycans of bacteria (59). These interactions induce the activation of innate as well as adaptive immune system and lead to production of chemokines and cytokines that induce the recruitment of immune cells into the lung.

LPS is the most extensively studied member of pattern recognition molecules and it is ubiquitous in environment including allergens. LPS, a major component of gram negative bacteria, is known to contribute in asthma exacerbation, decrease in pulmonary function, and persistent wheezing (55,60–65). A survey of endotoxin in houses showed strong relationship between levels of household LPS and asthma exacerbations (60). Occupational exposure to dust has been known to be associated with decreased pulmonary functions and several studies revealed that the concentrations of LPS in the dust, but not the dust itself, was the reason of the decrease in

pulmonary function (61,62). Also, clinical studies showed that Inhalation of LPS was directly associated with a reduced lung function in healthy subjects (63,64). Moreover, a study in Denmark showed that colonization of Gram-negative bacteria *Haemophilus influenzae* and *Moraxella caterrhalis* in pharynx of new babies is associated with an increased persistent wheezing and asthma by age 5 (65).

Here, we used LPS *in vitro* experiment to investigate about its effect on eotaxins produced by IL-13 (Fig.3).

LPS Detection and Signaling

LPS binds to LPS-binding protein forming a molecular complex, which interacts with the surface molecule cluster of differentiation 14 (CD14). CD14 helps this molecular complex to be sensed by TLR-4 on the surface of cells such as epithelial cells, monocytes, macrophages and dendritic cells (DCs) (51,66,67). TLR-4 can be localized either at the cell surface or the endosomal compartments, depending on the cell type (68). In airway epithelium, TLR-4 mostly express on the apical and also have basolateral and endosomal distribution (55,69,70). TLR-4 activation recruits the adaptor protein myeloid differentiation primary response gene 88 (MyD88) and forms a MyD88-TLR complex that will activate several signaling pathways including ubiquitination and phosphorylation. Briefly, MyD88 activates interleukin-1 receptor-associated kinase 4 (IRAK4), which in turn induces IRAK1 phosphorylation. Following IRAK1 phosphorylation both IRAK1 and IRAK4 leave the MyD88-TLR complex and bind with TNF receptor associated factor 6 (TRAF6) leading to its ubiquitination. Ubiquitination of TRAF6 leads to activation of TGF-β activated kinase 1 (TAK1) and then TAK1 binds to the IκB kinase (IKK) complex. This results in phosphorylation of IκB and activation and nucleus translocation of nuclear factor kappa B (NF-κB) (Fig.1) (51,71–74).

NF-κB is ubiquitous transcription factor and activated in response to cytokines, mitogens, physical and oxidative stress, infection, and microbial products such as LPS. It is necessary for directing of several cytokines (e.g. TNF, GM-CSF, IL-6, IL-8, IL-12...etc.), chemokines (e.g. CXCL2), adhesion molecules (e.g. ICAM-1) and other proinflammatory proteins to be transcribed (75–79). In the airway epithelium, NF-kB expression found to be high in human asthmatics as well as in murine model of allergic airway inflammation (80–82). Finally, NF-κB activation in the airways of allergen-challenged mice is attenuated by TLR-4 gene deletion, suggesting that the importance of TLR-4 and its contribution to NF-κB signaling in asthma (83).

LPS can also signal independently of MyD88 and NF-κB. In this case, TLR-4 activation by LPS recruits the adaptor protein TIR domain-containing adaptor protein inducing interferon-β (TRIF) and leads to type I interferons production (84). TRIF-dependent pathway involves the recruitment of the adaptor proteins TRIF and TRIF-related Adaptor Molecule (TRAM). TRAM-TRIF signals activate the transcription factor Interferon Regulatory Factor-3 (IRF3) via TNF receptor-associated factor 3 (TRAF3). IRF3 activation induces the production of type I interferons (IFN-β) and IL-10 (84,85).

The determination of LPS signaling may be influenced by the presence of CD14 and smooth or rough lipid A (component part of LPS). In the presence of CD14, LPS can induce TLR-4 activation via both pathways (TRIF-dependent pathway and Myd88 dependent pathway). In the absence of CD14, smooth lipid A fails to induce TLR-4 activation, while rough lipid A induces signaling via only MyD88 dependent pathway (86).

Moreover, Guillot et al. demonstrated that human bronchial epithelial cell lines (BEAS-2B) and human alveolar epithelial cell line A549 constitutively expressed TLR-4 and they also showed

the intracellular localization of this receptor (55). They also established that LPS-induced stimulation of these cells is dependent on the activation of TLR-4 signaling and intermediates MyD88 adaptor protein and NF-kB activation and not the other pathway (TRIF-dependent pathway) (55).

Taken together, epithelial cells play important role in innate and adoptive immune response and contribute in many aspects of allergic asthma.

Neutrophils

Neutrophils are the most abundant innate immune cell in the body. They are the first immune cells to rapidly migrate to the site of infection to help fight infection through ingesting microorganisms (phagocytosis) and releasing enzymes that kill the microorganisms. Their migration and activation lead them to release elastase, and myeloperoxidase that help to kill invading pathogens. They also form neutrophil extracellular traps (NETs) that also kill pathogens extracellularly (36,37). Neutrophils express FcεRI and play an important role in allergic asthma pathogenesis (87). During allergen exposure, neutrophils infiltrate the lungs where they release an array of inflammatory mediators and cytokines. Neutrophil infiltration is associated with lung function impairment and the severity of asthma (88). Neutrophil numbers are not increased in airway secretions from patients with mild and moderate asthma, but Wenzel and coworkers have shown that neutrophil numbers are higher than normal in airway lavage from patients with severe asthma (89).

The relationship between neutrophil infiltration and bronchoconstriction in asthma was investigated by Shaw and colleagues in almost 1,100 patients with asthma (90). They found a high number of neutrophils and eosinophils were associated with a low pre-bronchodilator first

second of forced expiratory volume (FEV1) and high numbers of neutrophils were associated with a low post- bronchodilator FEV1(90).

Taken together, these studies demonstrate neutrophilic infiltration in the airway associated with severe asthma and indicate a possible link between neutrophilic infiltration and bronchoconstriction in asthma.

Dendritic Cells

DCs are the primary professional antigen presenting cells (APCs). They can be found in areas exposed to antigens including allergens. DCs can take up allergen from epithelial cells or directly uptake the allergen in case of disrupted epithelial cells, migrate to the lymph nodes and present the allergens to T cells and become activated and start their differentiation (32). DCs are regulated by cytokines released by epithelial cells in response to allergen exposure, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), thymic stromal lymphopoietin (TSLP), and IL-33. GM-CSF promotes the maturation of DCs and enhances their inflammatory functions. IL-33 and TSLP direct DC function to promote T_H2 polarization. TSLP-activated DCs induce the expression of PGD2 receptor on T_H2 cells in asthmatic airways, a mechanism through which DCs promote the expansion of T_H2 memory cells and maintenance of T_H2 commitment (91). The function of TSLP can be enhanced by IL-25 released by epithelial cells, eosinophils, and basophils after allergen exposure. Systemic depletion of DCs resulted in abrogated airway inflammation, mucus production and AHR in OVA challenge mice (92). Another study also showed that selective depletion of lung DCs in mice led to the elimination of features of asthma including eosinophilic inflammation, goblet cell hyperplasia, and AHR. Moreover, injection of

DCs in these mice restored these defects. However, injection with other APCs such as macrophages failed to restore these defects (93).

Taken together, these results suggest a unique and vital role of DCs in allergic asthma development and exacerbation.

Mast Cells

Mast cells are derived from CD34+ progenitor cells circulating in peripheral blood and mature in the tissues (94,95). They can be found in surface, submucosa, and deep in the airway (35). They express the high affinity FcεRI (33). During allergen exposure, allergen binds to IgE on the surface of mast cells and in turn activates mast cells to degranulate. Activated mast cells release inflammatory mediators such as tryptase, heparin, histamine, prostaglandin D2 (PGD2), and leukotriene C4 (LTC4) and cause bronchoconstriction and vasodilation (33,35). Mast cells also secrete proinflammatory cytokines after activation such as IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, TNF, transforming growth factor beta (TGF-β) and chemokines such as CCL2 (Monocyte chemotactic protein 1; MCP-1), CCL3 (macrophage inflammatory protein- 1α; MIP-1α), CCL4 (MIP-1β), CCL17 (thymus and activation-regulated chemokine; TARC) and CCL22 (Macrophage-derived chemokine; MDC) (33,95–97) .

Taken together, these proinflammatory mediators, cytokines, and chemokines play major roles in characteristic features of allergic asthma

Basophils

Basophils are also derived from CD34+ progenitor cells found in cord blood and peripheral blood (94). Like mast cells, basophils express FcεRI and interactions between allergen and IgE

on surface of basophils stimulates the cells to release inflammatory mediators including histamine that eventually leads to bronchoconstriction and vasodilation (98). Basophils play a critical role in allergic asthma. They can act as APCs and differ from other APCs such as DCs due to their ability to produce IL-4 (99,100). Depletion of basophils impaired IL-4 production, whereas adoptive transfer of basophils in MHCII-deficient mice restored IL-4 production by CD4 T cells (34,101,102). Furthermore, basophils promote inflammation by releasing chemokines such as CCL3, CCL5 CCL7, CCL8, CCL11, CCL13, CCL24 and CCL26 that attract basophils and other pro-inflammatory cells into asthmatic lungs (103,104).

Taken together, these proinflammatory mediators, cytokines, and chemokines play major roles in causing allergic asthma and especially in IL-4 production, and suggest that basophils play a critical role in allergic asthma.

CD4+ T cells and T helper 2 (T_H2) Cells

$CD4^+$ T cells are considered the essential regulators of the immune responses especially in allergic asthma. Upon allergen presentation to naïve $CD4^+$ T cells, these cells will proliferate and differentiate into T_H2 cells effector cells that are specialized in terms of the cytokines that they secrete in allergic asthma (105). T_H2 cells play important role in the immune response against allergens. Increased presence of T_H2 cells in allergic asthmatics categorized this disease as a T_H2 cell-driven disease (106). Cytokines produced by T_H2 cells such as IL-4, IL-5, IL-9, and IL-13 have been primarily implicated in causing features of asthma including airway inflammation, AHR, and mucus production (107).

Interleukin-4 (IL-4)

IL-4 is a 17,492 dalton polypeptide with pleiotropic effects on many cell types including T cells, basophils, and mast cells (108,109). IL-4 binds to a heterodimeric receptor composed of IL-4 receptor α -chain (IL-4Rα), which has high affinity to IL-4, and IL-2 receptor common γ -chain, (IL2R γc), which is shared with IL-2, IL-9, IL-7, and IL-15 receptors (110–112). IL-4 can also bind to another heterodimeric receptor composed of IL-4Rα and IL-13Rα1, which is shared with IL-13 (113). IL-4 plays a pivotal role in the nature of the immune response. In naïve CD4+ T cells, IL-4 binds to its receptor and induces proliferation and differentiation into T_H2 cells and starts type 2 immune responses (114). IL-4 can instruct B cells to produce IgG1 and IgE in mice (115).

Interleukin -13 (IL-13)

IL-13 is a 15,816 dalton polypeptide with pleiotropic effects on many cell types including B cells, epithelial cells and monocytes (108). It was identified in 1993 by molecular cloning from activated human T-lymphocytes and described as a T cell - derived cytokine that can inhibit inflammatory cytokine production (116,117), however, subsequent studies indicated that IL-13 possesses several unique effector roles which can result in recruitment of eosinophils, activation of macrophages, increased airway epithelial cell permeability and mucus production (118–120). The human *IL-13* gene has been implicated in the induction of AHR and eosinophilic airway inflammation (121,122).

IL-13 receptor (IL-13R) is a heterodimer consisting of IL-13 receptor alpha 1 (IL-13Rα1) and IL-4Rα. (113). This receptor is shared with IL-4, but IL-$4^{-/-}$, and IL-$4R^{-/-}$ knockout mice

confirmed a non-redundant role for IL-13 in host immune responses (123). IL-13 receptor alpha 2 (IL-13Rα2) is another receptor for IL-13 and has been suggested to be "a decoy receptor". This receptor binds only to IL-13 and exists in a membrane-bound and a soluble form in mice. To date, no known soluble form of IL-13α2 has been detected in humans (124). Recent reports, however have shown that IL-13α2 has the ability to mediate IL-13 signaling. In a murine model of bleomycin-induced fibrosis, *in vivo* silencing of IL-13Rα2 with gene-specific small interfering RNAs (siRNAs) resulted in significantly reduced TGF-β1 production and collagen deposition in the lungs of mice. A study of IL-13 signaling in colorectal cancer demonstrated that IL-13Rα2 can serve dual functions as both a decoy receptor and a mediator of mitogen-activated protein kinase signaling cytokine production (124–127).

IL-13 Signaling

IL-13R is localized to the basolateral surface of epithelial cells (128). IL-13 binds to the IL-13Rα1 subunit and recruits the IL-4Rα subunit to form the stable heterodimer, which is found on cells of non-hematopoietic origin (129). This dimerization will activate Janus Kinase-2 (JAK-2) that is constitutively associated with IL-13Rα1 subunit (130). JAK-2 will auto-phosphorylate and recruit STAT6 to the receptor complex. STAT6 is then phosphorylated and activated by JAK-2, resulting in translocation of STAT6 to the nucleus for binding to specific DNA elements in the promoter regions of IL-13 responsive genes and initiation of transcription (Fig.2) (119,126,129).

