Exposure to Allergens and Proinflammatory Mediators Modulate Airway Epithelial Cell Innate Responses, Metabolism, and Physiology

by

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Abstract

Lungs and airways health and protection depend on the integrity of airway epithelium. Insulin is a growth hormone that through activation of several signaling pathways play a central role to maintain cellular growth and activate metabolism. Airway epithelium is in constant exposure to inhaled agents like pathogens, allergens, pollutants, and particular matters. Airborne allergens like cockroach or house dust mite (HDM) contain proteases that can interact with airway epithelium and initiate immune responses through activation of airway epithelial cells and innate immune cells. Airway epithelium cytokines and innate immune cells promote activation of adaptive immune cells like Th2 lymphocytes that produce proinflammatory mediators like interleukin-4 (IL-4), IL-13, and IL-5. T helper 2 (Th2) cytokines amplify the immune responses and stimulate airway epithelial cells to release CCL chemotactic factors/eotaxins that attract eosinophils to the site of inflammation. Airway epithelium exposure to inhaled irritants and excessive inflammatory responses is known to disrupt epithelium integrity and exacerbate inflammatory responses. Th2 inflammation in asthma is associated with epithelial injury, epithelial remodeling, and metabolic alterations. Increased expression of inflammatory mediators tumor necrosis factor (TNF) and IL-6 in metabolically active tissues have been associated with cellular damage and insulin resistance. Inducible AECs local inflammation was shown to trigger systemic inflammation, insulin resistance, and impaired glucose metabolism, and the severity of inflammation correlated with impaired glucose metabolism. Several studies showed that insulin resistance increased the risk of aeroallergen sensitization and developing asthma-like symptoms. In this project we hypothesized that exposure of airway epithelial cells (AECs) to allergens and proinflammatory mediators may modulate Th2 immune cell responses, induce insulin resistance and metabolic shifts. First, we examined the effect of cockroach and house dust mite on IL-13 and IL-4-induced inflammatory

responses. AECs were cultured and stimulated with cockroach or house dust mite, IL-13, IL-4, or a combination of an allergen and IL-13 or IL-4. IL-13 and IL-4 induced effects were measured. Then, we tested the effect of cockroach, house dust mite, TNF or IL-6-induced inflammatory responses on physiological epithelial changes and insulin-induced effects. Epithelial proliferation, resistance, energetic phenotype changes, and insulin induced activation of PI3K/Akt were examined. We showed that serine proteases of cockroach extract prevented IL-13-induced expression of eosinophil chemokine CCL26 from AECs. Depletion of CCL26 was previously shown to delay resolution of airway allergic inflammation which may result in prolonged eosinophilia. Prolonged inflammation was linked to altered metabolism and insulin action in several studies. We showed for the first time that house duct mite and TNF modulated insulin effects in AECs. TNF and HDM changed insulin-induced ATP production in AECs. Additionally, TNF reduced insulin-induced Akt phosphorylation, reduced epithelial barrier function and recovery after injury. Our data suggest that cockroach serine proteases and TNF may interfere with the Th2-mediated proinflammatory effects, regulate AECs energetic phenotype, and induce insulin resistance. These inflammatory and metabolic changes exacerbate the pathogenesis of asthma which may alter immune cell responses.

Preface

Airway epithelium is the first innate barrier that contributes to host protection by clearing inhaled agents and interacts with antigens to regulate innate and adaptive immune responses. Inhaled aeroallergens and their proteases are major contributors for allergenicity through airway epithelium activation and Th2 mediated inflammation. Chronic inflammation is known to induce epithelial damage and reduce insulin sensitivity in metabolically active tissues. This thesis includes work that was done to examine the effect of aeroallergens and proinflammatory mediators in immune cell responses and insulin-induced metabolic and biological effects in airway epithelial cells.

This thesis is an original work of Khadija Rashed Alzahrani. I have conducted all the experimental work and data analysis presented in this thesis, and I have recognized below the experiments done in collaboration with other colleagues. I was also responsible for preparing this manuscript.

Chapter 1 is a general introduction of my two projects. I introduced the airway epithelium structure and function in homeostasis and disease. The role airway epithelium and immune cells play in airway inflammatory diseases. Introduced the connection between inflammation and insulin resistance and metabolic shifts, which lead to the rationale of studying the role of inflammation in the modulation of Th2 responses, epithelial changes, and disrupted insulin action.

Chapter 2 of this thesis is published as Alzahrani KR, Gomez-Cardona E, Gandhi VD, Palikhe NS, Laratta C, Julien O, Vliagoftis H. “German cockroach extract prevents IL-13-induced CCL26 expression in airway epithelial cells through IL-13 degradation”. *FASEB J.* 2024 Mar 15;38(5):e23531. DOI: 10.1096/fj.202300828RRR. PMID: 38466220. Khadija Alzahrani, Vivek Ghandi, and Harissios Vliagoftis have designed the project. Vivek Ghandi and Cherryl Laratta have set up the first experiments (not presented in this thesis). Nami Shrestha have performed flow cytometry experiments and helped with data analysis from those experiments. Erik Gomez-Cardona performed experiments and data analysis of mass spectrometry. Khadija Alzahrani has performed the rest of experiments and data analysis. Khadija Alzahrani, Erik Gomez-Cardona, Olivier Julien, and Harissios Vliagoftis interpreted the data. Khadija Rashed Alzahrani prepared the first draft of the manuscript and revised the manuscript with Harissios Vliagoftis. All authors have read and approved the final manuscript.

Chapter 3 contains the data from experiments to study the effect of aeroallergens and proinflammatory mediators on airway epithelial cell on insulin resistance and modulation of energetic phenotype. Experiments and data analysis in this chapter were all performed by Khadija Alzahrani.

Chapter 4 is a general discussion of the thesis for all the findings in thesis and how that may contribute to the pathogenesis of epithelium inflammation and future dissertations.

Dedication

I dedicate my PhD thesis to my beloved parents, my siblings, my husband, and my little angels. They have been my source of support, strength, inspiration, and blessings. Their prayers surrounded me all the time and reinforced me through my ups and downs.

To my colleagues who were supportive, inspiring, and caring through my journey. Their support and guidance helped to bring difficulties to relief.

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List of abbreviations:

2DG: 2-deoxyglucose

2DG6P: 2-deoxyglucose-6-phosphate

8WIE: 8 well, 1 electrode

ABI: all but no insulin

AECs: airway epithelial cells

ALI: air liquid interface

AR-2 CP: PAR-2 control peptide

ARG2: arginase 2

ASL: airway surface liquid

BAL: bronchoalveolar lavage

BEBM: Bronchial Epithelial Basal Media

BEGM or GM: Bronchial Epithelial Growth Media or growth media

BMI: body mass index

BSA: bovine serum albumin

CCCP: carbonyl cyanide 3-chlorophenylhydrazone

CCL26: C-C motif chemokine ligand 26

CCR-3: C-C chemokine receptor -3

CCR3: CC motif chemokine receptor

CE: cockroach extract

CE: cockroach extract

COPD: chronic obstructive pulmonary disease

COX: cytochrome c oxidase

CX_3CR_1: C-X3-C motif chemokine receptor

Drp1: dynamin-related protein

ECAR: extracellular acidification rate

ECIS: Electrical Cell-substrate Impedance Sensing

ECM: extracellular matrix

ELISA: Enzyme linked immunosorbent assay

EMT: epithelial-mesenchymal transition

ETC: electron transfer chain

FADD: Fas-associated death domain

FeNO: fractional exhaled nitic oxide

FEV1: forced expiratory volume in 1 second

Fis1: fission 1

G6P: glucose-6-phosphate

G6PDH: glucose-6-phosphate dehydrogenase

GKs: glucokinases

GLUTs: glucose transporters

HDM: house dust mite

HDM: house dust mite

HICE: heat inactivated cockroach extract

HKs: hexokinases

HOMA-IR: homeostatic model assessment for insulin resistance

IGF-1: insulin-like growth factor 1

IGF-BPs: IGF-binding proteins

IGF-IR: IGF-I receptor

IKK2: I$\kappa\beta$ kinase 2

IL-4: interleukin-4

ILC2: type 2 innate lymphoid cells

iNOS: inducible nitric oxide synthase

IR: insulin receptor

IRR: insulin receptor-related receptor

IRS: insulin receptor substrate

MFI: median of fluorescence intensity

Mfn1: mitofusions 1

MHC: major histocompatibility complex

MMLV: Moloney Murine Leukemia Virus

MMP: Mitochondrial membrane potential

MMPs: matrix metalloproteases

MPC: mitochondrial pyruvate carrier

MS: mass spectrometry

mTOR: mammalian target for rapamycin

NFκB: nuclear factor kappa B

NHBE: normal human bronchial epithelial

NLR3: NOD-like receptor protein 3

NO: nitic oxide

OCR: oxygen consumption rate

OXPHOS: oxidative phosphorylation

PAR-2 AP: PAR-2 activating peptide

PAR-2: protease activated receptor -2

PASMCs: pulmonary artery smooth muscle cells

PDGF: platelet-derived growth factor

PDH: pyruvate dehydrogenase

PDK2: PDH kinase-2

PI3K: phosphatidylinositol-3-kinase

PRRs: pattern recognition receptors

pSTAT6: phosphorylated STAT6

ROS: reactive oxygen species

ROT/AA: rotenone + antimycin A

SBTI: soybean trypsin inhibitor

SGLT: sodium-coupled glucose transporter

SOCS-3: suppressor of cytokine signaling -3

SOCS3: suppressor of cytokine signaling 3

STAT-6: signal transducer and activator of transcription 6

T2DM: type 2 diabetes millets

TGFβ: transforming growth factor β

TMRM: tetramethylrhodamine methyl ester

TNF-RI: TNF receptor-I

TNF: tumor necrosis factor

TNS: trypsin neutralizing solution

TRADD: TNF receptor-associated death domain

TRAF2: TNF receptor-associated factor 2

TSLP: thymic stromal lymphopoietin

UCPs: uncoupling proteins

WC: waist circumference

WHR: waist-to-hip ratio

ZO-1: zonula occludin -1

Chapter 1:

Introduction

1. Airway epithelium structure and function in homeostasis:

The airways are covered with a continuous sheet of epithelial cells. By single cell RNA sequencing, many AEC types have been identified and presented here from most to least abundant: basal cells, suprabasal cells with co-expression of basal and secretory cell markers suggesting its differentiation from basal and secretory cells, secretory cells including club and goblet cells, multi-ciliated cells, and the precursors for ciliated cells, deuterosomal cells [1,2]. Airway epithelium protects and maintains airway homeostasis and functions to clear inhaled allergens, pathogens, and particular matter [3,4]. Epithelium protection is mainly mediated through the epithelium physical barrier, secretion of mucus (mucins), and antimicrobial peptides [5–7]. Mucins include MUC5AC and MUC5B which are components of the airway surface liquid (ASL) are produced by epithelium secretory cells and submucosal glands forming the first thin layer of fluid (ASL) lining the epithelium surface and contribute to clear inhaled agents [7]. AECs also express small antimicrobial peptides which clear invading microbes like bacteria, viruses, and fungi through disruption of cell walls via microbial permeabilization and seize of nutrients [6]. Between antimicrobial peptides, airway epithelium produces mainly two types: defensins and cathelicidins [5].

Airway epithelium and mucociliary clearance requires an intact physical barrier that clears and protects against inhaled agents [3,4,8]. Cell-to-cell connections through epithelial junctions promote epithelial barrier, prevent invasions from the exogenous environment, enable cell-to-cell communication, and establish epithelial polarity which influence epithelial resistance and reduce permeability [9]. Epithelial junctions are composed of tight junctions, adherens junctions, and desmosomes [9]. Paracellular permeability is regulated through tight junctions which are located apically and composed of occludin, claudins, and junction-adhesion-molecules [10]. High epithelial resistance was shown to be associated with continuous circumferential expression of zonula occludin -1 (ZO-1) protein [11]. ZO-1 is intracellular protein that anchor occludin and claudin to the cell cytoskeleton. Adherens junctions connect adjacent cells and promote cell-cell contact through E-cadherin [9]. The E-cadherin intracellular domain is stable through formation of a complex with catenin, actin cytoskeleton, and microtubule network [9]. This dynamic complex maintains the cell shape, regulation of(ASL), enables cilia motion, and important for cellular division and proliferation [12–14].

Airway epithelium is additionally equipped with pattern recognition receptors (PRRs) that can sense and recognise pathogens, allergens, and damaged cell that can activate epithelial cells and trigger secretion of various pro- and anti-inflammatory mediators such as cytokines, chemokines, and antimicrobial peptides that recruit and activate inflammatory cells [15–18]. AECs act in concert with resident and attracted immune cells to regulate immune response [15].

Innate immunity and adaptive immunity of the airways bridge through dendritic cells, the professional antigen presenting cells [19,20]. Exposure to inhaled agents that activate dendritic cells lead to the migration of dendritic cells to the draining regional lymph nodes where they activate T lymphocytes [20] . Dendritic cell activation can also be triggered by AECs through epithelial cytokine thymic stromal lymphopoietin (TSLP). TSLP was shown to stimulate dendritic cell-mediated activation of Th2 cells, type 2 immune response regulator, differentiation and production of Th2 cytokines [21]. Another epithelium cytokine that is an important effector for Th2 responses is IL-33. IL-33 was shown to enhance the production of Th2 cytokines [21]. Th2 cytokines IL-4 and IL-13 can further activate AECs and induced chemotactic factors like eotaxins: eotaxin-1/ C-C motif chemokine ligand 11 (CCL11), eotaxin-2 /CCL24, and eotaxin-3/CCL26 [22]. CCL26 expressed by AECs is the most effective trigger for activation and recruitment of eosinophils through C-C chemokine receptor -3 (CCR-3) activation [23–25]. CCL26 was also shown to activate another receptor CX_3CR_1 on macrophages to facilitate clearance of eosinophils [26].

Th2 cells are in turn migrate to the B cell follicle [20]. Th2 cytokines IL-4 and IL-13 and the direct interaction of Th2 and B cells trigger B cell immunoglobulin class switching [27]. Dendritic cells interactions with airway epithelial cells and antigen presentation and increased expression of inflammatory mediators amplify the airway immune responses [20] (Illustration 1.1.). Presence of tissue resident memory T cells aimed to subsequently encountered antigens generate a prompt immune response [28]. Macrophages are the most abundant immune cell in the airways [29,30] and functions to maintain epithelium homeostasis through phagocytosing airborne particles, microbes, and dead cells [31–34].

Macrophages can also modulate airway epithelium responses and send immunosuppression signals to prevent excessive inflammation and epithelial damage besides other signals via gap junctions to maintain tissue integrity [35]. Proinflammatory responses have been also detected in macrophages like high expression of inducible nitric oxide synthase (iNOS), IL-1β, and TNF in response to LPS

or inflammatory cytokines stimulation [36–38]. A complex crosstalk exists between AECs and immune cells to maintain host homeostasis. AEC signals to immune cells can modulate immune cell responses to antigens. In contrary, immune cells can modulate AECs physiology and phenotype.

2. Airway epithelium remodeling in inflammatory diseases:

In airway inflammatory diseases, high concentrations of plasma proteins in asthmatics bronchoalveolar lavage (BAL) and sputum are indicating tissue injury and insufficient repair which are orchestrating the inflammatory responses [13,39,40]. Accumulating evidence suggested that airway epithelium of asthmatics is structurally and biochemically impaired [13,39,41,42]. It was shown that asthmatic epithelium undergoes alterations of structural cells which was defined later as airway remodeling [43]. Airway remodeling is characterized by modifications of the normal composition and the structural organization of tissues compared to healthy tissues due to chronic injury and inflammation [44]. Airway remodeling in asthma involves epithelial changes, increased smooth muscle mass, enhanced activation of fibroblasts/myofibroblasts, abnormal deposition of extracellular matrix (ECM) proteins like collagen and fibronectin, and subepithelial fibrosis [44]. Epithelial-mesenchymal transition (EMT) is a key event in airway remodeling where epithelial cells acquire mesenchymal phenotype, loss their polarity, cell-to-cell adhesion, and increase their migratory capacity [45]. Epithelial changes detected in asthmatics have included shedding of epithelium, loss of ciliated cells, hyperplasia of secretory cells, and increased expression of cytokines and chemokines [43,44,46,47]. Similarly, allergen sensitized and challenged experimental model of asthma used to examine epithelial regeneration showed shedding of epithelium and patchy damage, which is suggesting local epithelial damage at the sites of injury [46,48]. Besides epithelial damage, several studies showed impaired barrier function of airway epithelium in asthmatics with less expression of tight junctions and impaired repair after injury [13,47,49]. Notably, epithelial cell brushings from asthmatics cultured in air liquid interface (ALI) were unable to form efficient tight junctions over several passages, and that was associated with decreased baseline epithelial resistance and leakage [50].

Inflammation is believed to be a key driving force for airway remodeling. Eosinophil granule-associated proteins, mainly the transforming growth factor β (TGFβ) a key player in remodeling, and mast cells along Th2 cells and their cytokines are also believed to orchestrate airway

remodeling [51–54]. TGFβ has been shown to promote fibroblasts differentiation to myofibroblasts and enhance expression of matrix metalloproteases (MMPs) major contributor to EMT and epithelial ECM alterations [55–58]; all are contributors to epithelium fibrosis. Additionally, Th2 cytokines IL-4, IL-13, IL-5, and IL-9 were shown to increase mucus expression, influence subepithelial fibrosis, and epithelium hypertrophy [59]. Th2 cytokines IL-4 and IL-13 disrupted epithelial barrier function of cultured human bronchial epithelial cells [60]. IL-4 and IL-13 reduced transepithelial resistance, increased epithelial permeability to dextran, and reduced expression of occludin, ZO-1, and β-catenin, and E-cadherin; none of these effected were detected in response to epithelial cytokines TSLP, IL-33, or IL-25 [60]. IL-4 and IL-13 have also been shown to enhance epithelial expression of TGFβ which can augment subepithelial fibrosis [61]. Disruption of epithelial barrier function and decreased expression of tight junction proteins ZO-1, occludin, and E-cadherin was also observed in epithelia cells exposed to TNF and IL-6 [62–64]. These two cytokines are among asthma cytokines that initiate and maintain airway inflammation in asthma [65–67].

Environmental irritants like pollutants and allergens can also induce epithelial damage on their own and elevate the release of proinflammatory mediators that can contribute to airway remodeling. Environmental irritant like cigarette smoke can also induce injury of differentiated asthmatic epithelial cells and cause further reduction of epithelial resistance at concentrations much lower than that required to induce similar injury in differentiated normal epithelial cells [68]. Similar defects were detected in asthmatic epithelial cells exposed to Alternaria extract which increased IL-8 and TNF release and reduced epithelial resistance [69]. These changes were prevented by heat-inactivated extract [69]. These data are indicating inflammatory environment and exposure to irritants can induce epithelial injury and compromise epithelium function, and that can be further impaired by profound inflammatory responses and insufficient repair.

2.1 Allergic Asthma:

Asthma is a chronic airway inflammatory disorder with multiple pathological mechanisms and clinical presentations [40,70,71]. Asthma is usually associated with excessive inflammation and bronchoconstriction in response to inhaled irritants like smoke, particulate matter, pathogens like viruses, and aeroallergens like cockroach, house dust mite, animal dander, pollens, and moulds [40,70,71]. Interaction of inhaled irritants with airway epithelium activate airway epithelium, induced immune hyperreactivity, bronchoconstriction, and reduced airway function; these changes are

presented mainly as reduction of forced expiratory volume in 1 second (FEV1) in response to methacholine challenge [72,73]. These changes lead to clinical presentations include airflow obstruction, cough, dyspnea, wheezing, phlegm, and exacerbations [72,73]. Half of adult asthmatics are atopic which refers to higher tendency to develop exaggerated allergic reactions and production of allergen-specific IgE [72]. There are several biomarkers of asthma including eosinophils, neutrophils, IgE, periostin, and fractional exhaled nitic oxide (FeNO) [74]. These biomarkers help in categorize and management of asthma phenotype in diagnosis and treatment [74].

Lung biopsies from asthmatics including non-atopic showed accumulation of eosinophil, mast cells, and $CD4^+$ T lymphocytes with IL-4 and/or IL-5 in the epithelium and lamina propria [75,76]. Clinical and research approaches showed asthma as a Th2 driven inflammation with increased type-2-cytokines IL-4, IL-13, IL-5, and eosinophilic airway inflammation [77]. However, in some asthmatics who respond poorly to steroids, airway infiltration is composed mainly of neutrophils this endotype of asthma is low in Th2 driven inflammation and known as T2-low [77,78]. T2-low asthma is characterized mainly by activation of Th1 and/or Th17 and the mechanism underlying the recruitment and infiltration of neutrophils is not well understood [77]. Meanwhile, the pathogenesis of T2-low asthma was shown to involve activation of NOD-like receptor protein 3 (NLRP3) and increased IL-1β [79]. The Th1-high and Th-17-high inflammation are marked by increased IFN-γ and IL-17 respectively [80,81] . In summary, understanding the airway inflammatory microenvironment will provide more insight to understand the underlying molecular mechanisms of airway inflammatory diseases and develop more therapeutic targets.

3. Airway epithelium activation and role of type 2 cytokines in allergic asthma:

3.1. Role of allergens in airway epithelium activation:

Allergens are defined as foreign proteins or glycoproteins that can trigger specific IgE production and allergic responses [82]. Proteolytic or proteases activity in some inhaled allergens, such as house dust mite (HDM), cockroach, and fungal allergens, were shown to be major contributors to allergenicity and responsible for allergic potency besides allergen repetitive motifs [83]; both proteases and repetitive motifs can activate B cell receptor crosslinking, dendritic cells, and

epithelial cells PRRs like TLRs that are promoting Th2 activation [82]. Proteases can also express their activity through cleavage of protease activated receptor -2 (PAR-2) which is elevated in asthmatic epithelium [84–86]. Mucosal exposure to cockroach extract (CE) *in vivo* increased eosinophilic airway inflammation, airway hyperresponsiveness, and allergen specific IgG1 [84]. These inflammatory responses were abolished with use of heat-inactivated CE, CE pretreatment with trypsin inhibitor, or anti-PAR-2 blocking Ab [84]. OVA-challenged wild type mice showed lumen infiltration of inflammatory cells eosinophils, neutrophils, and lymphocytes and increased hyperreactivity to methacholine, and overexpression of PAR-2 exacerbated both lumen infiltration and hyperreactivity [87]. These effects were diminished by deletion of PAR-2 and resulted in reduced antigen specific-IgE [87]. These results suggest that asthmatics may be more susceptible to inflammatory exacerbations upon exposure to PAR-2 agonists due to overexpression of PAR-2.

Collectively, airway epithelium can recognize and interact with inhaled allergens that drives activation of NFκB and increase expression of epithelial mediators that attract adaptive immune cells to the site of injury. These inflammatory responses were shown to be associated with epithelial damage, increased permeability, and dysregulated proliferation. These changes make asthmatics vulnerable to further penetration of allergens and pathogens with profound inflammation.

3.2. Role of type 2 cytokines in asthma:

Constitutive activation of transcription factors like nuclear factor kappa B (NFκB) and signal transducer and activator of transcription -1 and -6 (STAT-1 and -6) were detected in asthmatic epithelial cells compared to healthy controls [88–90]. Constitutive activation of NFκB, STAT-1 and -6 were associated with increased expression of inflammatory cytokines, which may contribute to epithelial damage [91] and tissue accumulation of Th2 cells [88–90]. On the other hand, allergen sensitized and challenged transgenic mice of suppressed epithelial NFκB showed that suppression of epithelial NFκB was sufficient to alleviate inflammatory responses [90], besides marked reduction of tissue infiltration with eosinophils, neutrophils, and lymphocytes compared to the wild type [90]. Suppression of epithelial NFκB also showed decreased expression of Th2 cytokines IL-5 and IL-13 and attenuated antigen specific IgE production [90]. Similar data was shown in animal models genetically deficient for Th2 cytokines [92–95]. In asthmatic patients, Th2 high inflammation with

increased Th2 cytokines IL-4, IL-13, and IL-5 and eosinophilia were detected in BAL and blood compared to healthy controls in various studies [96–98]. These data showed a central role of airway epithelium NFκB activation in maximal recruitment and activation of T and B lymphocytes which exacerbate airway inflammation. Increased expression of Th2 inflammatory mediators were shown to regulate expression TLRs in normal AECs [99]; indicating bi-directional regulation of Th2 and Th2 cytokines in activation of innate and adaptive immunity in airway inflammation.

Th2-associated cytokines or as known recently type 2 cytokines because they are also expressed by other immune cells, beside Th2 cells, like mast cells, type 2 innate lymphoid cells (ILC2s), and basophils [100,101]. IL-4 was defined first as B cell growth factor [102]. IL-4 stimulates expression of B cell major histocompatibility complex (MHC) calls II, surface IgM, and low affinity IgE receptor; in addition to stimulation of immunoglobulin isotype class switching from IgM to IgE [27,103,104]. Besides direct effect of IL-4 on IgE production, IL-4 was shown to promote Th2 differentiation and develop Th2-mediated responses [105]. IL-13 is another Th2 cytokine that is closely related to IL-4 and share some properties. Overexpression of IL-13 in the lungs induced inflammation, subepithelial fibrosis, mucus and eotaxin production [106]. Both IL-4 and IL-13 activate AECs and upregulate epithelial-chemotactic factor eotaxin-3/CCL26 expression which attract eosinophils to inflamed airways [23,107]. IL-4 and IL-13 can also activate B cells synthesis of IgE and IgG and promote expression of adhesion molecules that facilitate eosinophil migration to site of inflammation [108–110]. It was also shown that IL-4 and IL-13 induce goblet cell proliferation and mucus production [111–113], promote fibroblasts transformation to myo-fibroblasts [114], increase proliferation of airway smooth muscle [115], and cause airway hyperresponsiveness [116,117]. These data are suggesting that IL-4 and IL-13 can exacerbate airway inflammation through increased production of mucus, collagen deposition, and contraction of airways. However, the role of IL-4 in IgE production was not strictly required for airway hyperreactivity [105]. Evidently, overexpression of IL-4 induced lymphocytic and eosinophilic inflammation but not hyperreactivity [118], Similarly, the role of IL-13 in inflammatory diseases was questioned in some studies that showed IL-13 exposure to mast cell endogenous serine proteases during degranulation resulted in degradation of IL-13 [119,120] which may decrease IL-13 biological effects. These data are suggesting an elusive effector role of IL-4 and IL-13 in asthma. IL-5 has been shown to has a limited scale of activity that is limited to eosinophils in human. IL-5 regulates eosinophiles differentiation, recruitment, survival, and degranulation [121]. Constitutive expression of IL-5 in

transgenic mice showed profound and enduring eosinophilia in blood, peritoneal exudate, spleen, lung, lymph nodes, and bone marrow [122]. In contrast, sensitized and challenged animal model with inactive IL-5 gene vanished eosinophilia, lung inflammation and damage, airway hyperreactivity [123]. Reconstitution of IL-5 restored allergen-induced eosinophilia and airway dysfunction [123]. These data are suggesting that IL-5 is sufficient to induce eosinophilia. IL-9 is another Th2 cytokine that governs allergen-induced mast cell as shown in human asthmatic lung tissue where mast cells were the main IL-9 receptor expressing cell population [124]. IL-9 promotes mast cell proliferation and differentiation by regulating the expression of bone marrow derived mast cell protease transcripts.

Administration of anti-IL-9 to animal model of allergic asthma reduced mast cell number, expression of TGFβ, and had protective effect against airway remodeling, [124]. In conclusion, Th2 cytokines play a key role in orchestrating, preserving, and amplifying inflammatory responses in asthma.

4. Connecting inflammation with metabolic changes:

Insulin is a key growth hormone that regulate metabolism and maintain normal levels of glucose and lipid beside its stimulation of cell growth and proliferation [125–128]. Insulin functions through binding to its cell surface receptor that is a glycosylated, disulfide-linked $\alpha 2\beta 2$ tetramer including insulin-like growth factor 1 (IGF1) receptor and insulin receptor-related receptor (IRR) [129,130]. Binding of insulin to the insulin receptor activates its intrinsic tyrosine kinase activity leading to receptor autophosphorylation and subsequent phosphorylation of several downstream substrates [126]. The best described substrates are insulin receptor substrate (IRSs) proteins, Shc, SH2B2 (APS), and Cbl [131–135]. Tyrosine phosphorylation of IRS activates phosphatidylinositol-3-kinase (PI3K) and leads to the activation of Akt kinase [136,137]. Insulin activation of PI3K/Akt in hepatocytes is critical in the regulation of glycogen and lipid accumulation besides glucose homeostasis [138]. Activation of RAS/MAP kinase pathway through phosphorylation of Shc substrate regulates expression of cell growth, division, and differentiation genes; besides regulation of cell cycle, wound healing, integrin signaling, and cell migration [139–141]. Additionally, phosphorylation of SH2B2 and Cbl activate G proteins[133,135]. Insulin-induced G protein activation triggers actin cytoskeleton changes, exocyst complex assembly, and glucose transport [142]. Briefly, insulin activation of these substrates regulates a variety of transcription factors, GTPase- activating

proteins, and other kinases that are involved in metabolism and cell growth [143]. Pathophysiology of metabolic syndrome is mainly mediated by insulin resistance which result in defective cellular responsiveness to insulin and presented as reduction or inhibition of insulin-induced effects. Insulin resistance is part of many metabolic disorders like metabolic syndrome and type 2 diabetes millets (T2DM) [143–146]. The underlying molecular mechanisms causing insulin resistance has not been fully elucidated [145,147].

Inflammation modulates metabolism and was shown to play a role in the pathogenesis of insulin resistance [148–151]. Chronic inflammation of adipose tissue is associated with increased production of proinflammatory mediators TNF, and IL-6 [152–155]. TNF induced insulin resistance via inhibition of insulin receptor binding to IRS-1 which prevents insulin activation of PI3K/Akt pathway [156–158]. Biochemical or genetic blockage of TNF improved insulin sensitivity [153,159,160]. Interestingly, lack of TNF and TNF receptors did not prevent obesity in high-fat diet fed mice, but showed lower insulin levels, and remarkable insulin and glucose tolerance which improved insulin sensitivity compared to TNF $^{+/+}$ obese mice [153]. On the other hand, IL-6 induced insulin resistance through IL-6-induced suppressor of cytokine signaling -3 (SOCS-3) protein which can directly interact with IRS-1 and downregulate insulin action [155]. Inhibition of IL-6 improved insulin sensitivity in human [161] and animal model [162].