IL-13 in Asthma

The role of IL-13 in asthma has been studied extensively. Although IL-4 drives T_H2 cell differentiation and development, it does not appear to be necessary for allergic asthma, which

suggests an important role for another cytokine (later recognized as IL-13) (131). Abundant evidence has shown that IL-13 directs many of the processes involved in allergic asthma. In atopic mild asthmatic patients, IL-13 expression was increased in bronchoalveolar lavage (BAL) fluid and cells after nasal allergen challenge (132). As mentioned above, in a genome-wide association study (GWAS), multiple IL-13 polymorphisms have been identified and suggested to be associated with asthma susceptibility, bronchial hyperresponsiveness, and increased IgE levels (75). After allergen is inhaled, T_H2 cells and innate lymphoid cells (ILC-2) secrete IL-13, which influences many cells involved in allergic asthma such as B cells, epithelial cells, and smooth muscle cells. In B cells, IL-13 can induce proliferation and increase IgE and IgG4 production in human (133). In airway epithelial cells, IL-13 can increase mucus secretion, airway permeability, and VEGF accumulation in the airway (122,131,132). IL-13 can also stimulate airway epithelial cells to produce eosinophil chemotactic factors such as eotaxins, leading to eosinophil infiltration in the airways, worsened airway inflammation and asthma exacerbations (Fig.3) (118,134). In smooth muscle cells, IL-13 can also mediate airway hyperresponsiveness (135).

Taken together, these findings have demonstrated that asthma is associated with T_H2 immune response and that IL 13 is an important cytokine involved in allergic asthma.

Interleukin -5 (IL-5)

IL-5 is 15,238 dalton polypeptide with pleiotropic effects on many cell types including basophils and eosinophils in humans (108,136,137). IL-5 was originally described as 'T-cell replacing factor' that is secreted from T cells to stimulate antibody production from activated B cells (138). The receptor for IL-5 consists of IL-5 receptor alpha (IL-5Rα), with low affinity to IL-5 and

common beta-chain (βc), which is shared with IL-3 and GM-CSF. IL-5, produced by Th2 cells, is essential for proliferation, and differentiation, maturation of eosinophils in the bone marrow and their release into the blood in mice and humans (137,139). Mepolizumab, reslizumab, and benralizumab, which are antibodies against IL-5 or IL-5R, induce decreases in blood and sputum eosinophils and in the rate of exacerbations, and in turn improve on lung function especially in asthma, corticosteroid-requiring asthma (140).

Interleukin -9 (IL-9)

IL-9 is 15,909 dalton polypeptide with pleiotropic effects on many cell types. The major source of IL-9 is T_H2 cells and it can also be produced by mast cells (108,141). The IL-9 receptor (IL-9R) is composed of two subunits, IL-9R alpha chain (IL-9Rα), that binds to IL-9, and the IL2R γc, which is common to the IL-2, IL-4, IL-7 and IL-15 receptors (142). Since IL-9 shares its receptor with IL-2 and IL-4, it has been suggested that IL-9 can function similarly to IL-2 and IL-4. IL-9 promotes CD4+ T cells and Th2 cytokine production. Recent studies showed IL-9 has variable effects on regulatory T cell development (143).

B Lymphocytes

B cells are part of the adaptive immune system and can serve as APCs. They are derived from CD34+ progenitor cells in bone marrow and migrate to lymphoid organs such as lymph nodes and spleen (144). B cells express B cell receptors (BCRs) on their cell membrane, which bind to antigens and initiate specific antibody responses (144,145). B cells undergo an isotype switch to IgE to become IgE-producing plasma cells B cells which play an important role in allergic

sensitization. T_H2 cells induce the switching process by IL-4 or IL-13. Also, interaction of the cell surface marker CD40 with its ligand (CD40L) expressed on activated T cells induces the switching process. Once IgE-positive B cells are formed, they can differentiate into IgE-producing plasma cells. Finally, some plasma cells stay in the spleen while others return to the bone marrow or invade inflamed tissues, where they survive from several months to a lifetime in survival niches as resident, immobile cells (115,146) .

Eosinophils

Eosinophils were first observed by Wharton Jones in 1846 in unstained preparations of peripheral blood and were then described by Paul Ehrlich in 1879 in blood films stained using acidic dyes (147). He observed that these cells that contain many granules have high affinity for a pink synthetic dye (called eosin) that is produced by the action of bromine on fluorescein (147). Eosinophils are part of the innate immune system and derived from CD34+ progenitor cells. The transcription factor GATA1 is critical for their differentiation and maturation in bone marrow induced by cytokines such as IL-3, IL-5, and GM-CSF (137,148). IL-5 is the most selective for differentiation, proliferation, maturation, and survival of eosinophils, while IL-3 and GM-CSF alone cannot induce eosinophil development (137). Scientists observed the connection between the presence of eosinophils in BAL fluid and allergic asthma, and since then have tried to identify the role of eosinophils in asthma. Airway epithelial cells, fibroblast cells, and smooth muscle cells can recruit eosinophils to the site of inflammation when they are stimulated with IL-4 and/or IL-13 (134,149–153). Eosinophils can exacerbate asthma by degranulation and release of proinflammatory molecules including: reactive oxygen species, eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin

(EDN), cytokines, and chemokines all thought to be important in the underlying AHR and local inflammation, tissue damage, and necrosis (154–156).

Upon allergen exposure, high numbers of eosinophils infiltrate the airway and the magnitude of their recruitment is associated with the severity of disease (157). Eosinophils also express MHC II and can act as APC, uptake antigens and present them to T cells in lymph nodes (158).

Chemotaxis of Eosinophils

There are several chemokines able to induce migration and activation of eosinophils, including eotaxins, CCL7, CCL13, and CCL5 (159–162). However, eotaxins are more effective eosinophil chemoattractant than CCL5 and more selective for eosinophil recruitment *in vivo* (163,164). Eotaxins are CC chemokines, produced by airway epithelial cells, airway smooth muscle cells, vascular endothelial cells, and macrophages (150–152). They are potent chemoattractants for eosinophils and recruit eosinophils by stimulating CC chemokine receptor 3 (CCR3), a G-protein-coupled receptor (GPCR) on the eosinophil surface. CCR3 expression is restricted to cells involved in allergic inflammation such as Th2 cells, eosinophils, mast cells and basophils. Eotaxins also signal exclusively through CCR3 (165,166).

Eotaxin-1/CCL11

CCL11 was firstly identified in a guinea-pig model of airway allergic inflammation and named eotaxin (163). Later on it was renamed eotaxin-1 to differentiate it from two chemokines with similar functions, eotaxin-2 (CCL24) and eotaxin-3 (CCL26). CCL11 is constitutively expressed in the lung of healthy individuals and its levels increase in the lung and sputum following

allergen challenge (167). In both animals and humans CCL11 accounts for early recruitment of eosinophils in the lungs following allergen challenge (almost 6 hr of recruitment) but not for the sustained recruitment beyond 24 hr (151). $CCL11^{-/-}$ knockout mice show reduction in the number of eosinophils recruited in the lung after allergen challenge, but this reduction is moderate, and there are still enough eosinophils recruited to the lung to cause disease (168). CCL11 neutralizing antibodies showed similar results, with decreased numbers of eosinophils in the bloodstream and the lung of allergen-challenged mice, but not complete inhibition of airway inflammation (169). This indicates CCL11 is not alone in the process of eosinophil recruitment and other family members of chemotactic factors such as CCL24 and CCL26 may be important.

Eotaxin-2/CCL24

CCL24 has physiological activity similar to that of CCL11 and shares almost 39% structural homology with it (165,166). Ying and colleagues found a correlation between 24-hr tissue eosinophilia and CCL24 expression in human allergen-induced cutaneous responses, suggesting that this cytokine may be important in late-stage allergen-induced inflammation. Increased CCL24 expression has also been shown in atopic and non-atopic asthmatics (170).

Eotaxin-3/CCL26

CCL26 also has physiological activity similar to that of CCL11 and CCL24. CCL26 levels were high in patients with atopic dermatitis, chronic rhinosinusitis, eosinophilic esophagitis, and Churg-Strauss vasculitis, all diseases where eosinophils play a significant pathogenetic role. This finding suggests an important role for CCL26 in the pathogenesis of eosinophilic diseases (171–174). In asthmatic patients, Berkman and colleagues found that expression of CCL26 (not

CCL11 or CCL24 mRNA) was increased 24 hr after allergen challenge, suggesting that this chemokine may account for continuing eosinophil recruitment (167). In atopic dermatitis patients, CCL26 serum level but not CCL24 was found to be significantly high in these patients (172). In an *ex-vivo* study, CCL26 was found to be more effective on eosinophil migration in asthmatics than CCL11 or CCL24 (175). As for CCL11 and CCL24, CCL26 can be induced by IL-4 and IL-13 through activation of the IL-4Rα /STAT-6 pathway *in vitro* and can be attenuated by the glucocorticoid budesonide (176,177).

Taken together, these results suggest a significant role of eotaxins, especially CCL26, in asthmatics and the recruitment of eosinophils to the airway.

1.4 Rationale, Hypothesis, and Objectives

1.4.1 Rationale

Eosinophils are the hallmark of certain phenotypes of asthma and play a central role in the pathogenesis of asthma. They are found in increased numbers in blood and sputum in severe asthma and asthma exacerbations (178). Many studies (such as studies targeting IL-5 and its receptor) have used different approaches to decrease eosinophilic infiltration in asthmatics. There is evidence that bacteria are clinically relevant contributors to asthma exacerbations (179–182). A study by Bass demonstrated a decrease in the eosinophil count in blood in experimentally induced bacterial infection in mice (181). Moreover, typhoid infection is well described to be associated with peripheral blood eosinopenia (low eosinophils) (182). A study by Lipkin of 75 adult patients with positive blood cultures demonstrated that as the number of positive blood culture results per patient increased, the percentage of eosinophils in the peripheral blood

decreased (183). The rapid decrease in the number of eosinophils after bacterial infection suggests the ability of bacteria to prevent eosinophils recruitment through inhibiting eosinophil chemoattractant, such as eotaxins. Since human bronchial epithelium is a major source of eotaxins and their secretion results in the attraction of eosinophils into airways and they are frequently exposed to pathogens including LPS, we used an experimental model of *in vitro* cultured human bronchial epithelial cell line (BEAS-2B) to study the effects of LPS in IL-13 -induced CCL26 induction (Figure 3).

1.4.2 Hypothesis

We hypothesize that LPS, a product of gram negative bacteria, prevents the release of eotaxins from the airways epithelium and through this mechanism may decrease eosinophil accumulation in the airways.

1.4.3 Objectives

1. Study the effects of IL-13 on the production of eotaxins by human airway epithelial cells.
2. Study the interaction between LPS and IL-13 and the induction of CCL26 from airway epithelial cells.
3. Study the mechanisms mediating the inhibitory effect of LPS on IL-13 -induced CCL26 production

Figure 1

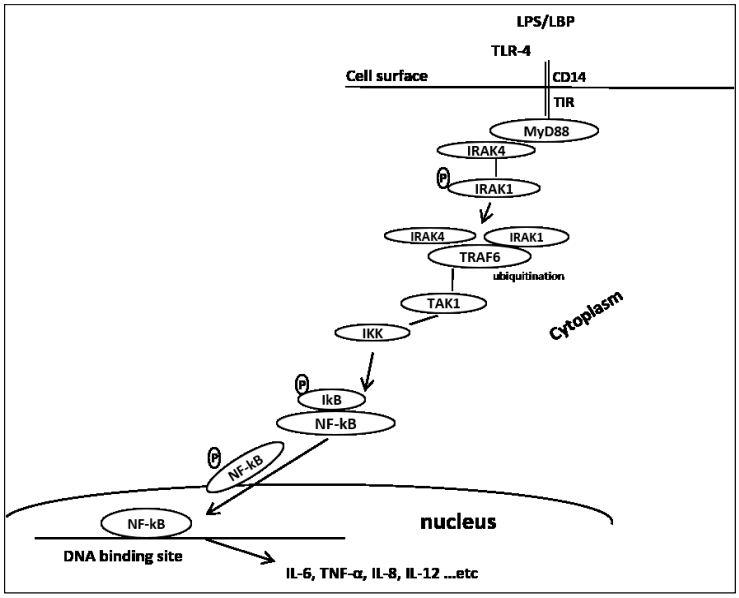


Figure 1: LPS Signaling Pathway. LPS is detected by TLR-4 on the cell surface and recruit the adaptor protein MyD88 that will activate several signaling pathways including ubiquitination and phosphorylation. Ultimately, LPS activates NF-κB, a transcription factor, and induces transcription of proinflammatory mediators such as IL-6, TNF-α, and IL-8

Figure 2

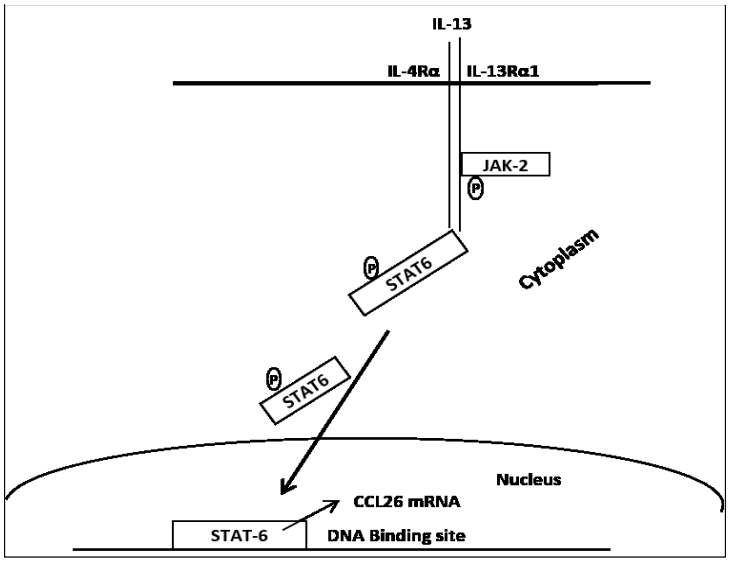


Figure 2: IL-13 signalling pathway. Il-13 binds IL-13Rα1 which dimrizes with IL-4Rα and then induces JAK-2 to binds to their cytoplamic tail. JAK-2 autophosphorylates and recruit STAT6. STAT6 will phosphorylated by JAK-2 and then translocates into the nuclous, which will induce CCL26 mRNA transcription.