Some chronic inflammatory conditions have been associated with more complicated metabolic changes and cellular damage that might be related to insulin resistance. Increased TNF in rheumatoid arthritis and systemic lupus erythematosus showed lower level of high-density lipoproteins, higher cholesterol, and impaired peripheral glucose metabolism compared to healthy control [163–166]. Similarly, metabolic syndrome is associated with metabolic abnormalities including insulin resistance, glucose intolerance, obesity, hypertension, and dyslipidemia [167]. Inflammation with each of these abnormalities was shown to increase the risk of diabetes and cardiovascular diseases [168]. In T2DM, hyperglycaemia was shown to cause mitochondrial dysfunction through increased formation of reactive oxygen species (ROS) which induce oxidative stress [169,170]. Oxidative stress stimulates stress-responsive intracellular signaling and induces cellular damage [169,170]. Further damage could happen with glucose toxicity in hyperglycaemia in which non-enzymatic attachment of glucose and its toxic derivatives with proteins, lipids, nucleic acids through glycation, that leads to the formation of advanced glycation ends products [171,172]. Advanced

glycation end products promote inflammation, increase production of proinflammatory cytokines, generation of free radicals, and binding to glycation end product receptors on immune cells which influence recognition, uptake, and antigen presentation by dendritic cells and monocytes [173–176]. Taken together, chronic inflammation and insulin resistance impair insulin-induced effects, cause complicated metabolic and pathologic changes, and trigger tissue damage.

4.1. Metabolic changes in airway inflammatory diseases:

Epidemiological data shows more severe asthma in obese patients [177–179]. It was also shown that obesity is connected to allergic asthma through inflammatory pathways [179–181], which may contribute to impaired airway function. Normal human bronchial epithelial cells are dependent on mitochondrial oxidative phosphorylation (OXPHOS) for ATP supply [182]. Mitochondrial aerobic oxidation uses glycolysis end products, glutamine, and amino acids in the presence of oxygen and H_2O to produce ATP [183] (Illustration 1.2). Several studies showed metabolic abnormalities in airway inflammatory diseases [184–186]. A metabolomics study used BAL obtained from sensitized and challenged mice experimental model of asthma revealed altered metabolic signatures including energy, carbohydrate, lipid, and creatinine relate metabolites [187]. Additionally, hyperglycaemia was detected in nondiabetic children and adult patients with asthma or COPD exacerbations [184,185] that was accompanied with hyperinsulinemia which was not sufficient to restore normal glucose level [186]. Other studies showed that asthma patients with high circulating level of IL-6 experience more severe asthma than those without increased circulating IL-6 level [188]. IL-6 high patients had high body mass index (BMI) and higher prevalence of diabetes [188]. They also had worse lung function and more frequent asthma exacerbations [188]. IL-6 is regulated by IL-1β and increased IL-6 is very likely suggesting activated antigen presenting cells that increase production of IL-1β. Some allergens like that in HDM are known to induce asthma via TLR4 activation which suggest increased IL-6 production[189]. These data are suggesting that patients with increased levels of IL-6 and TNF may experience more severe asthma, worsen prognosis, and modulations of glucose and lipid metabolic patterns.

Impaired insulin action may extend to alter mitochondrial function and capacity for oxidation. Insulin regulation of glycolysis provides metabolic derivatives such as pyruvate for mitochondrial OXPHOS; besides, insulin signaling is essential for synthesis of mitochondrial DNA and proteins

[190,191]. These connections are suggesting that insulin resistance may result in defected mitochondrial function. TNF was shown to regulate pyruvate dehydrogenase (PDH), PDH regulates the influx of the glycolysis product pyruvate into the mitochondria for OXPHOS [192]. In pulmonary arterial hypertension, PDH activity is reduced in pulmonary artery smooth muscle cells (PASMCs), and similar level of reduction in PDH activity was detected in primary PASMCs from healthy donors after exposure to TNF [192]. Reduced PDH activity was associated with reduced hexokinase-II mitochondrial association, which suggest subsequent modulation of glycolysis, and mitochondrial function [192]. Another study showed that TNF treated human airway smooth muscle cells showed increased mitochondrial volume density, and mitochondrial DNA copy through activation of mitochondrial transcription factors NRFs and TFAM [193]. During mitochondrial respiration tolerated levels of free radicals like ROS are generated, however oxidative stress due to increased level of ROS may alter the metabolism, disrupt cellular homeostasis, modulate mitochondrial structure and function, and function as signal transducer that modulate cytokines transcription [194–197]. Mitochondria can regulate its capacity and overcome stress through dynamic changes in its structure and morphology [198]. Mitochondrial networking (fusion) and fragmentation (fission) play a critical role in maintaining functional mitochondria when cells undergo stress [198]. Fusion ameliorates the effect of stress through merging the content of partially damaged and intact mitochondria to maximize mitochondrial capacity [198,199]. Fusion can take place normally during increased demand for OXPHOX, for example during cell proliferation, and is regulated by mitofusions 1 and 2 (Mfn 1, Mfn2) 193,197–200. Fission allows the removal of damaged mitochondria and can facilitate apoptosis [198,199]. Fission takes place normally during cell division to create new mitochondria and during increased demand for glycolysis and is regulated by mitochondrial fission 1 factor (Fis1) and recruited cytosolic dynamin-related protein 1 (Drp1) [194,198–201]. Exposure of AECs and airway smooth muscle cells to cigarette smoke was shown to increase Drp1 and decrease Mfn which suggest enhanced fission [202,203]. This structural change resulted in decreased OXPHOS and metabolic shift toward glycolysis [204]. Examining and imaging of lung tissue of chronic obstructive pulmonary disease (COPD) patients showed dysmorphic mitochondria, and decreased mitochondrial biogenesis though both fission and fusion [205–207], which very likely indicates severe stress. In contrast, airway smooth muscle in asthma showed increased Drp1 and decreased Mfn indicating fission [208]. These data are suggesting that airway inflammatory diseases and exposure of the lungs to inflammatory mediators or environmental

irritants may modulate mitochondrial structure and function which exacerbate lung inflammation in several ways.

Rational:

Airway epithelium has an essential role in protecting the host against inhaled stimuli. In airway inflammatory diseases, epithelium activation and interaction with inhaled agents like aeroallergens provoke expression of epithelium mediators that activate and attract immune cells leading to immune cell infiltration in the lung tissue and profound inflammation. Aeroallergens proteases, Th2 inflammation and elevated Th2 cytokines impair airway function and disrupt epithelium integrity. Airway epithelium damage and dysfunction lead to chronic inflammation in airway inflammatory diseases. Inflammatory responses and increased expression of proinflammatory mediators can modulate metabolism and inhibit insulin action. Hyperglycaemia, reduced OXPHOS, and mitochondrial dysfunction are among the metabolic changes detected in airway inflammatory diseases. Energy, carbohydrate, lipid, and amino acid related metabolites are altered as observed in metabolomic analysis of asthmatics and asthma experimental models. Several observational studies showed connections between inflammation, risk of asthma development, aeroallergen sensitization and insulin deprivation or insulin resistance. However, the effect of AECs exposure to aeroallergens and proinflammatory mediators on the modulation of immune responses, insulin action, and the contribution of that on the disruption of epithelium barrier function was not conducted.

Hypothesis:

Exposure of airway epithelial cells to allergens and proinflammatory mediators may modulate immune cell responses, insulin action, and induce metabolic shifts.

Objectives:

1. Study the interaction of allergens with asthma cytokines to evaluate epithelial changes and immune cell responses
2. Investigate the effects of allergens and asthma cytokines on epithelial energetic phenotype through measuring key parameters of cell energy metabolism: mitochondrial respiration and glycolysis
3. Examine insulin-induced effects in AECs in response to inflammation
4. Evaluate airway epithelial mitochondrial changes in inflammation

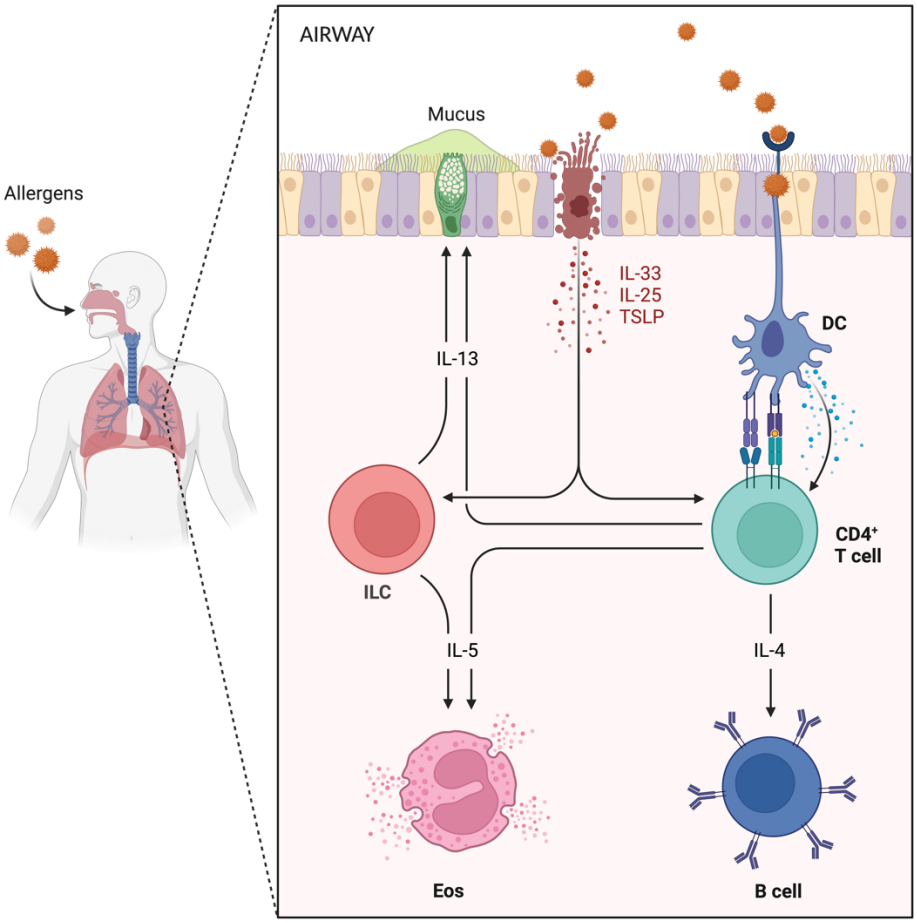

Illustration 1.1: allergic airway inflammatory responses. Created with BioRender.com

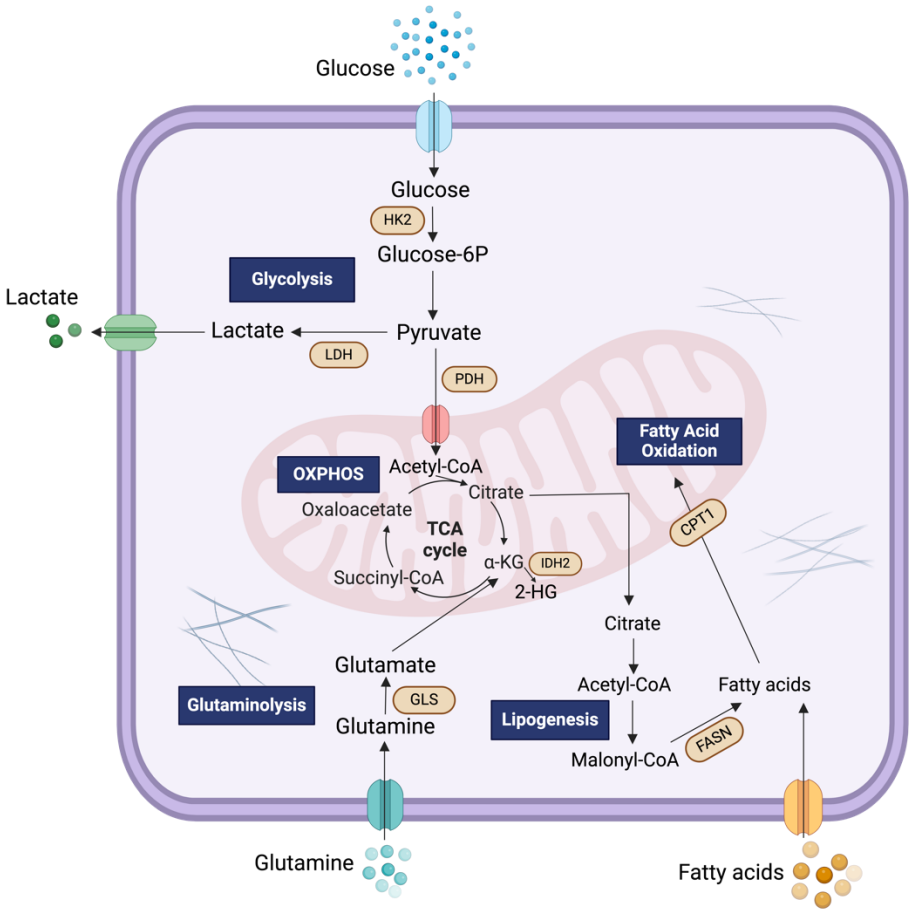

Illustration 1.2: cellular metabolic pathways including glycolysis, glutaminolysis, lipogenesis, TCA cycle, fatty acid oxidation (FAO). Dominant nutrient transporters and metabolic enzymes are emphasized (brown). HK2, hexokinase-2; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; GLS, glutaminase; FASN, fatty acid synthase; CPT1, carnitine palmitoyltransferase I; IDH2, isocitrate dehydrogenase 2; α-KG, alpha-ketoglutarate. Created with BioRender.com

Chapter 2:

German Cockroach Extract Prevents IL-13-induced CCL26 Expression in Airway Epithelial Cells Through IL-13 Degradation

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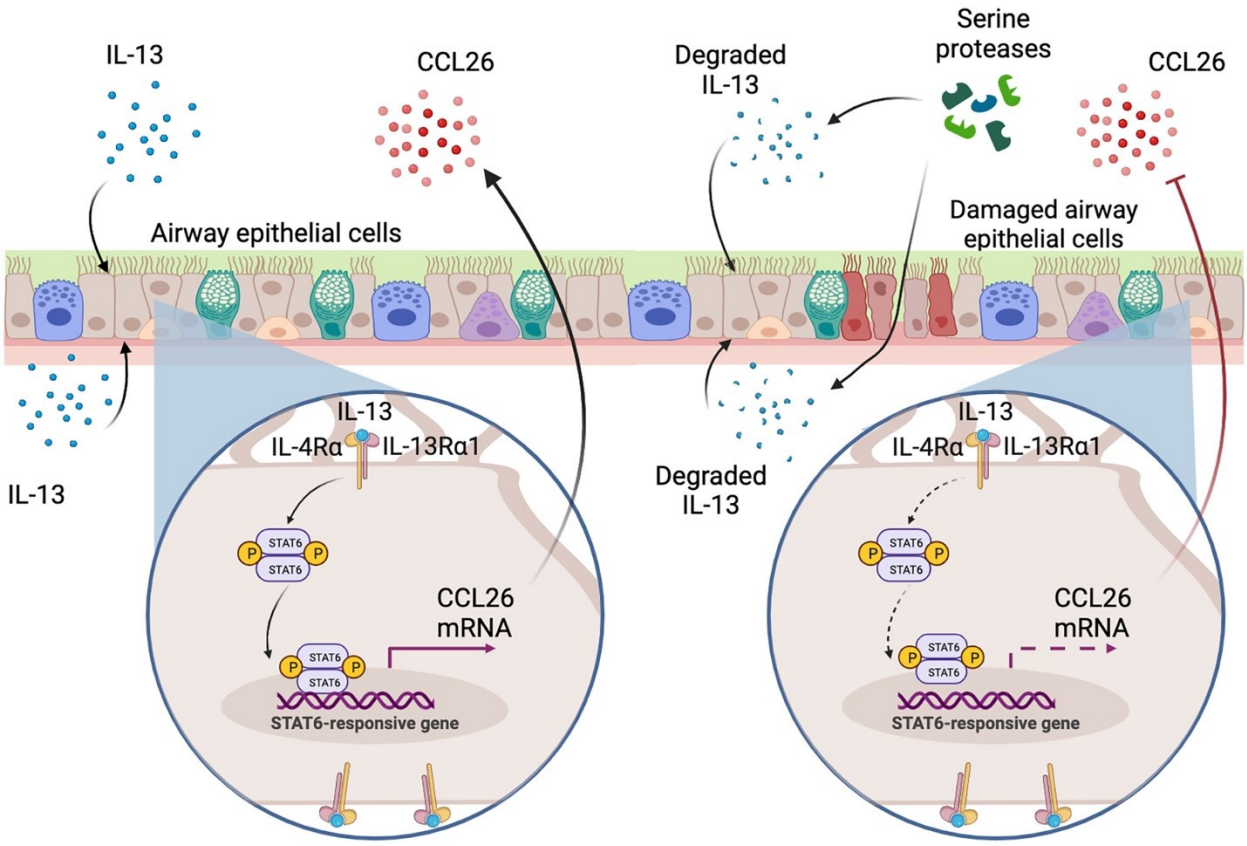
Graphical Abstract:



Airway epithelial cell stimulation with interluikin-13 (IL-13) induced upregulation of CC motif chemokine ligand 26 (CCL26) mRNA and protein release. Cockroach extract serine proteases decreased IL-13-induced CCL26 through degradation of IL-13.

Abstract:

Inhaled aeroallergens can directly activate AECs. Exposure to cockroach allergens is a strong risk factor for asthma. Cockroach allergens mediate some of their effects through their serine protease activity; protease activity is also a major contributor to allergenicity. The Th2 cytokine IL-13 induces upregulation of the eosinophil chemotactic factor CCL26. CCL26 induces eosinophil migration in allergic inflammation. In this work we studied the effect of cockroach proteases on IL-13-induced effects. Immersed cultures of the human bronchial epithelial cell line BEAS-2B and ALI cultures of primary normal human bronchial epithelial (NHBE) cells were stimulated with IL-13, *Blattella Germanica* cockroach extract (CE) or both. IL-13-induced genes were analyzed with qRT-PCR. IL-13 induced upregulation of CCL26, periostin, and IL-13Rα2 in bronchial epithelial cells which were decreased by CE. CE was heat inactivated (HICE) or pre-incubated with protease inhibitors. HICE and CE preincubated with serine protease inhibitors did not prevent IL-13-induced CCL26 upregulation. CE degraded IL-13 and specific cleavage sites were identified. CE also decreased IL-4-induced CCL26 upregulation and degraded IL-4. Other serine proteases such as bovine trypsin and HDM serine proteases did not have the same effects on IL-13-induced CCL26. We conclude that CE serine proteases antagonize IL-13-induced effects in AECs and this CE effect is mediated primarily through proteolytic cleavage of IL-13. IL-13 cleavage by cockroach serine proteases may modulate CCL26 mediated effects in allergic airway inflammation by interfering directly with the pro-inflammatory effects of IL-13 *in vivo*.

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

Introduction:

The airway epithelium has an essential and highly regulated role as the physical innate barrier that interacts with and clears inhaled particles and irritants to maintain normal airway function [209]. Growing evidence suggest that airway epithelium dysfunction orchestrates airway inflammatory diseases [210]. Airborne allergens, such as house dust mite, cockroach, fungal, or pollen allergens, can interact and activate the airway epithelium resulting in the release of pro- and anti-inflammatory factors, such as cytokines, chemokines, antioxidants, and antimicrobial peptides [211–214]. In turn, these pro-inflammatory factors may also enhance the production of allergen specific IgE to the inhaled allergens [215]. Allergic airway inflammation is characterized by eosinophilic infiltration of the lung tissues and airways [216–218] and the presence of Th2 cytokines, such as IL-4, IL-13, and IL-5 [219,220].

Increased levels of IL-13 have been detected in blood, sputum, and BAL fluid of subjects with asthma compared to healthy individuals [221–223]. IL-13 is secreted by multiple cell types including, but not limited to, activated Th2 cells, B lymphocytes, mast cells, and ILC2s [98,224–226]. IL-13 has a central role in asthma pathogenesis through its multiple effects. IL-13 regulates B cell class switching to IgE [227], facilitates inflammatory cell migration into the lung and other tissues through upregulation of adhesion molecules and chemotactic factors [228], promotes goblet cell proliferation and mucus production [111], and stimulates airway remodeling [228] and airway hyperresponsiveness [116].

Regarding eosinophilic inflammation, IL-13 promotes recruitment and activation of eosinophils in the airways through the release of eotaxins from the airway epithelium [23]. Among eotaxins, Eotaxin-3/CCL26 has been shown to be the most effective chemoattractant for eosinophils in asthma through CC motif chemokine receptor (CCR3) ligation [107,229]. CCL26 has been shown recently to also be a ligand for C-X3-C motif chemokine receptor (CX_3CR_1) expressed on myeloid cells[26,230]. CCL26 is the highest expressed eotaxin in cultured airway epithelial cells obtained from healthy donors upon stimulation with IL-13 or IL-4 [231]. CCL26 is also the most potent eotaxin for chemotaxis of eosinophils from patients with asthma and induces both CCR3-dependent and CCR3-independent eosinophil chemotaxis [229].

Exposure to cockroach allergens is one of the strongest risk factors for the development of allergic asthma in low-income urban populations [232–234]. Cockroach allergens have been detected in dust of 85% of homes in inner-city US; exposure to high levels of cockroach allergens is associated with higher risk of asthma-related health problems and development of allergic asthma in cockroach-sensitized patients [232]. Multiple cockroach allergens and other proteins of cockroach origin have been identified to possess proteolytic activity [235,236], which is believed to be a major contributor to their allergenicity [237]. We have shown that inhibition of cockroach enzymatic activity by heat inactivation, or specific inhibitors, prevents mucosal sensitization in a murine model of allergic airway inflammation [84]; the same has been shown for cockroach allergens by others [238], as well as for other allergens, such as house dust mite and aspergillus allergens [239,240]. Some of the pro-inflammatory effects of cockroach allergens may be the result of direct activation of airway epithelial cells by these allergens [241–243].

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

Methods:

Cell culture and activation:

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

Primary NHBE cells from two different normal donors, were purchased from Lonza. NHBE cells were cultured for a maximum of five passages with BEGM in multi-well plates pre-coated overnight with 30 μg/ml bovine collagen type I dissolved in BMEM and used for experiments during passage four and five only. NHBE Cells were cultured BEGM (as described above) with the addition of gentamicin, amphotericin, and retinoic acid (Lonza SingleQuots). ALI cultures of NHBE were done as described [244].

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

To inhibit CE proteases, CE was HICE at 65ºC for 30 minutes or incubated for 30 minutes at room temperature with each one of the following protease inhibitors separately: E-64 (Sigma) a cysteine

protease inhibitor at final concentration 10 μM, aprotinin (Roche Applied Science, Mannheim, Germany) a serine protease inhibitor and very effective against trypsin-like proteases at final concentration 2 μg/ml, or pepstatin A (Sigma) an acid proteases inhibitor at final concentration 1μg/ml. CE protease activity was also examined using trypsin inhibitor from
Glycine max (soybean trypsin inhibitor SBTI) at final concentration of 0.5-1 μg/ml (Sigma). The concentrations of inhibitors used were selected based on the effective working concentration recommended by the manufacturer of each inhibitor and based on previous studies [245–247]. To study PAR-2 activation, the PAR-2 activating peptide (PAR-2 AP) SLIGRL-NH2 and the control peptide (PAR-2 CP) LRGILS-NH2 were used at a concentration of 50 μM for both, as described previously [248,249]. PAR-2 peptides were generously provided by Dr. M Hollenberg, University of Calgary.
For comparative studies, different concentrations of trypsin from bovine pancreas (Sigma) or HDM extract (Greer Laboratories) were used with IL-13 instead of CE. Recombinant human IL-4 (rhIL-4) (R&D Systems) was also substituted for IL-13 in some experiments. Cell viability was evaluated 24 hours after stimulation using a 0.4% trypan blue (Life Technologies) exclusion test [250]. All the conditions presented in all the figures of this manuscript are done in duplicates in each independent experiment except for the experiments with ALI. cultures.

Reverse transcription and quantitative polymerase chain reaction:

Airway epithelial cell total RNA was isolated using either the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) or TRIzol (Life Technologies). 0.5 μg total RNA was reverse transcribed using 0.5μg/reaction of Oligo(dT) 12-18 as primer (Invitrogen, Waltham, MA, USA) and 200 U/reaction of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) in a 20 μl reaction volume.
GAPDH (Hs02658911_g1), CCL26 (Hs00171146) and IL-13Rα2 (Hs00152924_m1) gene expression assays (Thermo Fisher Scientific) were used to perform qRT-PCR. Amplification was performed for 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Samples were analyzed in triplicate using Applied Biosystems QuantStudio Real-Time PCR System (Thermo Fisher Scientific). The mRNA levels of tested genes were normalized to the housekeeping gene GAPDH and analyzed using the $2^{-\Delta\Delta CT}$ method [251] and expressed as fold change of stimulated over unstimulated cells.

Enzyme linked immunosorbent assay (ELISA):

CCL26 protein was measured in cell supernatants using the DuoSet ELISA kit for human CCL26/Eotaxin-3 (R&D Systems) according to the manufacturer's instructions. Seven-point serial 2-fold dilution standard curve of recombinant human CCL26 with highest concentration of 4000 and lowest 62.5 pg/ml was used to calculate concentrations in cell supernatants. IL-8 protein was measured using the DuoSet ELISA kit for human IL-8 (R&D Systems) according to the manufacturer's instructions. Seven-point serial 2-fold dilution standard curve of recombinant human IL-8 with highest concentration of 2000 and lowest 31.3 pg/ml was used to calculate concentrations in cell supernatants. Optical density was measured using a Power Wave XS (BioTek, Winooski, VT, USA) plate reader.

Quantification of CE trypsin-like activity:

Trypsin-like activity was measured using the fluorogenic peptide substrate butoxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin (AMC)·HCl (QAR-AMC) (Bachem, King of Prussia, PA, USA). The assay was performed as described previously [84]. Trypsin from bovine pancreas with known N-benzoyl-L-arginine ethyl ester (BAEE) U/mg activity was used to make a five point 5-fold dilution standard curve to calculate serine protease activity of unknown samples. Activity of protease solutions is shown as BAEE U/mg.

Detection of Human IL-13Rα1 by Flow Cytometry:

Resting and CE activated BEAS-2B cells were harvested with trypsin, centrifuged for 5 minutes at 300g, and resuspended (1×10^6 cells/tube) in 2 ml of FACS buffer (0.5% BSA, 0.1% NaN3 and 3% FBS in 1x PBS). Mouse IgG control antibody (50 μg/10^6 cells) (Invitrogen) and human FcR blocking reagent (1 μg/10^6 cells) (Miltenyi Biotec, Auburn, CA, USA) were added to minimize non-specific binding of antibodies. Cells were then incubated with monoclonal mouse anti-human IL-13Rα1 APC-conjugated antibody (Monoclonal Mouse IgG_{2B} Clone # 419718) or APC-conjugated mouse IgG2b isotype control (both from R&D Systems) at concentration 0.5 μg/10^6 cells. Flow cytometry was performed using LSR Fortessa-II (BD Bioscience, Franklin Lakes, NJ, USA). Results were analyzed using FlowJo (TreeStar, Ashland, OR, USA).

Western blot:

Cell lysates were separated on 10% SDS-PAGE Mini-Protean Precast gels (Bio-Rad, Hercules, CA, USA) and wet transferred to nitrocellulose membranes at 400 mA for 60 minutes at 4℃. Total STAT6 and phosphorylated STAT6 (pSTAT6) Tyr^{641} were detected using a monoclonal mouse anti-human STAT6 (BD Bioscience) or a polyclonal rabbit anti-human pSTAT6 (Cell Signaling, Danvers, MA, USA) antibodies respectively. Membranes were then blotted with IRDye-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (LI-COR, Lincoln, NE, USA) antibodies respectively.

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

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

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

Cleavage of IL-13 by CE was tested by incubating 10 ng of IL-13, IL-13 + 10 µg of CE, or IL-13 + CE pre-incubated with 10 µM aprotinin. All samples were loaded into a pre-cast gel (4-20%), resolved for ~0.5 cm, and stained with Coomassie blue silver. Fragments of IL-13 were N-terminally labeled with an in-gel acetylation protocol based on established work [252]. The addition of this chemical modification at the N-termini of the full-length protein and protein fragments is to facilitate the unambiguous identification of the cleavage events generated by CE. Gel bands were excised and cut into smaller pieces (~1 mm) and transferred to a round bottom 96 well plate. Gel pieces were de-stained twice with 100 mM NH_4HCO_3 containing 50% acetonitrile (ACN) at 37°C for 10 min. Reduction was performed by incubation with 10 mM 2-mercaptoethanol for 30 min at 37°C, followed by alkylation with 50 mM iodoacetamide in the same conditions. Gel fragments were dehydrated with 100% ACN and recovered in 100 µL of acetic acid and 20 µL of acetic anhydride. Samples were incubated for 5 hours at room temperature. Excess of labeling reagents was removed, and gel pieces were rinsed with deionized water. The gel was dried down

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

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

Statistics:

Results are presented as mean ± SEMs. To show differences between multiple groups, one-way and two-way ANOVA with multiple comparison analysis was used on GraphPad Prism;

significance is indicated on the figures as follows: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

Results:

1. *CE inhibited IL-13-induced CCL26 mRNA upregulation and CCL26 protein release in human airway epithelial cells:*

Stimulation of BEAS-2B cells with IL-13 (20 ng/ml) for 24 hours induced, as expected [253], CCL26 mRNA upregulation (Figure 2.1A). Co-stimulation of BEAS-2B cells with IL-13 and CE (5 μg/ml), however, decreased IL-13-induced CCL26 upregulation (Figure 2.1A) in a dose (Figure 2.1B) and time (Figure 2.1C) dependent manner. Stimulation of BEAS-2B cells with IL-13, CE or both for 24 hours did not affect cell viability (Figure 2.1D). CE (5 μg/ml) also inhibited IL-13-induced CCL26 protein release from BEAS-2B (Figure 2.1E). The inhibitory effect of CE was not specific for CCL26 induction by IL-13; CE also inhibited IL-13-induced IL-13Rα2 (Figure 2.1F) and periostin (Figure 2.1G) mRNA upregulation.

IL-13 also induced CCL26 mRNA expression and protein release from immersed NHBE cells, and CE prevented this induction at the mRNA and protein levels (Figure 2.1 H and I). We then tested the effects of CE on IL-13-induced CCL26 synthesis and release from NHBE ALI cultures. IL-13 added to the basolateral surface of the ALI cultures induced CCL26 mRNA and addition of CE on the basolateral side decreased this induction (Figure 2.1J) by at least 70%, while CE addition to the apical side did not affect this IL-13-mediated CCL26 induction. IL-13 added to the apical surface of these cultures induced a small CCL26 mRNA induction, approximately 10-fold increase versus >4000-fold increase when added basolaterally, and this induction decreased when CE was added to the apical surface (data not shown).

IL-13-induced CCL26 expression in epithelial cells is STAT6 dependent [254]. Ligation of IL-13R complex with IL-13 mediates pSTAT6-Tyr^{641} that peaks at 30 minutes [254–256]. IL-13-induced pSTAT6 in BEAS-2B cells after 30 minutes of activation was decreased in the presence of CE (Figure 2.2A and B).