Figure 3

Figure 3: IL-13 -mediated eosinophils recruitment in allergic asthma. IL-13, a T_H2 cytokine, plays an important role in allergic asthma. IL-13 induces epithelial cells to release eotaxins. Eotaxins will recruit eosinophils from blood into the lung and worsen the asthma symptoms.

Chapter 2

Materials and Methods

2.1 Materials

All materials/chemicals were used according to manufacturers' instructions as well as the instruction of Environmental, Health and Safety (EHS) of the University of Alberta and Work Hazardous Materials Information System (WHMIS).

2.1.1 Cell Culture:

- Bronchial Epithelial Cell Line (BEAS-2B), from American Type Culture Collection (ATCC® CRL-9609), Manassas, Virginia, United State of America (USA).
- Modified Eagle Medium (DMEM) 1 g/L D-Glucose, L-Glutamine, and 110 mg/L sodium pyruvate (Cat # 11885-092), Penicillin Streptomycin (Pen Strep) (Cat # 15140-122), and Fetal Bovine Serum (FBS), Hyclone Defined (cat # SH30070.03) from Gibco® by Thermo Fisher Scientific, Waltham, Massachusetts, USA.
- Trypsin/EDTA Solution (Cat # CC-5012), from Lonza, Allendale, New Jersey, USA.
- Phosphate Buffered Saline (PBS) pH 7.4 (cat # P-5368), from Sigma-Aldrich, St. Louis, Missouri, USA.
- Tissue Culture Flask –75 cm^2 (cat # 353136), 25 cm^2 (cat # 353108), and Tissue Culture Plate – 12 well (cat # 353043), from Falcon® by Thermo Fisher Scientific, Waltham, Massachusetts, USA.

2.1.2 Reverse Transcription and Quantitative Polymerase Chain Reaction (RT-qPCR):

- RNeasy® mini kit (cat # 74104) and QIAshredder (Cat # 79654), from Qiagen, Hilden, Germany.
- Axygen PCR® strip tubes PCR-0208-CP-C (cat # 321-10-061), from F Fisher Scientific by Thermo Fisher Scientific.
- Oligo $(dT)_{12-18}$ Primer (cat # 18418-012) , Deoxynucleotides (dNTPs) (cat # 10297018), 5 X First Strand Synthesis Buffer (cat # y02321), DTT (0.1 M) (cat # y00147), RNaseOut™ Recombinant Ribonuclease Inhibitor (cat # 10777-019), and M – MLV Reverse Transcriptase (cat # 28025-013) from Invitrogen by Thermo Fisher Scientific.
- TaqMan® Gene Expression Master Mix (cat # 4369016), TaqMan® gene Expression Assays CCL26 (cat # HS00171146-m1), IL-8 (cat # HS00174103-m1), and GAPDH (cat # HS02758991-g1), and UltraPure™ DNase/RNase-Free Distilled Water (cat # 10977015), from Applied Biosystems ™ by Thermo Fisher Scientific.

2.1.3 Enzyme-Linked Immunosorbent Assay (ELISA):

- Human CCL26/Eotaxin-3 (cat # DY346) kit contains: Capture Antibody (cat # 840501), Detection Antibody (cat # 840502), Standard (cat # 840503), and Streptavidin-HRP (cat # 890803), and Substrate Solution: Color Reagent A (cat # 895000), and Color Reagent B (cat # 895001), from R&D Systems, Inc.**,** Minneapolis, Minnesota, USA.

- Stop Solution (Sulfuric Acid solution 1N, H_2SO_4) (cat # SA212-4), Tween® 20 (cat # BP337-500) from F Fisher Scientific by Thermo Fisher Scientific.
- Microtest™ 96-Well ELISA Plate (Cat # 353279), from Falcon® by Thermo Fisher Scientific.
- Autowash II Microplate Washer WellWash ™ from Labsystems by Thermo Fisher Scientific.
- PowerWave XS Microplate Spectrophotometer from BioTek, from Winooski, Vermont, USA.
- Bovine Serum Albumin (BSA) (cat # A3059-50G), from Sigma-Aldrich.
- Reagent Diluent: 1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered
- Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2-7.4

2.1.4 Western Blot:

- Purified Mouse Anti-Stat6 (cat # 611290), from BD Transduction Laboratories™, San Jose, California, USA.
- Phospho-Stat6 (Tyr641) (cat # 9361), from Cell Signaling Technology®, Danvers, Massachusetts, USA.
- Anti-JAK2 (phosphor Y1007+Y1008) antibody (cat # ab32101), Anti-JAK2 antibody (cat # ab108596) from Abcam.
- IRDye® 800CW Goat (polyclonal) Anti-Rabbit IgG (H+L) (cat # 926-3221), IRDye® 680 Goat Anti- Mouse IgG (H+L) (cat # 926-3220), from Li -Cor, Lincoln, Nebraska, USA.
- Odyssey® Blocking Buffer (PBS) (cat # 927-40100), from Mandel Scientific, Guelph, Ontario, Canada.

- Mini-PROTEAN® TGX™ Gels 10 % (cat # 456-1034), Nitrocellulose Membranes, 0.2 µm (cat # 162-0112), from Bio-Rad Laboratories, Hercules, California, USA.
- 3 X Red Loading Buffer Pack (cat # B7709S), 30 X Reducing Agent 1.25 M DTT (cat # B7705S), from New England BioLabs® Inc, Ipswich, Massachusetts, USA.
- Tween® 20 (cat # BP337-500), from Fisher Biotech.
- RIPA Lysis Buffer System (Cat # sc-24948), from Santa Cruz Biotechnology, Dallas, Texas, USA that contains:
  - Vial 1: 1 X lysis Buffer: 1 X TBS, 1 % Nonidet P-40, 0.05 % Sodium deoxycholate, 0.1 % SDS, and 0.004 % sodium azide.
  - Vial 2: PMSF in DMSO.
  - Vial 3: Protease inhibitor cocktail (PIC) in DMSO.
  - Vial 4: Sodium Orthovanadate in water.

- **10 X Running Buffer, for 1 L:**
  - 30.3 g Tris – Base (cat # T1503-10KG), from Sigma-Aldrich.
  - 10 g SDS (cat # 161-0302), from Bio-Rad Laboratories.
  - 144.15 g Glycine (cat # G48-12), from Sigma-Aldrich.
  - pH between 8.3 – 8.9

- **1 X Transfer Buffer:**
- 9.09 g Tris – HCL (cat # BP153-1), from Fisher BioReagent® by Thermo Fisher Scientific.
- 43.2 g Glycine (cat # G48-12), from Sigma-Aldrich.

- 600 ml 100 % Methanol (cat # A452-4), from Fisher Chemical by Thermo Fisher Scientific.
- Top up to 3 L with double distilled H_2O.

2.1.5 Stimuli and Treatment:

- Human Recombinant IL-13 (cat # 213-ILB), from R&D Systems, Inc**.**
- Escherichia Coli (E. Coli) lipopolysaccharide (LPS) (cat #L4391-1MG), Sodium Orthovanadate (cat # 450243-10G), Cycloheximide Solution (cat # C4859-1ML), Actinomycin D (cat # A 4262), from Sigma-Aldrich.
- Curcumin (cat # 2841), Arctigenin (cat # 1777), Bengamide B (cat# 5273), from Tocris Cookson, Bristol, United Kingdom

2.2 Methods

2.2.1 Cell Culture:

BEAS-2B Cells aliquots (passage 39) were stored in liquid nitrogen. BEAS-2B cells were propagated (2 X 10^3 cells/ cm^2) in DMEM medium supplemented with 10,000 units/ml of Penicillin, 10,000 ug/ml of Streptomycin, and 10 % FBS. The cells were cultured at 37°C, 5% CO2, and 90% humidity, and fresh media was added one day after passage and then every two days.

2.2.2 Cell Stimulation:

Experiments were performed in 12- well plates using cells between passages 43 and 60. The stimulations/treatments were preceded with 24 hr of starvation by using DMEM media with no

FBS to avoid any effect of FBS on BEAS-2B cells' response. When BEAS-2B cells became confluent in T75 or T25 flasks, they were trypsinized, resuspended in a culture medium, and seeded into 12-well plate at a density of 5.0×10^4 cells per well. Upon 80-90 % confluency, the cells were serum deprived for 24 hr and then activated in fresh serum free media for up to 24 hr. Cells were activated with IL-13 (20 ng/ml), LPS (0.37 - 10 μg/ml) or the two in combination for 2, 6, or 24 hr.

To understand the mechanism of the LPS effect on IL-13 signaling, we added inhibitors of various signaling molecules activated by LPS or by IL-13 (e.g. curcumin, sodium orthovanadate, cycloheximide, actinomycin D … etc.) for the indicated time with or without washing the cells with PBS twice.

2.2.3 RNA Extraction, Reverse Transcription and Quantitative Polymerase Chain Reaction (RT-qPCR):

RNA Extraction: At the end of treatment in culture, the cells were washed with PBS and then 3.5 μl β-mercaptoethanol was added into 350 μl RLT buffer which contains a guanidine isothiocyanate and used to lyse the cells. The cell lysate was added into QIAshredder tubes and centrifuged at full speed for 2 min for shredding and homogenizing the lysate. 350 μl of 70% ethanol was added to the lysate for "perfect binding condition" as mentioned per manufacturer's instructions. The lysate was then loaded onto RNeasy mini spin column tubes and centrifuged for 15 sec at 8000 g. The flow through collection tube was discarded and 700 ul of buffer RW1 was added to the membrane/filter onto RNeasy spin column and centrifuged for 15 sec at 8000 g.

Again, the flow through collection tube was discarded and 500 µl of RPE buffer was added to the membrane/filter onto RNeasy spin column and centrifuged for 15 sec at 8000 g. The collection was discarded and another 500 µl of RPE buffer was added to the membrane/filter onto RNeasy spin column and centrifuged for 2 min at 8000 g. Finally, the collection tube was removed and replaced with a 2 ml eppendorf tube from Qiagen kit. 30 µl RNA/DNA free water was added to the RNeasy spin column connected with the eppendorf tube and centrifuged for 1 min at 8000 g. The concentration of RNA eluted in 30 µl RNA/DNA free water was measured using NanoDrop™ 2000/2000c Spectrophotometers from Thermo Fisher Scientific. 1 µl of concentrated RNA was pipetted directly onto pedestal of the NanoDrop Spectrophotometer to measure the RNA concentration.

Reverse Transcription: 0.5 µg of RNA was used for reverse transcription of complementary DNA (cDNA). 0.5 µg of oligo dT and 1µl of 10 mM dNTPs (final concentration - 0.5 mM dNTPs) with a specific amount of Ultra-Pure DNase/RNase-Free Distilled Water were added to make a total volume of 12 µl. The volume was heated to 65 °C for 5 min in PCR machine and cooled on ice. 4 µl first strand (FS) buffer, 2 µl Dithiothreitol (DTT), and 1 µl recombinant ribonuclease inhibitor (RNase out) were added and mixed into the volume and heated for 2 min 37 °C in a PTC-100 programmable thermal controller machine from Bio-Rad Laboratories. Later, 1 µl of Moloney Murine Leukemia Virus (MMLV) enzyme, which is reverse transcriptase, was added, mixed and heated into the volume for 50 min at 37 °C, and finally 50 min at 70 °C. cDNA is ready for PCR.

Quantitative Polymerase Chain Reaction: qPCR was performed using Taqman gene expression assay according to the manufacturer's instructions. Briefly, 2 ul of cDNA was mixed with 10 ul of gene expression master mix, 1 ul of Taqman gene expression assay and 7 µl of

sterile DNase/RNase free H_2O for a final total volume of 20 µl then aliquoted into PCR Axygen 8 - well strip tubes. Also, we did a no template (NTC) control, which they are samples without cDNA to detect any contamination of DNA. PCR was performed in an Eppendorf Master Cycler Real Plex machine from Thermo Fisher Scientific using 40 cycles of 95 °C for 15 sec, then 60 °C for a min.

2.2.4 Enzyme-Linked Immunosorbent Assay (ELISA):

The human CCL26/Eotaxin-3 DuoSet™ ELISA kit was used to measure CCL26 protein released from BEAS-2B cells. 96-Well ELISA Plates were coated by adding 100 µl of 1 µg/ml capture antibody, sealed with adhesive plate sealing, and incubated overnight at room temperature. Next day, the plate was washed 3 times with wash buffer using Autowash II Microplate Washer. 300 µl Reagent diluent was added to each well and incubated at room temperature for 60 min to block non-specific binding to the capture antibody. The plate was washed again and 100 µl of samples and standards in reagent diluent were added, covered, and incubated for 2 hr at room temperature. The plate was washed again and 100 µl of detection antibody was added into each well, covered, and incubated for 2 hr at room temperature. The ELISA plate was washed as previous described and 100 µl of 1:200 streptavidin-HRP in reagent diluent was added to each well, covered, and incubated in dark area for 20 min at room temperature. 100 µl of substrate solution was added to each well, covered, and incubated in dark area for 20 min at room temperature. Finally, 50 µl of sulfuric acid (stop solution) was added to each well, gently tapped and mixed for 2-5 min.

The plate was read by PowerWave XS Microplate Reader from Bio Tek to determine the optical density of each well and a seven point for standard curve (from 4000 pg to zero pg) was been made using graph prism to calculate the concentration of CCL26 in the samples.

2.2.5 Western Blotting:

At the end of the cell treatments, the 12-well plate was placed on ice immediately and media were aspirated.

Cell Lysate: 200 µl RIPA buffer containing Protease inhibitor cocktail (PIC) (1 ml of RIPA/20 µl of PIC) were added to each well to lyse the cells. The lysate was vortexed and incubated at 4 °C for 15 min for complete lysis. The lysate was then spun down at 16,000 g for 10 min to remove cell debris. Later, 150 µl of supernatant from lysated samples was transferred to 1.5 ml tubes and mixed with 75 µl of 3 X Red Loading buffer. The cell lysates were boiled for 5 min to break down the secondary structure of protein.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Gel Transfer and Immunoblot: The lysate was loaded in 10 % precast gel from Bio-Rad, and the gel was connected to Bio-Rad Power PAC 200 with 160 volts and constant amperage for 75 min to separate proteins based on size by gel electrophoresis. The proteins were then transferred into 0.2 µm Nitrocellulose Membranes by electrophoresis transfer using transfer buffer and Bio-Rad device with 400 mA and constant voltage for one hr at 4 °C. Following transfer, the membrane was blocked with 50 % odyssey blocking buffer in PBS for 1 hr at room temperature on VWR® Rocking Platform Shaker. The membrane was then incubated in primary antibody containing 50 % odyssey blocking buffer, 50 % PBS, and 0.05% Tween 20 at 4 °C overnight on the Shaker.

Next day, following three washings with washing buffer (0.05 % tween 20 in PBS) to remove unbound primary antibody, the membranes were incubated with the secondary antibody (diluted in 50 % odyssey blocking buffer, 50 % PBS, and 0.05% Tween 20) for 1 hr at room temperature on a shaker. Following the incubation, the membrane was washed again three times for 5 min/wash. Finally, the membrane was washed with PBS to remove remaining washing buffer and then placed on Li-Cor Odyssey Scanner and ImageJ software was used to quantify protein bands.

2.2.6 Statistics

Statistical analysis was performed using one-way analysis of variance (ANOVA) when there were three or more sets of data or Students t-test (two-tailed) when there were only two sets of data to determine whether there were any statistically significant differences by using the GraphPad Prism 5 software, GraphPad Software, San Diego, CA. Statistical significance was achieved when $P < 0.05$.

Chapter 3

Results

3.1 IL-13 Induced CCL26 but not CCL24 or CCL11 mRNA Upregulation in BEAS-2B Cells

It has been reported that airway epithelial cells produce several CCR3 ligands, such as CCL11/eotaxin-1, CCL24/eotaxin-2, and CCL26/eotaxin-3, both *in vitro* in response to T_H2 cytokines such as IL-13 and IL-4 and *in vivo* in response to allergen challenge or in asthma (151,167,169,184,185). To study whether IL-13 induces CCL11, CCL24 or CCL26 mRNA in the human bronchial epithelial cell line BEAS-2B, we stimulated BEAS-2B cells with IL-13 (20 ng/ml) for 24 hr. IL-13 strongly upregulated CCL26 mRNA (78.3 ± 3.9 fold over unstimulated cells, n= 13, $p < 0.001$) (Fig.4) while there was no detection of CCL11 or CCL24 mRNA in resting or IL-13 activated cells. Since CCL26 was the only eotaxin induced by IL-13 on BEAS-2B cells, we then studied in more detail IL-13-induced CCL26 mRNA upregulation in BEAS-2B cells.

3.2 LPS Inhibited IL-13 -Induced CCL26 mRNA Expression in BEAS-2B Cells in a Concentration and Time - Dependent Manner

LPS can induce a strong immune response and cause the release of critical proinflammatory cytokines through activation of TLR-4 in airway epithelium (186). To investigate the potential effect of LPS on eosinophilic chemoattractant induction by IL-13 stimulation, BEAS-2B cells were incubated with IL-13 (20 ng/ml), LPS (10 μg/ml) alone or together for 24 hr. LPS on its own had no effect on CCL26 mRNA levels in BEAS-2B cells, but inhibited the effect of IL-13 on CCL26 mRNA levels (Fig.5, A).

Moreover, using trypan blue dye exclusion, we showed that there were 5.2 % dead cells in control cells, 4.7 % in IL-13 -treated cells, 5.4% in LPS -treated cells, and 5.4% in IL-13+LPS -treated cells (experiment done once). These data indicate that the effect of LPS o0n CCL26 is not the result of cell toxicity.

A concentration of 10 μg /ml of LPS completely inhibited IL-13 -induced CCL26 mRNA expression. To identify the lowest concentration of LPS that mediates inhibition, we stimulated cells with IL-13 (20 ng/ml) in the presence of different LPS concentrations (10, 3.33, 1.11, and 0.37 μg/ml) for 24 hr. We found that concentrations of LPS from 10 to 1.11 μg/ml significantly inhibited IL-13 -induced CCL26 mRNA expression, while 0.37 μg /ml of LPS had no significant inhibitory effect (Fig.5, B).

In initial experiments, we found that the 24 hr of incubation of IL-13 and LPS together leaded to complete inhibition of CCL26 mRNA upregulation. We next studied the time course of this effect. BEAS-2B cells were treated with IL-13 and LPS (10 μg/ml) for 2, 6, or 24 hr. LPS induced significant inhibition of IL-13 -induced CCL26 mRNA at 2 and 6 hr, but only at 24 hr it inhibited CCL26 induction completely (Fig.5, C).

3.3 LPS Inhibited IL-13 -Induced CCL26 Protein Release from BEAS-2B Cells in a Concentration and Time - Dependent Manner

The observation that LPS inhibited IL-13 -induced CCL26 mRNA expression was further confirmed at the protein level. We analyzed CCL26 protein released in supernatants of IL-13 and/or LPS stimulated BEAS-2B cells by ELISA. IL-13 stimulation strongly induced CCL26 protein release (1462 ± 55.1 pg/ml, compared to undetectable levels of CCL26 protein

release in unstimulated cells, n=13, $p < 0.001$), while LPS alone did not induce CCL26 release. LPS was able to inhibit (93% ± 4.4 % inhibition) IL-13 -induced CCL26 release when the cells were stimulated with IL-13 and LPS simultaneously (Fig.6, A). We also stimulated cells with IL-13 (20 ng/ml) with different LPS doses (10, 3.33, 1.11, and 0.37 μg/ml) for 24 hr. Concentrations of LPS from 10 to 1.11 μg/ml inhibited IL-13 -induced CCL26 protein release, but 0.37 μg/ml of LPS mediated no inhibition (Fig.6, B).

LPS effect on IL-13 -induced CCL26 release following different co-incubation times was also evaluated. BEAS-2B cells were stimulated with LPS and IL-13 for 2 hr, 6 hr and 24 hr. Supernatants were collected and ELISA used to measure CCL26 release. LPS mediated significant inhibition of CCL26 protein release in the supernatant at all 3 time points tested (Fig.6, C).

These results support the qRT-PCR results that showed decreased CCL26 mRNA expression in the presence of LPS (Fig.5).

3.4 LPS Effect on IL-13 – Mediated Signaling

IL-13 signaling involves tyrosine phosphorylation and receptor association of a number of downstream molecules including Janus kinase 2 (JAK-2) and Signal transducer and activator of transcription 6 (STAT6) (Fig.2). We hypothesized that inhibition of IL-13 -induced CCL26 mRNA upregulation by LPS was associated with inhibition of IL-13 -induced JAK-2 and STAT6 phosphorylation. To accomplish this, BEAS-2B cells were treated with IL-13 (20 ng/ml), LPS (10 μg/ml) or both for different periods up to 30 min. Western blot analysis showed IL-13 -induced STAT6 phosphorylation was time dependent; within 5 min of activation slight

phosphorylation was observed, which peaked after 30 min of IL-13 stimulation (Fig.7, A). LPS alone did not induce STAT6 phosphorylation. However, when LPS was added to the cells together with IL-13 and incubated for 30 min, LPS reduced IL-13 -induced STAT6 phosphorylation (Fig.7, B and C).

Previous studies have demonstrated STAT6 is tyrosine phosphorylated by JAK-2 kinase following IL-13 receptor activation. We next determined whether LPS also inhibits IL-13 -induced JAK-2 phosphorylation. To investigate this postulate, BEAS-2B cells were treated with IL-13 (20 ng/ml), LPS (10 μg/ml) or both together for 30 min. Western blot analysis showed LPS alone did not induce JAK-2 phosphorylation. However, when LPS added together with IL-13 to the cells and incubated for for 30 min, LPS significantly reduced IL-13 -induced JAK-2 phosphorylation (Fig.7, D and E).

3.5 LPS Inhibited IL-13 -Induced CCL26 mRNA Upregulation when It is Added Together With IL-13 as Well as 30 Min prior to IL-13 stimulation.

We have shown that simultaneous stimulation with LPS and IL-13 inhibited IL-13 -induced CCL26 and also decreased IL-13 -mediated JAK-2/STAT6 phosphorylation. IL-13 induced CCL26 is dependent on STAT6 phosphorylation and we showed that IL-13 -induced STAT6 phosphorylation peaks after 30 min (187). Taken together, we hypothesized that to inhibit IL-13 -induced CCL26, LPS needed to be added simultaneously with IL-13 or by 30 min after IL-13 but not as late as 90 min after IL-13 when there is little phosphorylation of STAT6. To test this, BEAS-2B cells were incubated with one of either IL-13 for 30 and 90 min or LPS for 30 min and

then LPS was added to IL-13 -stimulated cells or IL-13 was added to LPS - stimulated cells and cells were incubated for a further 24 hr. We found that LPS inhibited CCL26 mRNA when it was added together with IL-13, as well as when added 30 min before or after IL-13 stimulation. However, LPS did not inhibited IL-13 -induced CCL26 mRNA when LPS was added to the culture media 90 min after the initiation of IL-13 stimulation (Fig.8).

3.6 NF-κB Antagonists Prevented The Inhibitory Effect of LPS on IL-13 - Mediated CCL26 mRNA Upregulation and Protein Release

NF-κB is a transcription factor and a central regulator of LPS effects. Thus, we postulated that LPS inhibition of IL-13 -induced CCL26 is mediated through NF-κB activation. To test this postulate, we incubated the cells with three different NF-κB inhibitors, curcumin (Curc.) (10 μM), bengamide B (Beng. B.) (1 μM), and arctigenin (Arc.) (1 μM), separately for 2 hr before adding IL-13 alone, LPS alone or together for 24 hr. NF-κB inhibitors reduced the effect of LPS on IL-13 -induced CCL26 mRNA upregulation (Fig.9). However, curcumin and Bengamide B also enhanced IL-13 -induced CCL26 mRNA upregulation (Fig.9, A, C). We also tested LPS - induced IL-8 mRNA upregulation, a known positive control for NF-κB activation when adding curcumin, an inhibitor of NF-κB. We found curcumin inhibited LPS -induced IL-8 mRNA upregulation (Fig.9, D).

These three NF-κB inhibitors also prevented the effect of LPS on IL-13 -induced CCL26 protein release (Fig.10). This result also supports the qPCR results (Fig.9).

3.7 Inhibition of New Protein Synthesis Prevented The Inhibitory Effect of LPS on IL-13 -Mediated CCL26 mRNA Upregulation.

NF-κB is a transcription factor activated by LPS. When NF-κB becomes activated, it translocates to the nucleus and activates gene transcription. We showed that antagonists to NF-κB prevented LPS from inhibiting IL-13 -induced CCL26. This observation may suggest that LPS-mediated NF-κB activation induced the synthesis of one or more proteins that are required for LPS-mediated CCL26 inhibition. To test this, BEAS-2B cells were incubated with the translational inhibitor cycloheximide (10 μg/ml), for 30 min before being stimulated with LPS and/or IL-13 for 24 hr. In the presence of cycloheximide, LPS did not inhibit IL-13 -induced CCL26 mRNA upregulation (Fig.11, A). We also did ELISA; just confirm there is no CCL26 protein release by IL-13 in presence of cycloheximide (Fig.11, B).