2. *The CE effect on IL-13-induced CCL26 upregulation is trypsin-like protease dependent:*

Multiple proteins of cockroach origin were identified to possess proteolytic activity [235,236]. We therefore tested whether the inhibition of IL-13-induced CCL26 mRNA upregulation by CE is

protease-dependent. Heat inactivation decreases CE protease activity [84] and HICE did not inhibit IL-13-induced CCL26 mRNA upregulation (Figure 2.3A). To identify the specific CE protease(s) responsible for inhibiting IL-13-induced CCL26 upregulation, CE was pre-incubated with E-64, aprotinin, or pepstatin A. In contrast with CE pre-incubated with E-64 or pepstatin A, CE pre-incubated with aprotinin, did not inhibit IL-13-induced CCL26 mRNA upregulation and protein release (Figure 2.3B and C), suggesting that the inhibitory effect of CE is mediated through serine proteases. A trypsin-like protease inhibitor, SBTI, also prevented the effect of CE on IL-13-induced CCL26 mRNA upregulation (Figure 2.3D), indicating that the CE effect is mediated by a trypsin-like serine protease. In support of these observations, heat-inactivation, SBTI and aprotinin all decreased trypsin-like protease activity of CE in our experiments (Figure 2.3E).

CE and certain purified CE serine proteases activate PAR-2 [235]. To test whether CE inhibition of IL-13-induced CCL26 mRNA upregulation might be mediated through PAR-2 activation, we treated BEAS-2B cells for 24 hours with IL-13 and either the PAR-2AP SLIGRL-NH2 or the CP LRGILS-NH2. These peptides have been used previously to study PAR-2 activation [257]. PAR-2 activation did not induce CCL26 mRNA upregulation on its own and had no effect on IL-13-induced CCL26 mRNA upregulation (Figure 2.3F), suggesting that CE inhibition of IL-13-induced CCL26 upregulation is most likely PAR-2 independent. We validated that PAR-2AP was able to activate BEAS-2B cells, as it induced IL-8 protein release (Figure 2.3G), as has been shown [258].

Since CE trypsin-like activity appeared to mediate the inhibition of IL-13-induced CCL26 mRNA upregulation in airway epithelial cells, we then tested whether this is a general effect of trypsin-like proteases. Bovine pancreatic trypsin, in concentrations that had similar trypsin-like activity to the concentrations of CE used in our experiments (Figure 2.3E), was not able to inhibit IL-13-induced CCL26 mRNA upregulation (Figure 2.3H). Similarly, HDM extract, that contains serine protease activity [245,259], did not inhibit IL-13-induced CCL26 upregulation (Figure 2.3I).

3. CE did not decrease IL-13Rα1 expression on airway epithelial cells:

IL-13 signaling in epithelial cells is mediated through the IL-13Rα1, that is a heterodimer of the IL-4Rα and IL-13Rα1 chains [260]. Alteration of IL-13R expression by CE, could potentially mediate the inhibitory effects of CE on IL-13-induced CCL26 mRNA upregulation. Incubation of BEAS-2B cells with CE for 24 hours did not alter IL-13Rα1 expression on the cell surface (Figure 2.4 A and B). In addition, as we discussed above (Figure 2.1F), CE did not alter IL-13Rα2 mRNA

expression, a decoy receptor that binds strongly to free IL-13 and could limit its activity, on its own, but in contrast, decreased IL-13-induced IL-13Rα2 mRNA upregulation.

4. CE proteolytic activity cleaves IL-13:

We next examined whether CE may cause proteolysis of IL-13 and therefore inhibit its induced effects. We detected IL-13 cleavage after incubation of recombinant IL-13 with CE for 15 minutes at 37℃ as evidenced by decreased intensity of the full-length IL-13 band and the generation of short forms of IL-13 in the 7.85-9.21 MW range (Figure 2.5A and B). HICE and BCE did not induce IL-13 cleavage. Inhibition of serine proteases with aprotinin did not affect cleavage in general but preserved more of degradation products, which were not as evident in the case of IL-13 incubated with fully active CE. It is interesting that inhibition of cysteine proteases with E64 prevented to some extent IL-13 degradation by CE and generated cleavage products of slightly different MW compared to products generated by CE incubated with aprotinin (Figure 2.5A and B). Quantification of the cleavage products is shown in (Figure 2.5C).

5. Identification of cleavage sites on IL-13 by mass spectrometry:

We then performed chemical modification and mass spectrometry analysis of the fragments generated upon incubation of IL-13 with CE or CE pretreated with aprotinin to identify the potential cleavage sites that may affect IL-13 activity in our assays [261,262]. Our chemoselective labeling approach allowed the identification of several N-terminally modified peptides that might correspond to cleavage events on the IL-13 sequence by the proteases in the CE. A total of 5, 4 and 10 acetylated peptides were detected for IL-13, IL-13+CE or IL-13 + CE pretreated with aprotinin, respectively (Figure 2.6A).

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

6. CE decreased IL-4-induced CCL26 upregulation and cleaved IL-4:

IL-13Rα1/IL-4Rα complex can be activated by either IL-13 or IL-4 [260]. CE also inhibited IL-4-induced CCL26 mRNA upregulation from BEAS-2B cells (Figure 2.7A) and mediated IL-4 cleavage that was evident within 5 minutes and led to almost complete degradation of IL-4 by 2 hours (Figure 2.7B and C).

Figures:

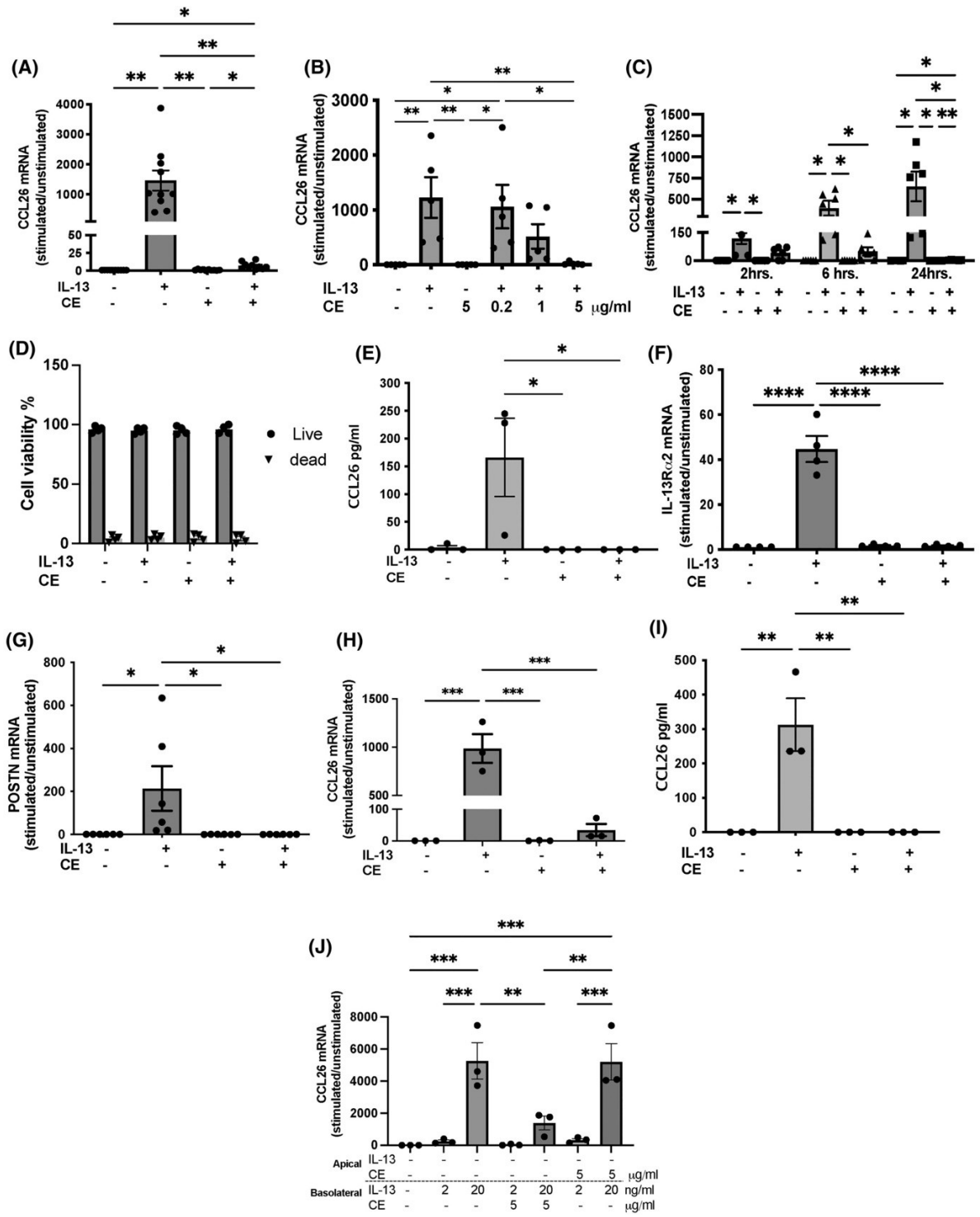


Figure 2.1: CE inhibited IL-13-induced CCL26 mRNA expression and protein release in human airway epithelial cells. (A) Fold induction of CCL26 mRNA following activation of BEAS-2B

cells with IL-13 (20 ng/ml), CE (5 μg/ml), or both for 24 hrs.; n=9. IL-13-induced CCL26 expression in BEAS-2B cells incubated with IL-13 (20 ng/ml) and various concentrations of CE ; n=4 (B) or CE (5 μg/ml) for different time points (C); n=3. (D) BEAS-2B cell viability 24h after activation with IL-13 (20 ng/ml) and/or CE (5 ug/ml); n=4. (E) IL-13-induced CCL26 protein release after 24 hrs. activation with IL-13 (20 ng/ml), CE (5 μg/ml), or both; n=3. (F) Inhibition of IL-13-induced IL-13Ra2 mRNA after 24 hrs activation with IL-13 (20 ng/ml), CE (5 μg/ml), or both; n=4. (G) Inhibition of IL-13-induced periostin mRNA after 24 hrs. activation with IL-13 (20 ng/ml), CE (5 μg/ml), or both; n=6. IL-13-induced CCL26 mRNA levels (H) and CCL26 protein release (I) 24 hrs. after activation of NHBE cells with IL-13 (20 ng/ml), CE (5 μg/ml), or both; n=3 each. (J) IL-13-induced CCL26 mRNA in stimulated ALI with IL-13 (2 and 20 ng/ml), CE (5ug/ml) or both in apical or basolateral side as indicated; n=3. Results shown are mean ± SEM. Results were analyzed with one-way ANOVA, multiple comparisons except in (C) two-way ANOVA multiple comparison was used, ns=non-significant, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

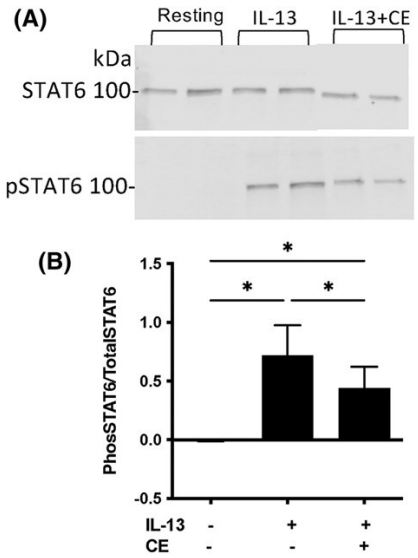


Figure 2.2: CE decreased IL-13-induced STAT6 phosphorylation. BEAS-2B cells were treated with IL-13 (20 ng/ml), CE (5 μg/ml), or both for 30 min. (A) Western blot of total STAT6 (top) and pSTAT6-Tyr^{641} (bottom); a representative of three experiments is shown. (B) Densitometric analysis of protein abundance determined as ratio of optical density signal of pSTAT6-Tyr^{641}/STAT6. Values are shown as mean ± SEM, n=3. Results were analyzed with one-way ANOVA, multiple comparisons, *p≤0.05.

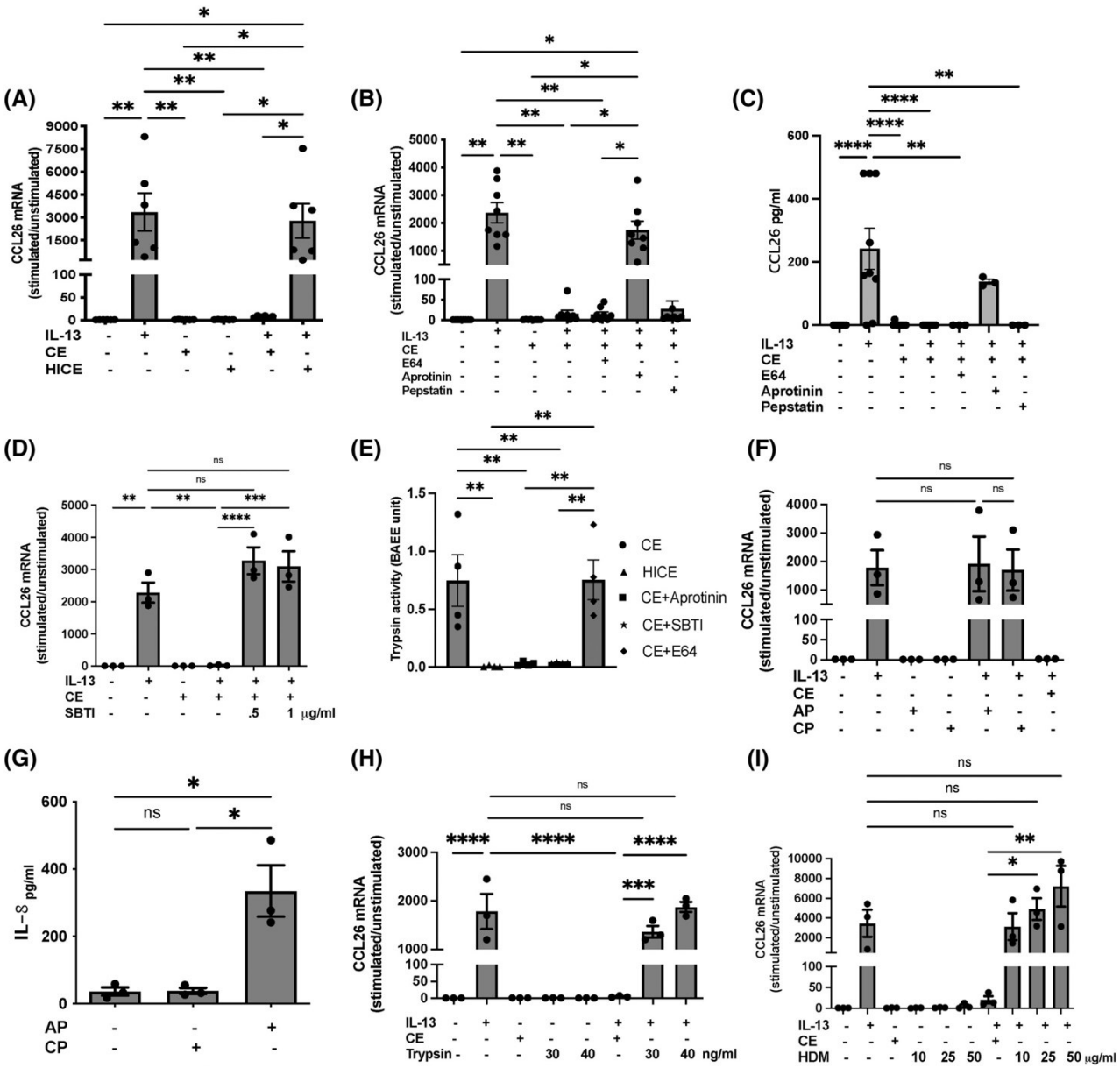


Figure 2.3: The inhibitory effect of CE on IL-13-induced CCL26 upregulation is trypsin-like protease dependent. (A) IL-13-induced CCL26 mRNA in BEAS-2B cells stimulated with IL-13 in the presence of CE or HICE for 24 hrs.; n=3. IL-13-induced CCL26 mRNA; n=4 (B) and protein release; n=3 (C) in cells treated with IL-13, CE or CE pre-incubated with protease inhibitors. (D) IL-13-induced CCL26 mRNA in cells treated with IL-13, or IL-13 with either CE or CE pre-incubated with SBTI; n=3. (E) Trypsin activity in BAEE unit in solutions of CE (5μg/ml), HICE (5μg/ml), or CE preincubated with aprotinin, SBTI, or E64; n=4. IL-13-induced CCL26 mRNA upregulation (F) and IL-8 protein release (G) in cells treated with IL-13, or IL-13 with either CE, PAR-2AP or PAR-2CP; n=3 each. (H) IL-13-induced CCL26 mRNA in cells treated with IL-13,

or IL-13 with either CE or bovine trypsin; n=3. (I) IL-13-induced CCL26 mRNA in cells treated with IL-13, or IL-13 with HDM for 24 hrs.; n=3. Results shown are mean ± SEM. Results were analyzed with one-way ANOVA, multiple comparisons, ns=non-significant, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

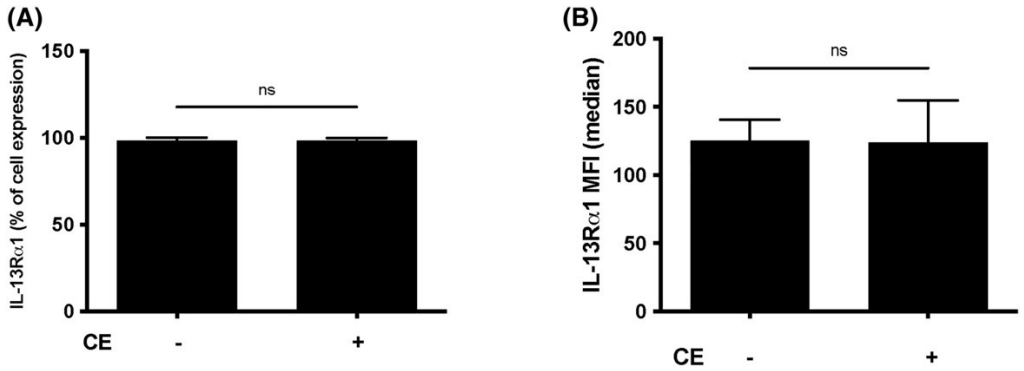


Figure 2.4: CE did not decrease IL-13Rα1 expression on the surface of airway epithelial cells. BEAS-2B cells were untreated or treated with CE (5μg/ml) for 24 hrs. (A) % of BEAS-2B cells expressing IL-13Ra1 on cell surface. (B) Median fluorescent intensity (MFI) of IL-13Ra expression on BEAS-2B cells. Results shown are mean ± SEM; n=3, ns=non-significant.

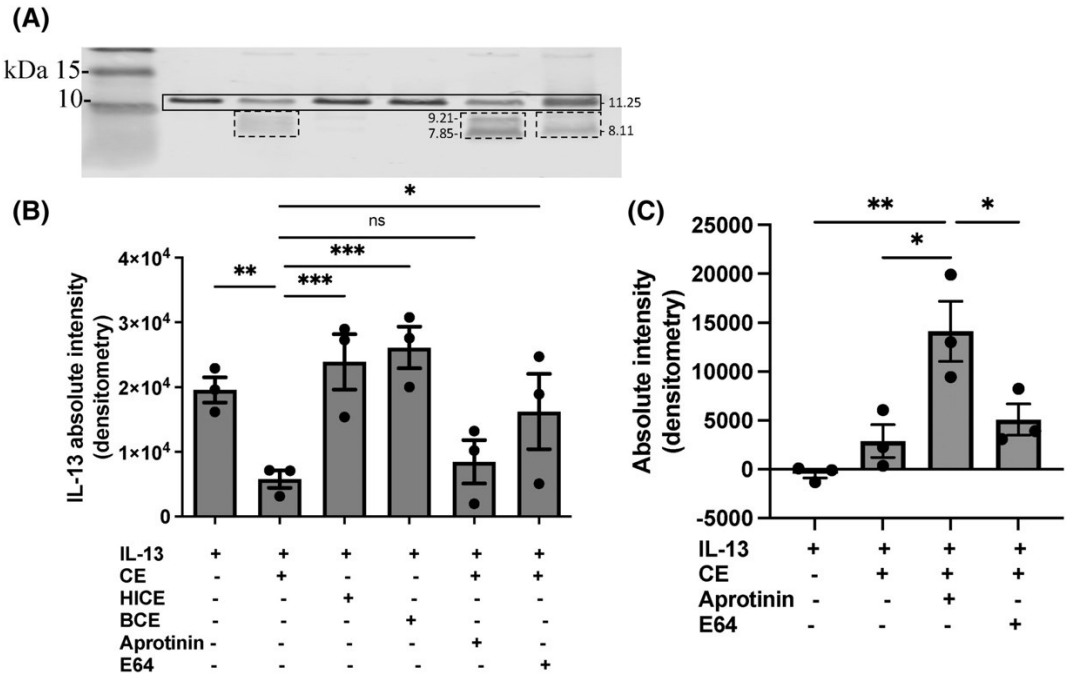


Figure 2.5: CE proteolytic activity cleaved IL-13. (A) Western blot of IL-13 or IL-13 treated with CE, HICE, BCE, or CE preincubated with aprotinin or E64; a representative of three experiments is shown. (B) Quantification of signal intensity of full-length IL-13 at 11.25 kDa (solid box) and (C) Quantification of IL-13 cleavage products signal intensity (dashed boxes); n=3. Results shown are mean ± SEM. Results were analyzed with one-way ANOVA, multiple comparisons, ns=non-significant, *p≤0.05, **p≤0.01, ***p≤0.001.

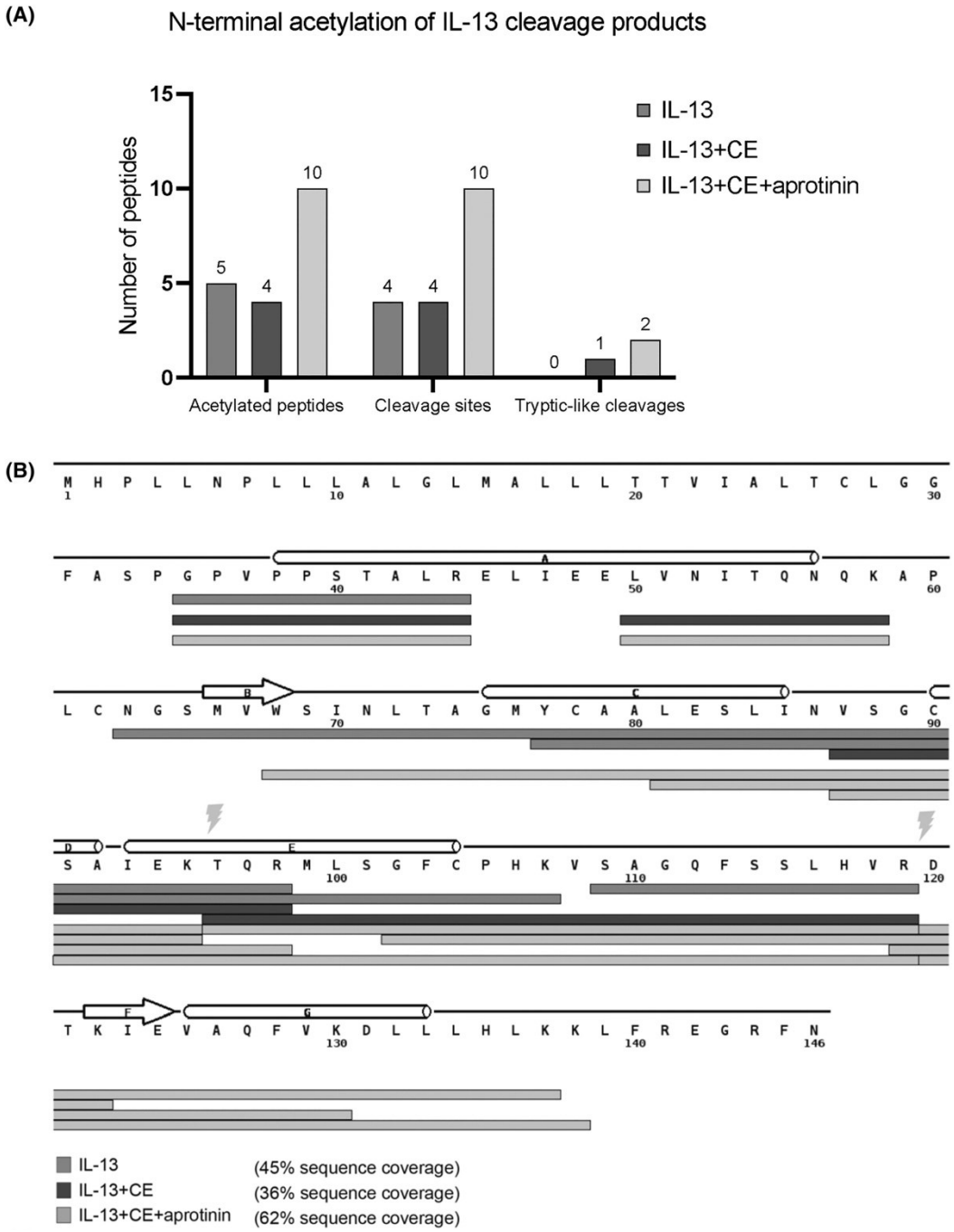


Figure 2.6: Identification of cleavage events in IL-13 by mass spectrometry. (A) Summary of the MS results from in-gel acetylation reaction. (B) Peptide map showing primary sequence of IL-13, secondary structural features, acetylated peptide distribution and sequence coverage. Two possible cleavage sites in the IL13 sequence show the P1=K/R specificity are highlighted (K95|T96 and R119|D120).

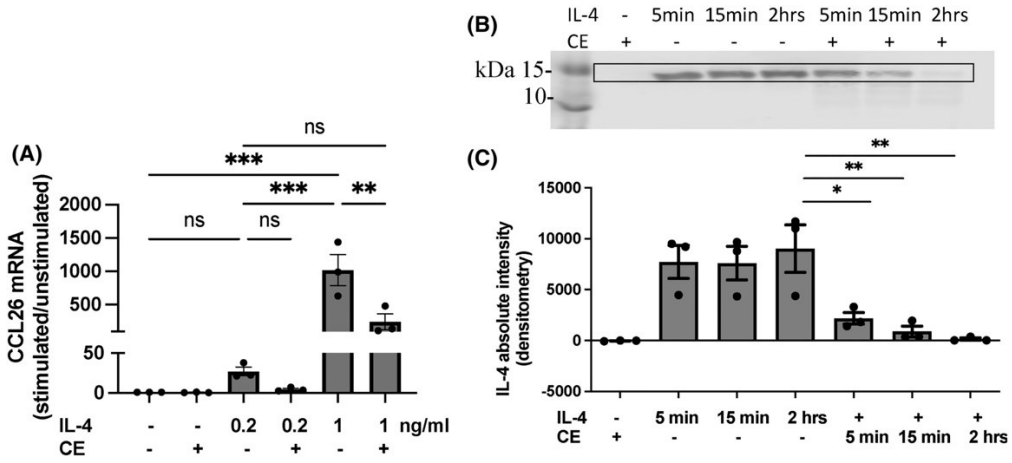


Figure 2.7: CE decreased IL-4-induced CCL26 upregulation and cleaved IL-4. (A) CCL26 mRNA in BEAS-2B cells after activation for 24 hrs. with IL-4 (0.2 or 1 ng/ml) or IL-4 with CE (5 μg/ml); n=3. (B) Western blot analysis of IL-4 or IL-4 treated with CE for 5, 15 min, and 2 hrs.; a representative of three experiments is shown. (C) Quantification of signal intensity of full-length IL-4 (solid box); n=3. Results shown are mean ± SEM. Results were analyzed with one-way ANOVA, multiple comparisons, ns=non-significant, *p≤0.05, **p≤0.01, ***p≤0.001.

Discussion:

In this study we showed that CE inhibited IL-13-induced CCL26 mRNA upregulation and protein release from bronchial epithelial cells in a time and dose dependent manner. CE antagonized all IL-13 effects on airway epithelial cells, since we showed that it also prevented upregulation of other IL-13-regulated genes, such as periostin and IL-13Rα2 and decreased IL-13-induced STAT6 phosphorylation. We also showed that this CE effect was trypsin-like protease dependent as it was prevented by heat inactivation of CE or pre-treatment of CE with serine and trypsin-like protease inhibitors. We present evidence that suggest this inhibition of the IL-13-induced effects is mediated, to a large degree, by degradation of IL-13 by CE serine proteases. The data we present from experiments that used ALI cultures of airway epithelial cells support the degradation scenario, since inhibition of IL-13-induced CCL26 was evident only when IL-13 and CE were added to the same side of the ALI cultures, and no inhibition was detected when IL-13 and CE were added in opposite sides of ALI. We finally showed that CE can also degrade IL-4 and inhibits IL-4-induced CCL26 upregulation in airway epithelial cells.

Since bovine trypsin, in concentrations with similar serine protease activity to that in CE, and HDM extract, another extract that contains allergens with serine protease activity [245,259], did not have the same effect with CE on IL-13-induced CCL26 expression, we conclude that the inhibitory effect of CE is not a general effect of serine proteases, but a specific effect of one or more serine proteases present in CE. Recently, our group described three distinct serine proteases from *Blattella Germanica* extract [235], the same extract we used for the experiments presented here. It is likely that one or more of these proteases is/are responsible for the effects we describe in this manuscript. Further work will be needed to identify the specific cockroach protease capable of inducing IL-13 degradation.

We showed here for the first time that CE can cleave IL-13 protein. IL-13 is also susceptible to degradation by mast cells proteases [119,120]. In one of these studies [120] mast cell degranulation was shown to decrease the biological effects of IL-13 through IL-13 degradation. If cockroach proteases can also cleave IL-13, as well as IL-4, in vivo, then inhaled cockroach proteases may be able to alter the biological activity of IL-13 in allergic diseases. A recent observation indicates that CCL26 may also facilitate clearance of eosinophilic inflammation through activation of CX_3CR1 expressing macrophages [26], indicating that degradation of IL-13 by CE may further aggravate

airway inflammation by delaying eosinophil clearance from the airways. Alternatively, we cannot exclude the possibility that exposure to high levels of cockroach proteases may limit the pro-inflammatory and pro-fibrotic effects of excessive IL-13 production during an allergic response, and possibly reduce tissue remodeling. Detailed in vivo studies in mice will be required to fully understand the biological significance of our results. Our ALI data also indicate that CE should have access to the basolateral epithelial surface to degrade IL-13 and prevent CCL26 induction; this is possible since it is known that epithelial barrier is damaged in allergic asthma [263] and allergen proteases can directly digest epithelial tight junction proteins and increase epithelial permeability [264,265].

The exact amount of CE allergens inhaled in everyday life is difficult to estimate. Quantitative measurement of airborne cockroach allergens in apartments with asthmatic children showed that the mean content of the cockroach allergen Bla g2 was 0.38 μg/g of total suspended particulates [266], while levels as high as 1.06 μg/g have been seen in settled dust in another study [267]. The mean of Bla g2 content in 13 *Blattella Germanica* cockroach extracts was 18.01 μg in a total protein content of 4.02 mg [268]. Based on these observations a 5 μg/ml solution of CE, as the one we use on our experiments, should contain around 0.022 μg of Bla g2, or the equivalent amount present in 0.05-0.021 g of dust. Whether it is possible to have exposure to this level of cockroach allergens/proteins in everyday life, or whether cockroach proteases may accumulate in the surface airway liquid and increase an individual's exposure, is something that needs further study. There is some evidence that high levels of inhaled allergens may protect from allergic sensitization [269]. Whether the same happens with cockroach allergen exposure and whether proteases are important for this effect, is not known.