3.8 Tyrosine phosphatase inhibitor did not prevent the inhibitory effect of LPS on IL-13 mediated CCL26 mRNA upregulation.

Above, we showed that LPS decreased tyrosine phosphorylation of JAK-2 and STAT6 in IL-13 mediated JAK-2/STAT6 phosphorylation. Therefore, we hypothesized activation of a tyrosine phosphatase activity might cause this reduction in tyrosine phosphorylation. To test this hypothesis, we incubated the cells with IL-13 alone or in combination with LPS for 24 hr in the presence or absence of tyrosine phosphatase inhibitor sodium orthovanadate (Na3VO4) (10 μM or 25 μM). As predicted, Na_3VO_4 enhanced IL-13 effect on CCL26 mRNA. However, in the presence of Na_3VO_4, LPS inhibited IL-13 -induced

CCL26 mRNA; indicating that tyrosine phosphatase activity was not the cause of inhibitory effect of LPS on CCL26 mRNA (Fig.12).

Figure 4

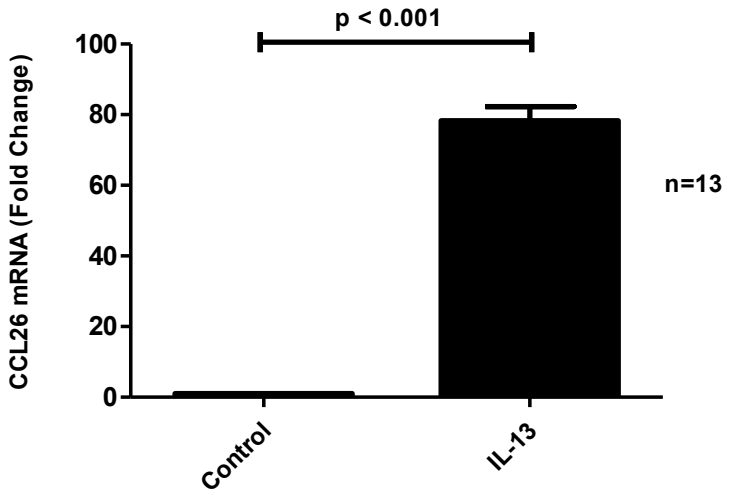
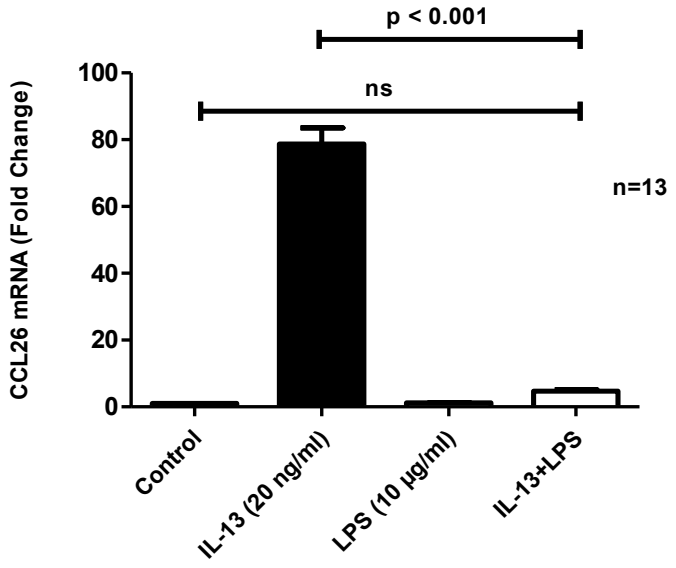


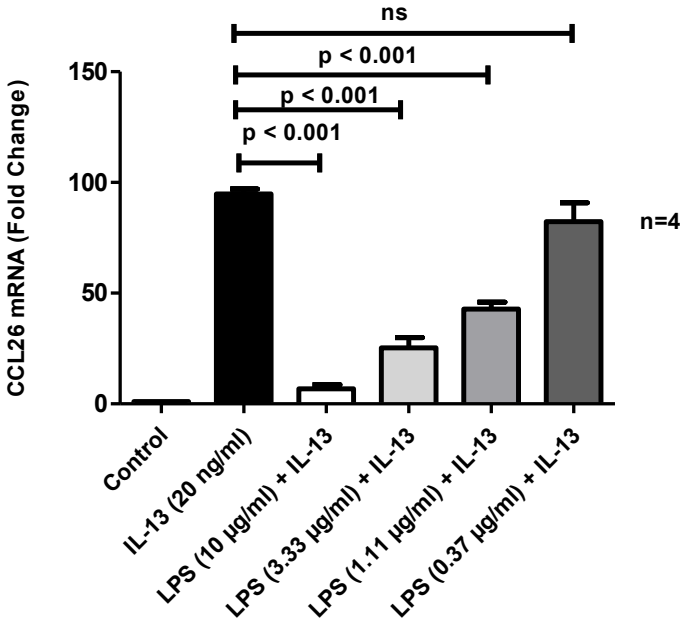
Figure 4: Effect of human recombinant IL-13 on CCL26/Eotaxin-3 mRNA expression by BEAS-2B, human airway epithelial cells. Cells were incubated in the presence or absence of IL-13 (20ng/ml) for 24 hr and then harvested and RNA isolated. CCL26 mRNA was determined by qRT-PCR. There was no detection of CCL11 or CCL24 mRNA in resting or IL-13 activated cells. Results shown are "mean ± SEM" from 13 independent experiments. P < 0. 001 compares the value in IL-13 -stimulated cells with the unstimulated cells. P value and statistical analysis have been done by paired t-test analysis.

Figure 5

A)


p < 0.001
ns
100
80
60
40
20
0
CCL26 mRNA (Fold Change)
n=13
Control
IL-13 (20 ng/ml)
LPS (10 µg/ml)
IL-13+LPS

B)


ns
p < 0.001
p < 0.001
p < 0.001
150
100
50
0
CCL26 mRNA (Fold Change)
n=4
Control
IL-13 (20 ng/ml)
LPS (10 µg/ml) + IL-13
LPS (3.33 µg/ml) + IL-13
LPS (1.11 µg/ml) + IL-13
LPS (0.37 µg/ml) + IL-13

C)

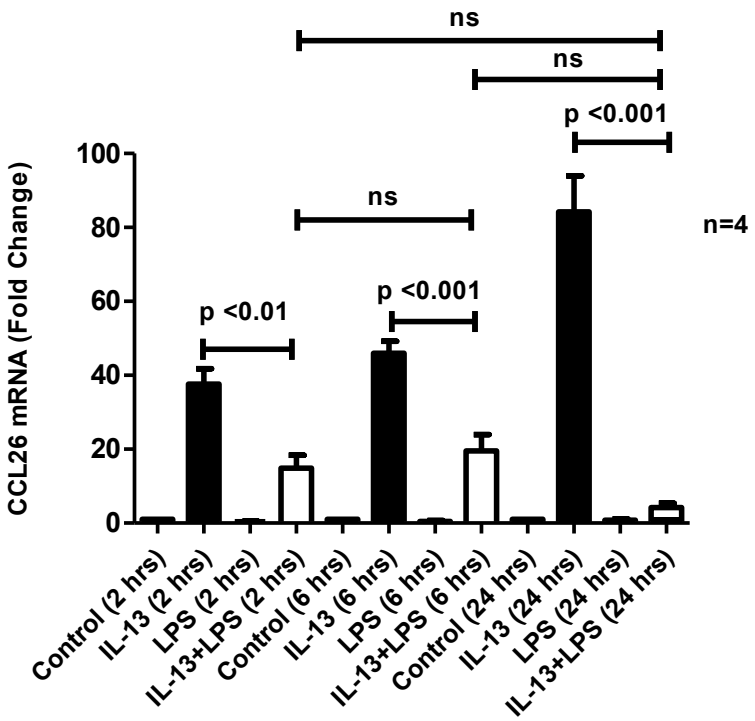
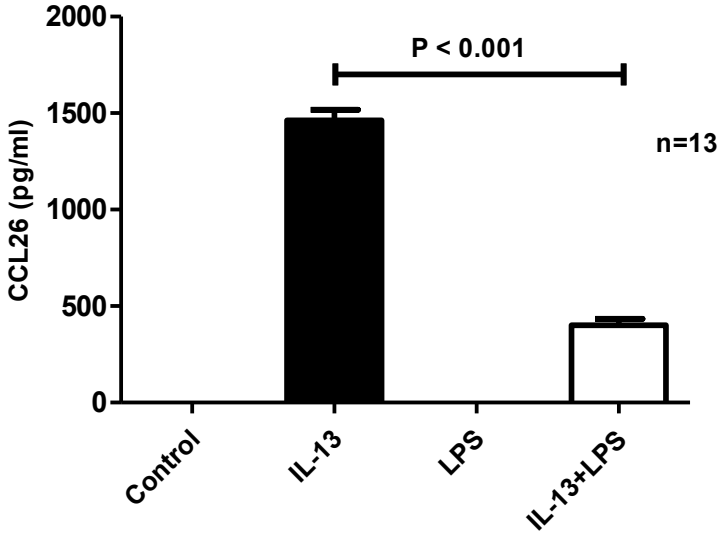


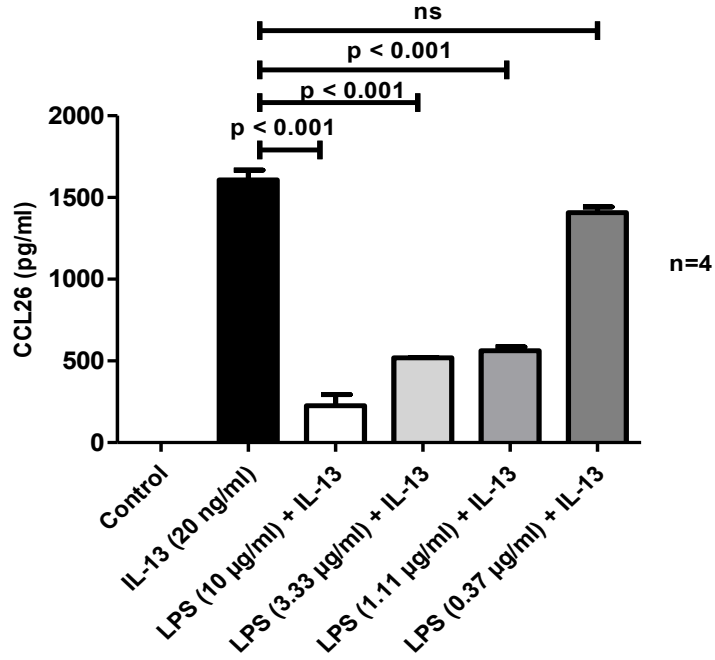
Figure 5: LPS effect on IL-13 -induced CCL26 mRNA in BEAS-2B cells. A) BEAS-2B cells were stimulated with IL-13 (20 ng/ml) alone or in combination with LPS (10 µg/ml) for 24 hr. B) Different LPS doses were used in combination with IL-13 (20 ng/ml). C) The cells were stimulated as described previously and incubated for 2 hr, 6 hr, and 24 hr. The cells were harvested and RNA isolated. CCL26 mRNA was determined by qRT-PCR. Results shown are mean ± SEM of values from 4 separate experiments. CCL26 mRNA was determined by qRT-PCR. P value and statistical analysis have been done by one-way ANOVA.