IL-13 cleavage by CE was completely inhibited by heat inactivation or boiling of the extract indicating the presence of heat labile proteases. Even though neutralization of serine proteases with aprotinin prevented CE-mediated inhibition of IL-13-induced CCL26 upregulation, it did not prevent cleavage of the full molecule, but large IL-13 fragments are more prominent in this case than when IL-13 is degraded by fully active CE proteases. Inhibition of cysteine proteases inhibited IL-13 cleavage but that was not enough to prevent CE-mediated inhibition of IL-13-induced CCL26 upregulation. These observations suggest that there is one or more specific cleavage sites responsible for abolishing IL-13-induced effects.

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

STAT6 phosphorylation at Tyr^{641} mediates STAT6 translocation to the nucleus and induces CCL26 mRNA transcription [254–256]. We have showed that cells activated with IL-13 in the presence of CE exhibit decreased pSTAT6-Tyr^{641} compared to cells activated with IL-13 alone. Early degradation of IL-13 by CE and decreased binding to IL-13R may be the reason for this difference. However, early pSTAT6-Tyr^{641} was detected, CCL26 expression is completely abolished. It is therefore likely that other signalling events mediated after prolonged activation of the IL-13R are required for sustained CCL26 induction. Phosphorylation of serine residue in transactivation domain (TAD) of STAT6 results in conformational changes and loss of STAT6 DNA binding [256] without affecting Tyr^{641} phosphorylation, STAT6 dimerization, or nuclear translocation. It would be interesting to test whether AEC activation by IL-13 and CE may induce STAT6 TAD domain serine phosphorylation leading to loss of STAT6 DNA binding and therefore complete inhibition of CCL26 mRNA expression. Though IL-13 degradation may explain the CE-mediated effects we describe here, we also tested whether other mechanisms that may be involved. One such mechanism might be PAR-2 activation by CE proteases [235]. However, PAR-2 activation by synthetic activating peptides did not inhibit the IL-13 effects on airway epithelial cells suggesting that the effect of CE on IL-13-induced CCL26 mRNA upregulation was independent of PAR-2 activation.

Conclusion:

Our study shows that CE serine proteases have the capacity to degrade IL-13 and decrease IL-13-mediated CCL26 induction *in vitro*, suggesting that CE may also have the ability to modulate allergic airway inflammation *in vivo* by interfering directly with the pro-inflammatory effects of IL-13 [270,271] or delay eosinophil clearance in allergic inflammation[26]. Further work is required to investigate the effect of the identified cleavage sites on IL-13 biological activity in the context of allergic airway inflammation.

Data availability statement:

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

Conflict of interest statement:

The authors declare no conflicts of interest.

Author contributions:

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

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Disclosures:

The authors declare no competing interests.

Chapter 3:

TNF and HDM Induce Insulin Resistance in Airway Epithelial Cells

Abstract:

Asthma, obesity, and metabolic syndrome are epidemiologically and mechanistically connected. All these conditions showed altered glucose metabolism and increased levels of proinflammatory mediators. The state of chronic inflammation and abnormal production of proinflammatory meditators in obesity and T2DM disrupts normal insulin signaling pathways which induces insulin resistance. In insulin resistance elevated levels of proinflammatory meditators results in attenuated insulin-induced biological effects, such as glucose uptake, metabolism, and cell growth. Increased prevalence of metabolic and airway allergic diseases was shown in several studies. Hyperglycaemia accompanied with hyperinsulinemia that does not restore normal glucose levels, and dysregulation of epithelium are known events in exacerbations of airway inflammatory diseases that indicate a state of insulin resistance. Exposure of airway epithelium to inhaled allergens and particular matter activates airway epithelium and induce production of proinflammatory mediators. TNF proinflammatory mediator, and activation of TLR2 and TLR4 by free-fatty acids were shown to reduce insulin sensitivity and induce insulin resistance. Exposure of AECs isolated from asthmatics to HDM altered glucose metabolism. We aimed to investigate the effect of TNF and HDM in insulin-induced biological effects in AECs. NHBE cells were exposed to TNF or HDM in presence or absence of insulin. Chronic exposure of NHBE cells to TNF reduced insulin-induced epithelial proliferation, epithelial barrier function, and recovery after injury. Decreased insulin-induced phosphorylation of Akt was also detected in presence of TNF. Both of TNF and HDM altered NHBE cells energetic phenotype and mitochondrial membrane potential. We concluded that induced insulin resistance in NHBE cells modulated energetic phenotype which may contribute to disruption of epithelial barrier function and reduced epithelial proliferation.

Introduction:

Chronic inflammation in obesity and T2DM is associated with insulin resistance in metabolically active tissues like hepatic, adipose, and skeletal muscle tissues [272–274]. Insulin resistance involves disruption of insulin-mediated metabolic and physiologic effects. Increased activation of proinflammatory transcription factor NF-κB plays a key role in this pathogenesis [275]. Chronic inflammation and increased expression of proinflammatory mediators TNF, IL-1, and IL-6 were observed in inflamed adipose tissues and linked to reduced insulin sensitivity and insulin resistance [152–155]. In obesity, free-fatty acids can also reduce insulin sensitivity through activation of TLR2 and TLR4 and genetic deficiency of these receptors improved insulin sensitivity [276,277].

In airway inflammatory diseases hyperglycaemia accompanied with hyperinsulinemia were detected in asthmatics during asthma exacerbations [184,186,278]. Chronically inflamed airways of asthma and COPD patients have increased production of proinflammatory mediators like IL-6 and TNF [279,280]. These inflammatory and metabolic changes might be related to reduced insulin action [272,281–283]. Altered glucose metabolism was detected in AECs isolated form asthmatics after exposure to HDM [284] which suggest a role of allergens in airways metabolic changes maybe through a mechanism involving activation of TLRs. Metabolomics studies of BAL and lung tissues revealed inflammatory-related metabolic alterations in experimental asthma and HDM-induced allergic asthma; the detected changes included alterations in energy, amino acid, and lipid metabolites [187,285].

Airway epithelium is the first innate barrier that functions as a regulator of inflammation and provides protection against inhaled pathogens, allergens, and particular matter [20]. Epithelium barrier dysfunction like loss of junctional proteins and de-differentiation is associated with increased expression of proinflammatory mediators [91] and believed to be a central feature in pulmonary inflammatory diseases [286,287]. Epithelial dysfunction and elevated expression of proinflammatory mediators may profound the risk of aeroallergen sensitization and the associated metabolic shifts. Deprivation of insulin has been a mechanism used in some studies to investigate the role of insulin in epithelial development. Interestingly, lack of insulin increased expression of PAR-2 in AECs [288], showed suppression of epithelial differentiation, and gene expression approximated that of asthmatic cells [41,289]. These data are connecting airway inflammatory diseases with insulin resistance through clinical observations, genetic changes, and altered metabolomics.

It is not known whether the metabolic changes detected in airway inflammatory diseases are due to or related to development of insulin resistance; to our knowledge there is no study in literature investigated insulin resistance and insulin resistance-induced metabolic changes in epithelial cells.

Insulin and insulin-like growth factors (IGFs) function:

Insulin is a homeostatic growth hormone that is involved in the regulation of multiple metabolic and physiologic processes through multiple pathways including regulation of glucose uptake [290] and metabolism [290], lipid metabolism [291,292], cell proliferation and differentiation [293,294], and epithelial barrier function [295] (Illustration 3.1). Reduced insulin sensitivity and insulin resistance result in dysregulation of these processes. There are IGF-I and IGF-II hormones that share some receptors besides multiple metabolic and developmental roles with insulin [296,297]. There are classical insulin-like receptors that are composed of IR, IGF-I receptor (IGF-IR), and IGF-IIR [298–300]. Activation of IGF-IR and IGF-IIR is highly regulated, since the IGF peptides are not in a free form in the circulation but in a complex with high-affinity IGF-binding proteins (IGF-BPs) [296,297,301]. IGF-BPs demonstrate high affinity to IGF-I and IGF-II but not to insulin [296,297,301]. The high molecular weight of the IGF-1/IGF-II and IGF-BPs has a key role to prevent the interaction of these complex to IR, which make low molecular weight circulating insulin free to interact with IR [296]. IGF-BPs can also regulate activation/inhibition of IGFs metabolic and proliferative actions [302]. IGF-I induces mitogenic responses, cell differentiation, and prevents cell death in multiple cell types [303,304]. IGF-I can also induce metabolic effect as glucose uptake without reducing free fatty acids [305]. IGF-II can stimulate IR and IGF-IR with different potencies [306,307]. IGF-II besides its role in cell proliferation it has an important supportive role in human embryonic stem cell growth [306,307]. These data are indicating that IGFs are capable of inducing metabolic and mitogenic responses; however, these responses are highly distinct and regulated without overlapping insulin induced effects.

Role of insulin in glucose uptake and metabolism:

Studies of expression of glucose transporters in airway epithelium are limited with discrepancies between mRNAs, protein, fresh isolated cells, and cells *in vitro* [308]. It also seems like the expression of glucose transporter (GLUT) mRNAs are dependent on the cell type, polarization, and culturing conditions. Glucose uptake is essential to provide energy to support cell key functions like proliferation, ion exchange, cilia movement and secretion of epithelial mediators [309].

There are 14 GLUTs (GLUT1-14) encoded by SLC2A gene family and sodium-coupled glucose transporters (SGLT 1, 2, and 4) encoded by SLC5A gene family regulating glucose uptake and transport across plasma membranes [310]. Glucose is normally transported across the cell membrane through GLUTs by passive diffusion down concentration gradient which is generated by glucose metabolising enzymes hexokinases (HKs) and glucokinases (GKs) [311].

In contrast, glucose is moved through SGLTs in active transport according to Na^+ gradient [311,312]. SGLTs allow glucose to be transported against its concentration gradient because the transmembrane Na^+ gradient is the driving force [311,312]. SGLT1-2 are expressed mainly in the kidney and intestine where they mediate glucose reabsorption [312]. Some studies showed SGLT expression in alveolar epithelial cells in animal models [313,314], but were not detected in human airway epithelial cells [315].

GLUT1 transcript was shown to be the highest expressed transporter in most cell types of the airway epithelium with high abundance in basal cells, ciliated cells, and secretory cells [316,317]. GLUT1 was primarily detected at the basolateral side of primary human bronchial epithelial cells in air-liquid interface [315,318]. GLUT2 mRNA was detected in non-polarized lung epithelial cell lysates in separated plasma membrane and intracellular protein fraction [319]. It was shown that GLUT4 is the insulin-sensitive glucose transporter and is predominantly expressed in muscle and adipose tissues [320]. GLUT4 transcript was also detected in non-polarized lung epithelial cells but was absent in polarized cells [319]. GLUT8 mRNA was shown to be at low abundant in lung tissue [321]. It was suggested that GLUT8 is a hexose intracellular transporter that is localized in lysosomal/ endosomal membranes rather than at plasma membrane. GLUT8 expression was correlated with increased levels of glucose and insulin in cultured cells which indicate its role to maintain cellular metabolic capacity and glucose homeostasis [322]. In human airway tissues from trachea, bronchioles, and alveolar regions, GLUT10 was detected in high abundance [315,323,324]. GLUT10 along with GLUT1 were identified as markers for ciliated cells [315,323,324]. Apical localization of GLUT10 in human bronchial epithelial cells cultured in air-liquid interface suggest its function in glucose uptake at the apical surface [308]. GLUT12 is another marker for ciliated cells in some studies with transcript abundant in ciliated cells [317,325–327]; with others defined GLUT12 as a marker for ciliated cells [325]. GLUT13 transcript was detected at low level in basal and secretory cells [328]; however, is not responsible for transport of glucose but instead it transports myoinositol.

Data about mRNA expression correlates poorly with protein abundance [329,330]. GLUT1 protein is expressed in fetal lung tissues and in rat epithelium and its expression was shown to be declined with lung development [331,332] with localization at the basolateral surface of differentiated human bronchial epithelial cells *in vitro* [315]. GLUT1 expression increased in squamous cell carcinoma and was associated with increased proliferation and decreased differentiation [332,333]; suggesting abnormal expression of GLUT1 in malignant AECs that is associated with proliferation and de-differentiation. Some studies showed that GLUT2 protein was present in human bronchial biopsies and cultured lung epithelial cells [319,334] in both apical and basolateral sides of polarized lung epithelial cells and human bronchial epithelial cells obtained by bronchoscopy [334,335]. GLUT2 detection in ciliated cells showed that it is co-localization with sweet taste receptor protein T1R3 which is suggesting a sensory role of GLUT2 in the airways [335]. Dynamic regulation of GLUT2 was shown in obesity with reduced expression and underneath cilia expression in ciliated cells from obese rats [335]. GLUT4 protein was present in intracellular fraction but not in plasma membrane in cell lysates of non-polarized human lung epithelial cell line H441 but was absent in polarized cells and normal human lung epithelial cells [319]. H441 cell line that was isolated form a donor with papillary adenocarcinoma of the lungs, which makes expression of GLUT4 uncertain in normal airway epithelium. GLUT10 protein was detected in the apical side of human bronchial epithelial cells [315,318] which is confirming the role of GLUT10 in glucose uptake across apical surface. GLUT12 was detected in bovine and human immortalized epithelial cells [336,337]. Other studies showed that GLUT12 was largely associated with Golgi network in murine [338] and intestinal epithelial cells [339]. Infusion of insulin triggered GLUT12 translocation to plasma membrane in human biopsies of skeletal muscle [340]. These results are suggesting that GLUT12 is another insulin-sensitive glucose transporter.

Glucose diffusion across the epithelium is determined by the GLUTs activity and epithelial permeability to glucose. Glucose concentration in airway surface liquid (ASL) is one tenth of the glucose concentration in plasma, which creates a driving force, due to gradient difference, for glucose to move from the plasma to ASL [315,318]. Movement of glucose to the ASL is mainly mediated through paracellular diffusion or flux [315] and transcellular pathways (Illustration 2.2). Epithelial cells tight junctions including adhesion molecules, claudins, and occludins determine epithelial permeability that discriminate between solutes based on their size and charge [341,342]. In

human AECs, it was shown that reduction in the abundance of claudin-1 and occludin increased glucose flux to ASL [343,344]; suggesting that claudin-1 and occludin are potentially responsible for the regulation of glucose flux in AECs. GLUTs expression in both apical and basolateral sides raise the potential for transcellular diffusion of glucose. Apical expression of GLUT10 and basolateral expression of GLUT1 in human AECs were shown to induce bidirectional flux of glucose [315]. These studies are suggesting that movement of glucose across the epithelium is a dynamic mechanism that can maintain glucose gradient difference and low glucose concentration in ASL. Moreover, another mechanism to maintain glucose gradient across the epithelium was identified [345]. It is known that GLUTs can only transport glucose down the concentration gradient and metabolism of intracellular glucose by HKs is a rapid rate-limiting process [346,347]. Rapid metabolism of AECs intracellular glucose maintains the driving force for glucose uptake across the apical and basolateral membranes (Illustration 3.2), and inhibition of GLUTs increased glucose in ASL [345].

Role of insulin in lipid metabolism:

Bronchial epithelial cells from asthmatics showed increased expression of fatty acid metabolism related genes, elevated levels of lipid species, and reduced expression of OXPHOS genes compared to healthy controls [41]. Insulin regulates phosphorylation and dephosphorylation of multiple metabolic enzymes; insulin also controls the gene expression of glycolysis and gluconeogenesis hepatic enzymes which can indirectly impact lipid metabolism [348,349]. Insulin enhances lipid metabolism by increasing transcription of lipogenesis, lipid synthesis, enzymes like fatty acid synthase and acetyl-CoA carboxylase [292]. Insulin also enhances transcription of glycolytic enzymes and increased glucose uptake [292]. Glucose uptake activates lipogenesis enzymes because metabolised glucose is stored mainly as lipid for energy supply suggesting that insulin regulation of glycolysis can indirectly impact lipid metabolism. Insulin also decreases lipolysis, breakdown of stored fats, by inhibiting lipase [350]. These metabolic shifts are influenced by inflammatory changes and work in a complex network that is adjusted to substrate availability to meet cellular demand for energy.