Figure 6

A)


2000
1500
1000
500
0
CCL26 (pg/ml)
P < 0.001
n=13
Control
IL-13
LPS
IL-13+LPS

B)


ns
p < 0.001
p < 0.001
p < 0.001
2000
1500
1000
500
0
CCL26 (pg/ml)
n=4
Control
IL-13 (20 ng/ml)
LPS (10 µg/ml) + IL-13
LPS (3.33 µg/ml) + IL-13
LPS (1.11 µg/ml) + IL-13
LPS (0.37 µg/ml) + IL-13

C)

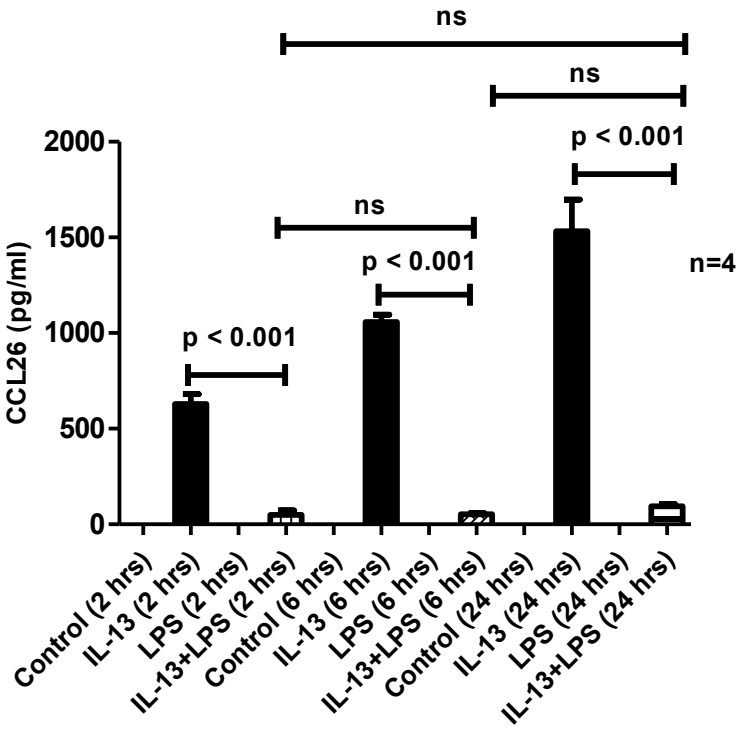
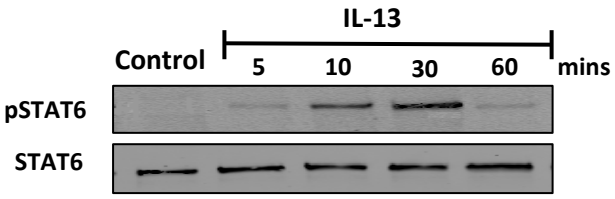


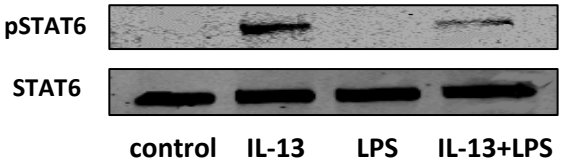
Figure 6: LPS effect on IL-13- induced CCL26 protein release in BEAS-2B cells supernatants. A) BEAS-2B cells were stimulated with IL-13 (20 ng/ml) alone or in combination with LPS (10 µg/ml) for 24 hr. B) Different LPS doses were used in combination with IL-13 (20 ng/ml). C) The cells were stimulated as described early and incubated for 2 hr, 6 hr, and 24 hr. The supernatants were collected and CCL26 was evaluated by ELISA. Results shown are mean ± SEM of values from 4 independent experiments. P value and statistical analysis have been done by one-way ANOVA analysis.

Figure 7

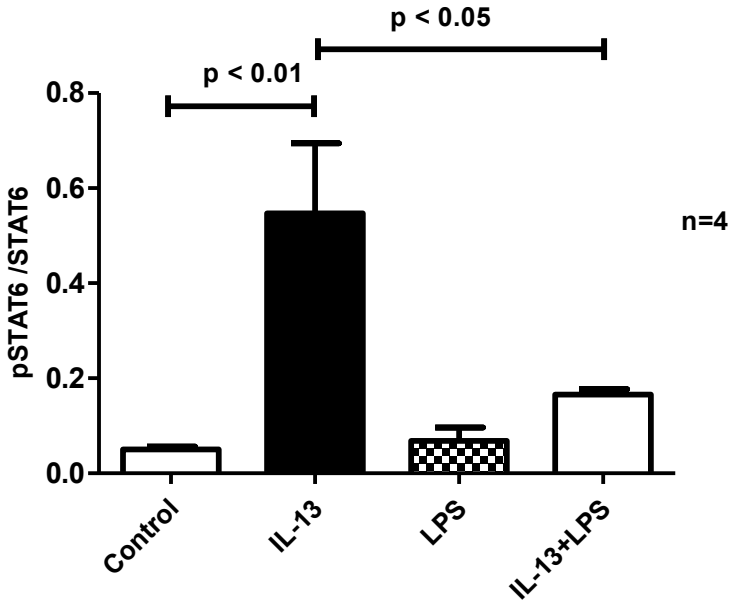
A)


IL-13
Control
5
10
30
60
mins
pSTAT6
STAT6

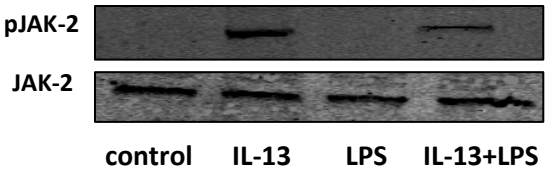
B)


pSTAT6
STAT6
control
IL-13
LPS
IL-13+LPS

C)


p < 0.05
p < 0.01
0.8
0.6
0.4
0.2
0.0
pSTAT6 /STAT6
n=4
Control
IL-13
LPS
IL-13+LPS

D)



E)

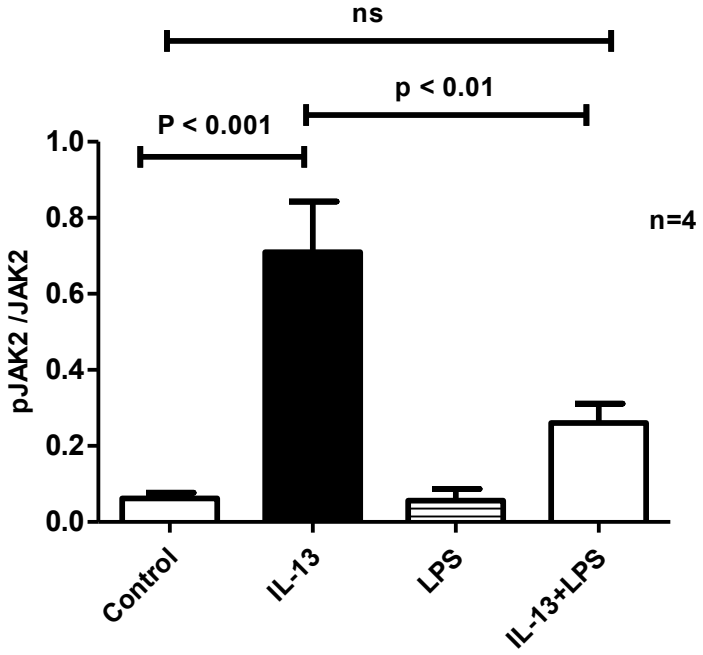


Figure 7: Phosphorylation of STAT6 or JAK2 by IL-13 in BEAS-2B cells. A) Representative gel of western blot analysis of IL-13 -induced tyrosine phosphorylation of STAT6 at different time points. B) Representative gel of western blot analysis of LPS and/or IL-13 -induced tyrosine phosphorylation of STAT6. C) Densitometric analysis of the ratio of phosphorylated STAT6 over total STAT6 in western blot experiments (n=4). D) Representative gel of western blot analysis of LPS and/or IL-13 -induced tyrosine phosphorylation of JAK2. E) Densitometric evaluation of the data in separated western experiments showing the ratio of phosphorylated STAT6 or JAK-2 from their total. The blots are representative of 4 independent experiments. P value compares the value in IL-13 alone with IL-13 in combination with LPS stimulation.

Figure 8

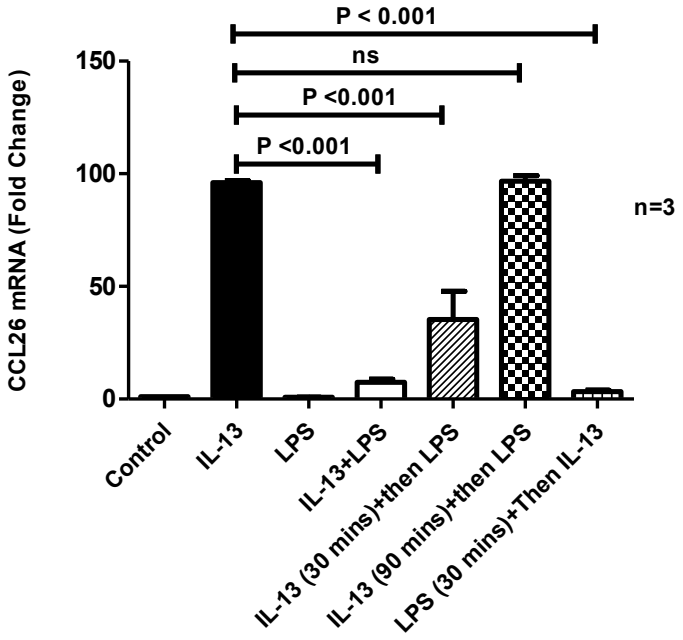
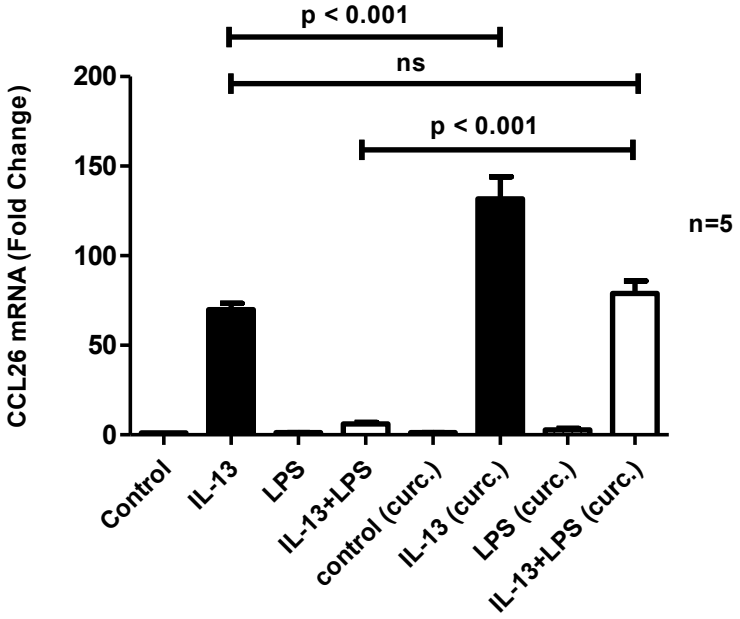


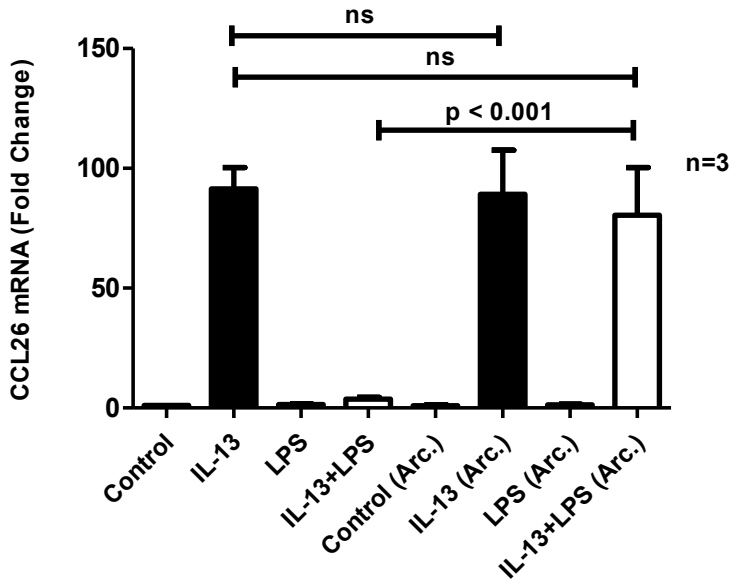
Figure 8: LPS effect on IL-13 -induced CCL26 mRNA in BEAS-2B cells. BEAS-2B cells were stimulated with IL-13 (20 ng/ml) for 30 or 90 min before adding LPS (10 μg/ml) or LPS for 30 min before adding IL-13 without changing the culture media for 24 hr. The cells were harvested and RNA isolated. CCL26 mRNA was determined by qRT-PCR. Results shown are mean ± SEM of values from 3 separate experiments. CCL26 mRNA was determined by qRT-PCR. P value and statistical analysis have been done by one-way ANOVA.

Figure 9

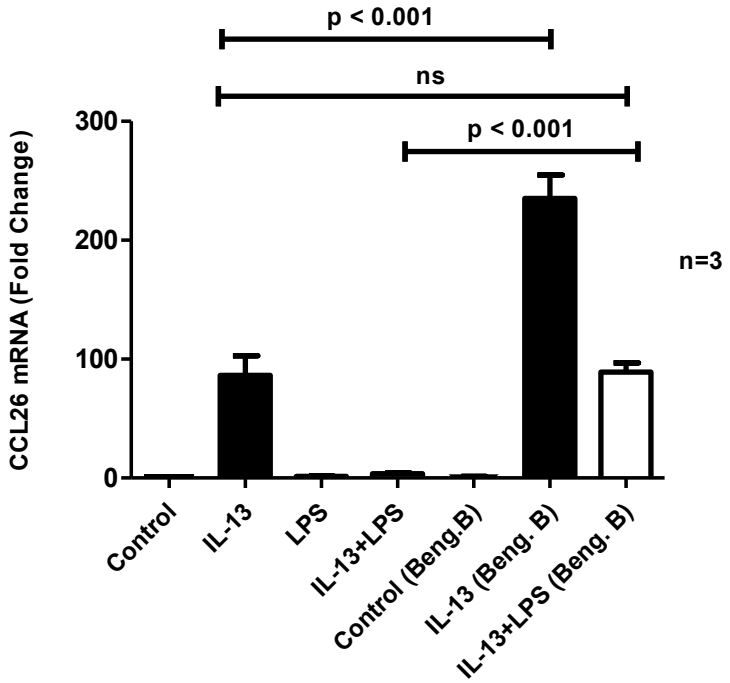
A)


p < 0.001
ns
p < 0.001
200
150
100
50
0
CCL26 mRNA (Fold Change)
n=5
Control
IL-13
LPS
IL-13+LPS
control (curc.)
IL-13 (curc.)
LPS (curc.)
IL-13+LPS (curc.)

B)


ns
ns
p < 0.001
150
100
50
0
CCL26 mRNA (Fold Change)
n=3
Control
IL-13
LPS
IL-13+LPS
Control (Arc.)
IL-13 (Arc.)
LPS (Arc.)
IL-13+LPS (Arc.)

C)



D)

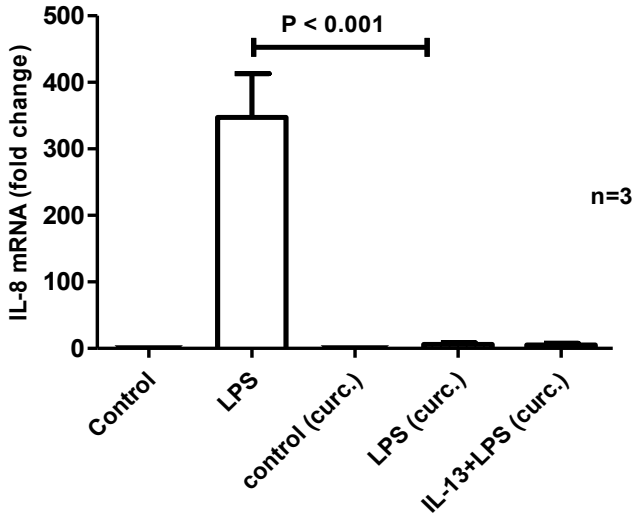
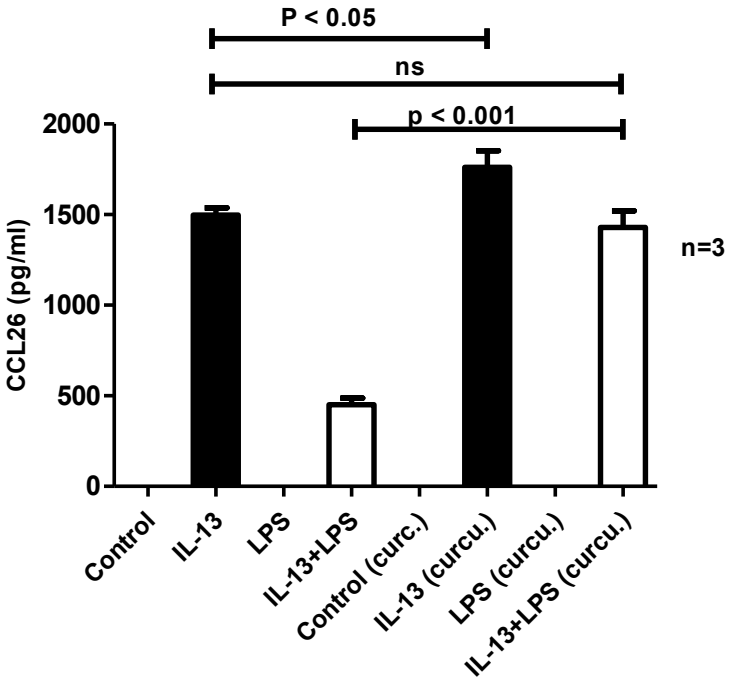


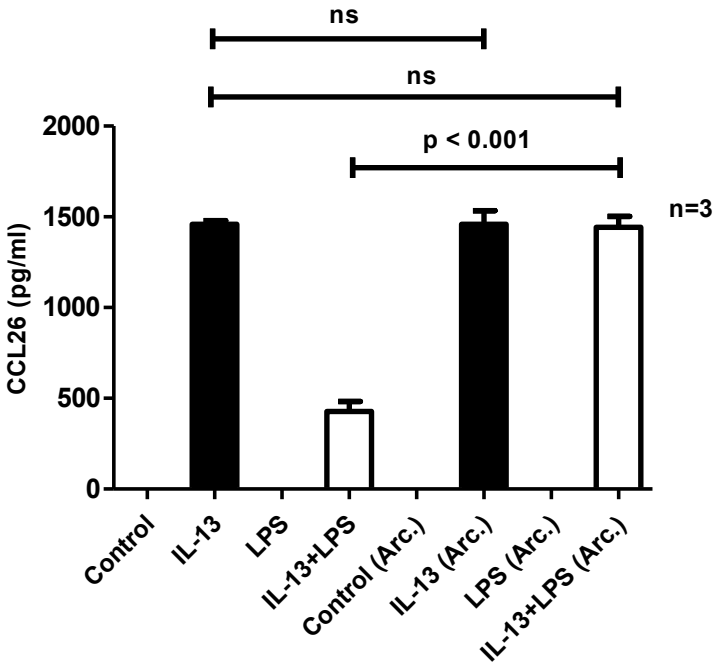
Figure 9: The effect of NF-κB inhibition by curcumin, arctigenin, and bengamide B on either LPS or IL-13 or both together in BEAS-2B cells. A, B, and C) BEAS-2B cells were pretreated with three different NF-kB inhibitors before LPS stimulation with or without IL-13. D) curcumin (curc) effect on LPS- induced IL-8 mRNA. CCL26 mRNA and IL-8 mRNA were determined by qRT-PCR Results shown are mean ± SEM of values from 5 independent experiments for curcumin results and 3 independent experiments for arctigenin (Arc) and bengamide B (Beng.B). No significant difference between IL-13 alone and IL-13+LPS that pretreated with NF-κB inhibitor.

Figure 10

A)


P < 0.05
ns
p < 0.001
2000
1500
1000
500
0
CCL26 (pg/ml)
n=3
Control
IL-13
LPS
IL-13+LPS
Control (curc.)
IL-13 (curcu.)
LPS (curcu.)
IL-13+LPS (curcu.)

B)


ns
ns
p < 0.001
2000
1500
1000
500
0
CCL26 (pg/ml)
n=3
Control
IL-13
LPS
IL-13+LPS
Control (Arc.)
IL-13 (Arc.)
LPS (Arc.)
IL-13+LPS (Arc.)

C)

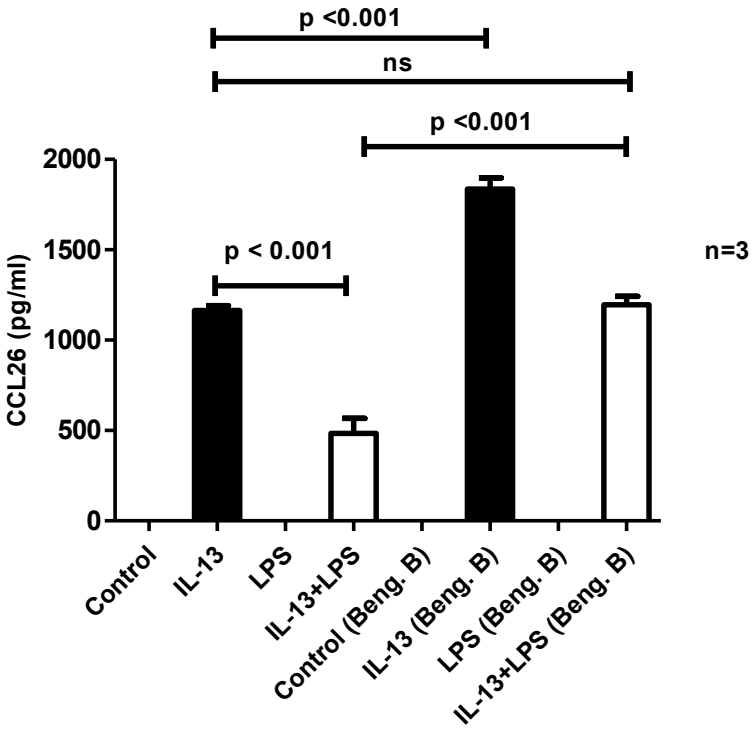
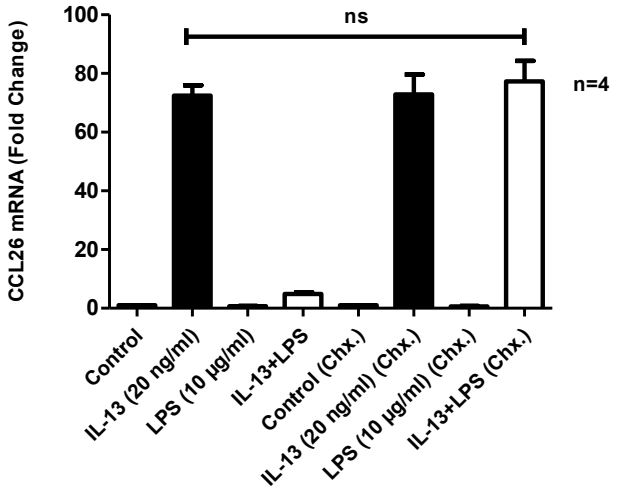


Figure 10: The effect of NF-κB inhibitors (curcumin, arctigenin, and bengamide b.) on LPS-stimulated BEAS-2B cells with or without IL-13. A, B, and C) BEAS-2B cells were pretreated with three different NF-κB inhibitors before LPS stimulation with or without IL-13. CCL26 release in supernatants was determined by ELISA. Results shown are mean ± SEM of values from 3 separate experiments. No significant P value between IL-13 alone and IL-13+LPS that pretreated with NF-κB inhibitor.

Figure 11

A)



B)

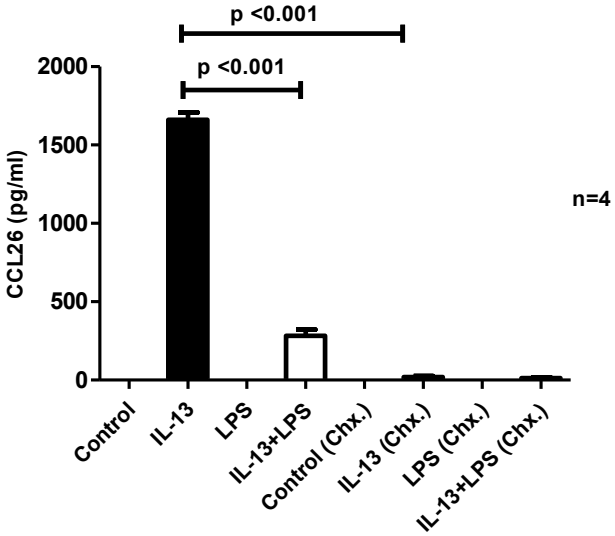


Figure 11: The effect of Protein synthesis inhibitor (cycloheximide) on LPS-stimulated BEAS-2B cells with or without IL-13. A) CCL26 mRNA was determined by qRT-PCR. B) CCL26 release in supernatants was determined by ELISA. Results shown are mean ± SEM of values from 4 separate experiments

Figure 12

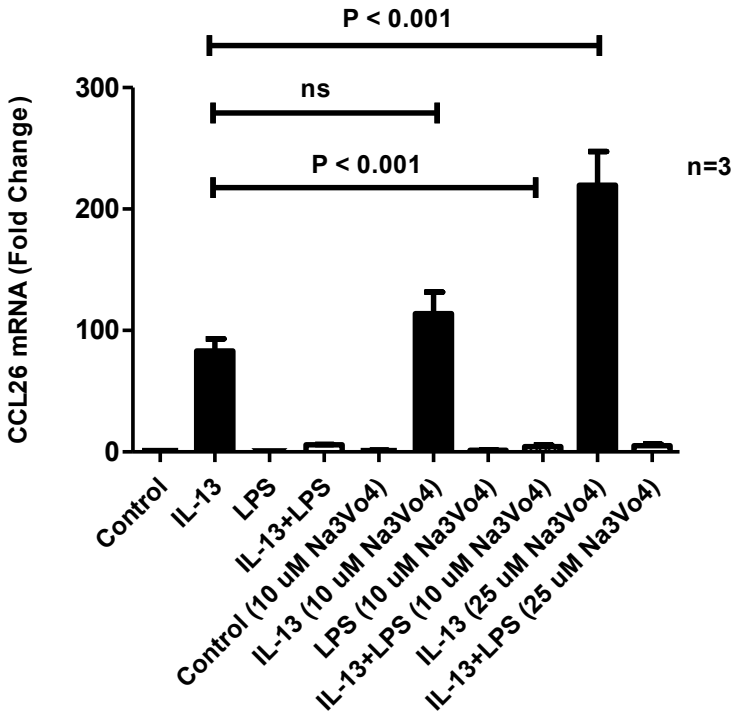


Figure 12: The effect of the tyrosine phosphatase inhibitor Na3Vo4 on LPS-stimulated BEAS-2B cells. The cells were stimulated as previously described with or without Na3Vo4. The cells were harvested and RNA isolated. CCL26 mRNA was determined by qRT-PCR. Results shown are mean ± SEM of values from 3 separate experiments.

Chapter 4

Discussion and Future Direction

4.1 Discussion

Since Paul Erlich's discovery of eosinophils in 1879, the infiltration of eosinophils into the lung has been observed in conjunction with allergic asthma and suggested to be a significant contributor in the pathogenesis of asthma. Since then, numerous studies have revealed diverse and complex functionality of eosinophils in allergic asthma including: their ability to present antigens, secrete an array of cytokines and chemokines, degranulate, release lipid mediators, and induce T cell polarization (155). Increased numbers of eosinophils in blood and sputum have also been linked with asthma exacerbation and worsening of asthma symptoms (155,188). As a consequence of the diverse role of eosinophils in asthma, they have been identified as a target for therapeutic intervention. Indeed, therapeutic approaches are developing to eliminate eosinophils in patients suffering from airway eosinophilic infiltration, especially patients with severe, and frequently exacerbating asthma (189,190). Anti-IL-5 antibodies, as an example, have been developed to reduce the number of eosinophils in both sputum and blood, resulting in a reduction in the frequency of exacerbations and in the need for treatment with systemic glucocorticoids (191,192). Since epithelial cells produce eotaxins, eosinophilic chemotactic factors in response to T_H2 cytokines such as IL-13 and IL-4, we used *in vitro* culture of human bronchial epithelial cells to test the effect of LPS on the induction of eotaxins by IL-13 stimulation.

We have established that LPS inhibits IL-13 -induced CCL26 production. We also showed that the LPS inhibitory effect is mediated by reduction of JAK-2 and STAT6 phosphorylation through LPS-mediated NF-κB activation. These results may help to develop strategies that will lead to decreased eosinophils in airway

Our laboratory observed that allergens such as house dust mite (HDM) and cockroach inhibited IL-13 -induced CCL26 in both primary human bronchial epithelial cells (NHBE) and

the human bronchial epithelial cell line (BEAS-2B) (unpublished data by K Alzahrani and V Gandhi). We generated two hypotheses to explain this observation. One postulate was that this inhibition is a result of allergen -derived proteases acting through PARs. Another postulate was that this inhibition is due to the presence of LPS in the allertgen extract preparations we have been using. This thesis has focused on the second hypothesis, that LPS from contaminating bacteria mediated the effect of allergens in our previous studies, and therefore we investigated the effects of LPS on IL-13 induced eotaxins upregulation in airway epithelial cells.

Immunohistochemical (IHC) studies for eotaxins demonstrated that the bronchial epithelium has greater eotaxin intensity than other tissues in humans and mice (153,193,194). In this study, we used BEAS-2B cells instead of NHBE for a number of reasons; BEAS-2B cells are a homogeneous clone without the differences that that we and others have observed between NHBE cells of different origin, they have a longer lifespan thatn NHBE cells, and they are also easier to grow and less costly and therefore they represent an excellent *in vitro* experimental model of human airway epithelium. Moreover, BEAS-2B cells possess typical epithelial morphology and many functional characteristics similar with primary epithelial cells, such as squamous differentiation and expression of important adhesion molecules (195,196). Several studies have shown that IL-4 and IL-13 can activate bronchial epithelial cells by directly inducing the production of a number of chemokines, including CCL11, CCL24, and CCL26 (184,185). IL-4 and IL-13 have overlapping functions due to a shared subunit in their receptor complexes (197). Numerous studies highlight important roles for IL-13 and its main subunit (the IL-13Rα1) in comparison with IL-4 in asthma. Studies in IL-13Rα1$^{-/-}$ mice established that certain features of asthma, including: eotaxin production, mucus production, airway resistance, fibrosis, and chitinase (pathogenic molecule associated with asthma severity) were completely

dependent on IL-13Rα1(198–201). Supernatant from T_H2 cells administrated intranasally to naive mice induced eotaxin production along with lung eosinophilia. A neutralizing anti-IL-13 antibody removed most of the eotaxin-inducing activity from T_H2 supernatants, suggesting that IL-13 is more potent than IL-4 at inducing eotaxin in mouse bronchial epithelium *in vivo* (200). Thus, we used IL-13 but not IL-4 to stimulate the BEAS-2B cells to induced eotaxins. We found CCL26 was the only eotaxin detected by qPCR after 24 hr in both resting and IL-13 - activated BEAS-2B cells. In contrast, other studies have shown production of CCL11, CCL24, as well as CCL26 by IL-13 in BEAS-2B cells (177,185). The exact reasons for these differences from our results are not clear, although they used the same method for detection (qPCR). In addition, they used different BEAS-2B cells passage numbers as well as different culture media and different time of stimulation (less than 24 hr) (177,185). These differences may explain the different results. Several studies have shown that CCL26 is produced in large amounts by human lung epithelial cells and also showed that CCL26 is significantly increased after 24 hr of allergen challenge (more IL-13 in BAL fluid) (167,185,202,203). These results support findings that CCL26 is more effective in recruiting eosinophils in human airways than CCL11 or CCL24 and thereafter we carried out our experiments with checking only CCL26.

Airway epithelium is a major portal of entry for pathogens and it employs several defense mechanisms (e.g. physical barrier..etc.) to eliminate airborne pathogens encountered in breathing, including LPS- contaminated airborne pathogens (41). Airway epithelium can detect LPS via TLR-4 and stimulate immune cells (72). Heterogeneity of LPS within a particular bacterial strain and it is contamination with other inflammatory components of the bacterial cell wall have complicated the use of LPS and choosing its working concentration. We chose 10 μg/ml of LPS based on previous studies of BEAS-2B cells that showed this concentration of LPS

can induce NF-κB activation and other LPS-mediated response (e.g. JNK, MAPK, PI3-kinase, and tyrosine kinases) without affect the viability of the cells (including membrane integrity and cell degeneration) (186,204,205). Thereafter, we used 10 μg/ml as well as different LPS concentrations and we showed only 10 μg/ml of LPS completely inhibited CCL26 mRNA by IL-13 stimulation and returned to the baseline (unstimulated cells). The viability of the LPS-treated cells with 10 μg/ml of LPS was determined and showed this concentration did not affect the viability.

In another hand, IL-13 when it was first described in 1993, it is known as a T cell - derived cytokine that can inhibit several inflammatory cytokine productions including that were produced by LPS stimulation (117,206). Numerous studies demonstrated that IL-13 has inhibitory effect on LPS induce IL-6, IL-8 and TNF as well as NF-κB activation. This is controversial with our finding where we found the opposite whereas LPS inhibited IL-13 -induced CCL26. Going back to those studies that showed inhibitory effect of IL-13 on LPS, they were different from the present study in the working concentration of LPS that they used. For example, Lotz al et. showed IL-13 inhibited LPS- mediated response by using 10 ng/ml of LPS in epithelial cells (207). Other studies by D'Andre et al. and Marie et al. showed IL-13 inhibited LPS- mediated response by used 1 ug/ml or 100 ng/ml of LPS in peripheral blood mononuclear cells (206,208). These studies along together with my study suggest this might be LPS concentration dependent and LPS concentration will determined the inhibitory effect of either LPS or the IL-13. This might explain that 370 ng/ml of LPS did not inhibit IL-13 -induced CCL26 and support the importance of high LPS concentration in order to overcome the inhibitory effect of IL-13 and cause inhibition of IL-13 -induced CCL26.

We also showed that inhibition of CCL26 mRNA upregulation and protein release can happen as early as 2 hr of adding IL-13 and LPS simultaneously, suggesting an early effect and crosstalk between LPS and IL-13 signaling pathways. To understand the mechanism of the inhibitory effect of LPS on IL-13 mediate signaling, we investigated the IL-13 signaling pathway. Dimerization of IL-13 receptor subunits enhances JAK-2 activity and leads to phosphorylation of tyrosine residues in the cytoplasmic domain of IL-13Rα1 (209,210). Then, these residues act as docking sites for STAT6. Studies of STAT6 wild type–transfected cells in mice showed significant increase in eotaxin protein secretion after IL-13 stimulation. Co-transfection with a mutant dominant negative STAT6 inhibited activation of the eotaxin promoter by IL-13 (177). These studies indicate that IL-13 stimulation of eotaxin expression in airway epithelial cells is dependent on STAT6 (177). Therefore, we investigated if the inhibitory effect of LPS on CCL26 production by IL-13 associated with inhibition of IL-13 -induced STAT6 activation. Western blot analysis revealed that LPS inhibited IL-13 -mediated STAT6 phosphorylation, suggesting that the mechanism of inhibition occurred in the cytoplasm and in early event of IL-13 signaling. STAT6 must be tyrosine phosphorylated by JAK-2 for STAT6 dimerization and nuclear translocation to occur (130). We also checked JAK-2 phosphorylation by western blot. Like STAT-6, LPS inhibited IL-13 mediated JAK-2 phosphorylation. We concluded that the inhibition of STAT6 phosphorylation was a result of inhibiting of JAK-2 phosphorylation. In addition, western blot for JAK-2 and STAT6 phosphorylation revealed the inhibition occurred early in the signaling pathway of IL-13, indicating a possible interaction between LPS signaling and IL-13 signaling pathways.

We continued studying the mechanism of LPS – mediated signaling cascades and we investigated the role of NF-κB, the central regulator of LPS effects and a prominent transcription factor. To determine the role of NF-κB in the LPS effect on IL-13 -induced CCL26, we first used curcumin to inhibit NF-κB activation. Curcumin, a component of the curry spice, recently received attention for its antioxidant, anti-inflammatory, and antitumor properties (211,212). The anti-inflammatory actions of curcumin seem to be closely related to the suppression of cytokine-mediated NF-κB activation by blocking IκB kinase (IKK) activity in mouse epithelial cells. Curcumin also can block c-Jun/ Activator protein 1 (AP-1) pathway in mouse fibroblasts (213,214). Here, we have shown that curcumin removed the inhibitory effect of LPS in IL-13 -induced CCL26. IL-8 mRNA upregulation by LPS is known to be dependent on NF-kB activation by LPS, therefore we checked the effect of curcumin on LPS induced IL-8 mRNA upregulation and we confirmed by qPCR that curcumin inhibited IL-8 mRNA upregulation. However, curcumin is considered to be general inhibitor for NF-κB and therefore, we used two other NF-κB inhibitors, bengamide B and arctigenin to clarify the role of NF-κB in this inhibitory effect. There are reports that bengamide B decreases IκBα phosphorylation and attenuates expression of TNF-α, IL-6 and MCP-1 by LPS stimulation (215). The anti-inflammatory actions of arctigenin were documented through inhibition of c-Jun/AP-1 activation, LPS-induced iNOS expression, LPS-induced TNF production, and NF-κB activation through inhibit IκBα phosphorylation and p65 nuclear translocation (216–218). Like curcumin, both arctigenin and bengamide B removed the inhibitory effect of LPS on IL-13 -induced CCL26 mRNA upregulation and protein release. These results reveal the importance of NF-κB in the regulation of IL-13 -induced CCL26 production and raise a question about the function of NF-κB independent of being a transcription factor. Shen et al. provided evidence that NF-κB and

STAT6 can bind to each other in studies of co-immunoprecipitation and glutathione *S*-transferase pull-down assays (219). However, it is unknown if this interaction between NF-κB and STAT6 occurs in the cytoplasm or the nucleus and therefore, it was important to determine if NF-κB functions as a transcription factor in this interaction and if new protein synthesis is a requirement for this NF-κB -mediated inhibition.

Cycloheximide is an inhibitor of protein synthesis and exerts its effect through interfering with the movement of mRNA and transfer RNA (tRNA) in the translational elongation phase (220). We tested if new protein synthesis was associated with the inhibitory effect of LPS on IL-13 -mediated signaling by using cycloheximide. Cycloheximide removed inhibitory effect of LPS on IL-13 - induced CCL26 mRNA upregulation, suggesting that LPS stimulation activated NF-κB leading to the synthesis of new protein and subsequent the inhibition of CCL26. Therefore, we hypothesized that this newly synthesized protein might be a tyrosine phosphatase which would dephosphorylate JAK-2 and STAT6. Thus, we used a tyrosine phosphatase inhibitor, sodium orthovanadate. However, sodium orthovanadate did not remove the inhibitory effect of LPS, suggesting that a phosphatase was not involved. The newly synthesized protein induced by LPS stimulation could be different type of phosphatase such as serine or threonine phosphatase or could be other non-phosphatase molecules.

4.3 Future Direction

I have studied the effect of LPS, on IL-13 -induced eosinophilic chemoattractant, CCL26, using a BEAS-2B cell-based *in vitro* model. Despite my research progress, many questions remain, and the ground is fertile for continued investigations. The question about the mechanism of the effect

of LPS on IL-13 induced CCL26 remains unanswered. We have shown a role of NF-κB in the process of LPS inhibition in IL-13 -induced CCL26 and we have evidence that new protein synthesis is involved.

However, it remains possible that a different phosphatase such as a serine - specific phosphatase, threonine-specific phosphatase, or histidine- specific phosphatase could be involved (221,222).

The NF-κB inhibitors which were used here differ in their specificity and sensitivity in inhibiting NF-κB molecule. Therefore, silencing NF-κB by short interfering RNA targeting NF-κB is necessary to solidify our results with NF-κB antagonists.

Suppressors of cytokine signaling 1 and 3 (SOCS -1 and SOCS -3) are known to be induced following IL-13 stimulation and work as negative regulators of IL-13 signalling and blocking JAK-2/STAT6 phosphorylation (223). It would be important to investigate the production of SOCS 1 and SOCS 3 following stimulation by either LPS or IL-13 or both together.

We also showed LPS inhibited IL-13 – mediated JAK-2 phosphorylation. It would be appropriate to determine the dimerization of the two subunits of IL-13 receptor was inhibited by LPS stimulation or not.

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