In human bronchial epithelial cell cultures, repair of airway epithelium required metabolic shift from glycolysis to fatty acid oxidation for differentiation of AECs from basal cells toward secretory and multi-ciliated cells [351]. Pharmacological or genetic impairment of fatty acid oxidation resulted

in impaired regeneration of a fully functional airway epithelium and enhanced fatty acid oxidation promoted epithelial repair [351]. These findings are supporting the central role of fatty acid oxidation in AECs differentiation and regeneration in normal conditions or in repair post airway insult. That might explain the metabolic shift toward fatty acid oxidation in asthmatics as a metabolic adaptation to support epithelial regeneration which may switch into pathological metabolic shift with persistent epithelial damage in chronic inflammatory diseases.

Role of insulin in cell proliferation and epithelial barrier function:

Insulin promotes cell proliferation and epithelial barrier function. In breast epithelial cell line, insulin induced phosphorylation of p85 and formation of a protein complex IRS-1/p85α^{PI3K}, and that was required for Akt and ERK1/2 activation [352]. Insulin mediated IRS-1/p85α^{PI3K} formation and Akt activation was critical for cellular survival, proliferation, and wound healing [352]. In contrast, insulin insufficiency or insulin resistance in diabetes is linked to reduced cell proliferation and impaired epithelial barrier function due to downregulation of PI3K/Akt pathway [295,353,354]. Downregulation of PI3K/Akt pathway either due to use of PI3K/Akt inhibitor [354] or in insulin resistance [295,353] reduced cell proliferation, epithelial barrier formation, and cellular spreading. A study showed that PI3K inhibition caused 80% reduction of proliferation in rat intestinal epithelial cells with cells accumulated in G1 phase of cell cycle profile [355]. Equally important, insulin activate MAPK/ERK pathway [356]; however, MAPK/ERK inhibition caused 20% reduction of proliferation with no alteration of cell cycle profile [355]. Similar findings were detected in proliferation of differentiated human intestinal epithelial cell lines [355]. These studies are suggesting more reliance of epithelial proliferation on PI3K/Akt, and indicating that activation of PI3K/Akt pathway, and MAPK/ERK at less extent, are required for epithelial cell proliferation and wound healing which are impaired in insulin resistance.

Increased expression of proinflammatory mediators may aggravate the disruption of epithelial barrier function. Epithelial exposure to IFNγ and TNF decreased expression of tight junction proteins occludin, ZO-1, and E-cadherin [63,357]. Deceased transepithelial resistance and increased expression of the channel-forming tight-junctional protein claudin-2, which disrupt epithelial barrier function, were detected in intestinal epithelial cells in response to TNF and IL-13 stimulation [62,358]. Similar effects were detected in response to IL-6 [64]. Increased expression of these proinflammatory mediators besides reduced insulin sensitivity would exacerbate epithelial damage

and trigger epithelial remodeling. Interestingly, inhaled aerosolized insulin was shown to ameliorate airway inflammatory responses and lung injury in experimental model of acute lung injury compared to intravenous administration and control group [359]. Therefore, insulin has a key role in airway healing after injury and that function is very likely mediated through airway epithelium.

Connecting chronic inflammation and insulin resistance:

Increased expression of proinflammatory cytokines disrupt insulin action [158,275,360,361]. TNF is a proinflammatory mediator that has been extensively studied for it is role in inducing insulin resistance [156,157,362,363]. TNF mediates its effects through ligation to its two distinct receptors TNF receptor-I (TNF-RI) and TNF-RII. These receptors are expressed on the membranes of all cell types except erythrocytes. TNF simulation activates TNF-RI binding to the TNF receptor-associated death domain (TRADD) which can activate apoptotic pathway through Fas-associated death domain (FADD), or proinflammatory pathway through activation of TNF receptor-associated factor 2 (TRAF2) and NF-κB. On the other hand, TNF-RII can signal only through TRAF2 associated pathway [364]. TNF-RI mediates the inhibitory effects of TNF on insulin signaling [365,366] whereas TNF-RII deficiency did not affect insulin sensitivity [160].

Fat, muscle, and liver tissues from obese rats showed decreased insulin-stimulated autophosphorylation of IR tyrosine kinase compared to lean rats [367]. Neutralization of TNF in obese rats improved insulin-stimulated IR autophosphorylation and tyrosine phosphorylation of IRS-1 in fat and muscle tissues of obese rats [367] as well as improved insulin-induced peripheral glucose uptake [368,369] compared to control rats. TNF and fatty acids were shown to induce insulin resistance through elevated phosphorylation of IRS-1 (Ser^{307}) (p-IRS-1 Ser^{307}) which reduce insulin-induced tyrosine phosphorylation [370,371] and subsequently reduce p-Akt (Ser^{473}) [370,372,373]. These studies are suggesting a central role of TNF and fatty acids in insulin resistance.

Free-fatty acids have also showed a role to induce insulin resistance *in vivo* through activation of TLR2 [276] and TLR4 [277]. TLR2 increased expression of inflammatory cytokines IL-1β, IL-6, and TNF and showed reduced insulin sensitivity. However, TLR4-induced insulin resistance in adipose tissue was through endogenous ligand, fetuin-A, activation of TLR4 and not direct activation of fatty acids to TLR4. Mutation or deletion of TLR2 [276] or TLR4 [277] improved glucose tolerance and

insulin sensitivity but did not change plasma levels of TNF and IL-6. There data are suggesting fatty acid-induced insulin resistance is independent of TNF and IL-6 expression.

Another proinflammatory cytokine that has been linked to insulin resistance is IL-6. In cultured hepatocytes, IL-6 stimulation reduced insulin-induced IR tyrosine phosphorylation, Akt activation, and reduced glycogen synthesis were detected [374]. The mammalian target for rapamycin (mTOR) served as a kinase for STAT3 and that increased the transcriptional activity of STAT3 and induced suppressor of cytokine signaling 3 (SOCS3) [374]. Overexpression of SOCS3 reduced IRS-1 protein level through proteosomal degradation in hepatocytes [375] and adipocytes [376] which cause subsequent reduction of p85 binding to IRS-1. SOCS3 can also functions as a negative regulator of insulin signaling. In adipocytes, insulin induced expression of SOCS3 through STAT5b, and SOCS3 inhibited insulin activation of STAT5b without modification of IR tyrosine phosphorylation. This mechanism is involving a very likely competitive binding of SOCS3 and STAT5b to the same insulin receptor motif [377,378] which reduce insulin-activated STAT5b. SOCS3-deficient adipocytes showed increased IRS-1 and IRS-2 phosphorylation, IRS-1 to p85 binding, PI3K/Akt activation, and glucose uptake [376]. Moreover, SOCS3 deficiency blocked the TNF inhibitory effect on insulin [376].

Other studies showed that insulin resistance has been attributed to oxidative stress [379–382]. Excessive ROS generation through p38 MAPK pathway in hepatocytes was shown to reduce insulin activation of Akt pathway through a mechanism involve increased p-IRS-1 Ser [307] [382]. Use of antioxidant or inhibition of p38 MAPK activation resulted in reduced p-IRS-1 Ser [307] but did not restore Akt activation [382]. Suggesting that the effect of excessive ROS on IRS-1 and Akt activation are not linked and there is another mechanism involved in Akt inactivation. Adipocytes prolonged exposure to H_2O_2 impaired insulin-induced GLUT4 translocation [383]. However, IR tyrosine phosphorylation binding to IRS-1 and PI3K activation was not prevented [383]. Suggesting that the effect of H_2O_2 on insulin-induced GLUT4 translocation is very likely through dysregulation of GLUT4 vesicular storage compartment mobilization.

Connecting airway inflammatory diseases with insulin resistance:

Metabolic diseases were shown to be more prevalent in allergic individuals [272,281–283]. Patients with metabolic syndrome were found to be at higher risk to develop asthma and showed decreased lung function [384,385]. These reports bridge metabolic disorders with airway inflammatory diseases,

mainly through a state of chronic inflammation. A study investigated the relationship between insulin resistance, obesity, and the development of asthma-like symptoms in adults showed that all obesity measures of BMI, waist circumference (WC), and waist-to-hip ratio (WHR) were associated with incident wheezing and asthma-like symptoms [282]. Insulin resistance evaluated by homeostatic model assessment for insulin resistance (HOMA-IR) was associated with increased risk of wheezing and asthma-like symptoms development in adults [282]. Moreover, insulin resistance was identified as a stronger predictor for wheezing and asthma-like symptoms incident than obesity [282]. A cross-sectional study for 3609 Danish individuals 30-60 years old investigated the association of obesity and insulin resistance with aeroallergen sensitization and asthma [283]. This study showed that obesity measures (BMI, WC, and WHR) increased the risk of aeroallergen sensitization and allergic and non-allergic asthma [283]. Insulin resistance was associated with increased risk of aeroallergen sensitization and allergic asthma [283].

In a cross-sectional study that included hospital admitted patients with an exacerbation of asthma or COPD, it was shown that 82% of admitted patients showed hyperglycemia [184]. Hyperglycaemia was also detected in 68% of nondiabetic and the prevalence of hyperglycemia was not different between patients with asthma or COPD [184]. Hyperglycaemia was also shown in children hospitalized with acute asthma [185]. Despite the inflammatory condition in those patients, it was not investigated weather hyperglycemia was due to insulin resistance or not. Airway inflammatory diseases have also been associated with mitochondrial dysfunction.

Mitochondria is the hub for aerobic oxidation of glycolysis end products, metabolic derivatives, and amino acid degradation [183]. In aerobic oxidation the mitochondrial is utilizing oxygen and H_2O to produce ATP [183]. Insulin regulates metabolism through PI3K/Akt pathway [386]. Glucose and lipid metabolism converge to ATP through OXPHOS in the mitochondria is essential to fulfill cellular energy. Insulin signaling is required for synthesis of mitochondrial DNA and proteins, and stimulation of mitochondrial oxidative capacity and ATP production [190,191]. Based on this metabolic network, insulin resistance was shown to be associated with mitochondrial dysfunction, characterized by reduced oxidative capacity and ATP production [387] which may exacerbate metabolic shifts.

Normal bronchial epithelial cells depend on mitochondrial OXPHOS for ATP supply [182]. Increased oxidative stress [388] and reduced OXPHOS [182,389] were detected in asthma, which can contribute to

mitochondrial dysfunction [388,390,391]. In a model of OVA-induced experimental allergic asthma it was observed mitochondrial dysfunction which included reduction of mitochondrial cytochrome c oxidase (COX) activity, reduced ATP production, and mitochondrial structural changes [389]. A transcriptomic analysis of bronchial epithelial cells from healthy donors and asthmatic patients showed reduced expression of OXPHOS genes in asthmatics and profound expression of fatty acid metabolism related genes [182]. Elevated levels of lipid species in bronchial epithelial cells from asthmatics was also confirmed [182]. In contrary, asthmatic epithelium bioenergetic showed increased mitochondrial oxygen consumption rate and glycolysis extracellular acidification rate [392]. Energetic phenotype in this case was supporting mitochondrial bioenergetic through overexpression of arginase 2 (ARG2) and increased metabolism of arginine, which contributed to production of nitic oxide (NO) and metabolic shift to OXPHOS with increased ATP production [392]. Increased arginine metabolism preserved cellular respiration, protected against hypoxia-inducible factors, and dampened Th2 proinflammatory signals [392]. ARG2 deficient mice showed decreased NO production, lower mitochondrial membrane potential, greater hypoxia sensing, and increased Th2 activation [392]; indicating that metabolic changes in asthma is in part related to arginine metabolism.

Taking together, these data are indicating that the pathogenesis of asthma is associated with metabolic adaption that could show bioenergetic differences based on differences in insulin action and mitochondrial functions.

Insulin resistance and immunity:

Insulin resistance is linked to altered function of immune cells and production of proinflammatory mediators. In a study aimed to investigate the association between metabolic status, obesity, and atopy, it was shown that insulin resistance was associated with increased levels of total and aeroallergen-specific IgE to house dust mite, cockroach, and dog in the test group [393]. Level of total allergen-specific IgE, specifically for cockroach-specific IgE and dog-specific IgE, were higher in obese participants compared to non-obese in general [393]. However, according to the participants metabolic condition, non-obese participants with highest quartile of insulin resistance were at higher risk of increased total IgE than non-obese metabolically healthy participants [393]. Additionally, non-obese participants with highest quartile of insulin resistance but not metabolically healthy obese phenotype was associated with atopy [393]. These data are suggesting

that insulin resistance rather than obesity is associated with atopy and increased IgE levels. Similar findings were detected in another study in a population of men and women aged 30-60 with no sex-differences observed [283]. Immune cells have also shown a role as metabolic controllers. Infiltration of B cells in visceral adipose tissues of high-fat diet feeding mice increased numbers and proportions of class-switched mature B cells, such as IgG^+ cells [394]. B^{null} high-fat diet mice were protected of this effect [394]. B^{null} high-fat diet mice have also shown lower fasting glucose, improved glucose tolerance, lower fasting insulin, and improved insulin sensitivity compared to high-fat diet wild type [394]. This study also showed that in high-fat diet fed mice B cells induced systemic and local, in visceral adipose tissues, inflammation, increased cytokines TNF and IFN-γ, and promoted visceral adipose tissue T cell activation [394]. Purified IgG from high-fat diet fed mice that was injected into B^{null} mice induced worsen glucose tolerance compared to normal fed mice [394]. These data are suggesting that B cell pathogenic function in this model is influenced by other immune cells like T cells, produce pathogenic IgG that mediate glucose intolerance, and induce systemic inflammation.

Rational:

Chronic inflammation and abnormal production of proinflammatory meditators in obesity and T2DM disrupts normal insulin signaling pathways and induce insulin resistance. Increased prevalence of metabolic and airway allergic diseases was shown in several studies. Airway epithelium health is essential to maintain airways physical barrier function, epithelial innate immunity, and airway clearance. It was shown that local airway epithelium inflammation and increased expression of proinflammatory cytokines TNF and IL-6 triggered systemic inflammation, insulin resistance, and reduced peripheral glucose uptake. These results are suggesting a regulatory role of airway epithelium inflammation and increased TNF and IL-6 in systemic insulin action. Insulin deprivation in normal human bronchial epithelial cells induced loss of epithelial differentiation that showed gene expression closely approximated gene expression in asthmatic epithelial brushing. These data indicated that epithelial changes detected in asthma is likely related to state of inflammation and disrupted insulin action. Insulin activation of PI3K/Akt pathway regulates cellular metabolism, growth, and survival, which would be abrogated in insulin resistance. We tested whether allergens, Th2 cytokines and other inflammatory mediators present in the airways in asthma can mediate insulin resistance to help us understand the role inflammation in the regulation of epithelium metabolic changes.

Hypothesis:

We hypothesized that exposure of AECs to TNF or HDM induces insulin resistance, which may impair epithelial barrier and mitochondrial functions.

Objectives:

1) Examine the effect of TNF on insulin-induced epithelial structure and physiology.
2) Evaluate the effect of TNF on insulin-induced activation of PI3K/Akt pathway.
3) Study metabolic shifts in allergen and TNFstimulated epithelial cells.
4) Evaluate mitochondrial changes in TNF or allergens stimulated epithelial cells in presence or absence of insulin.

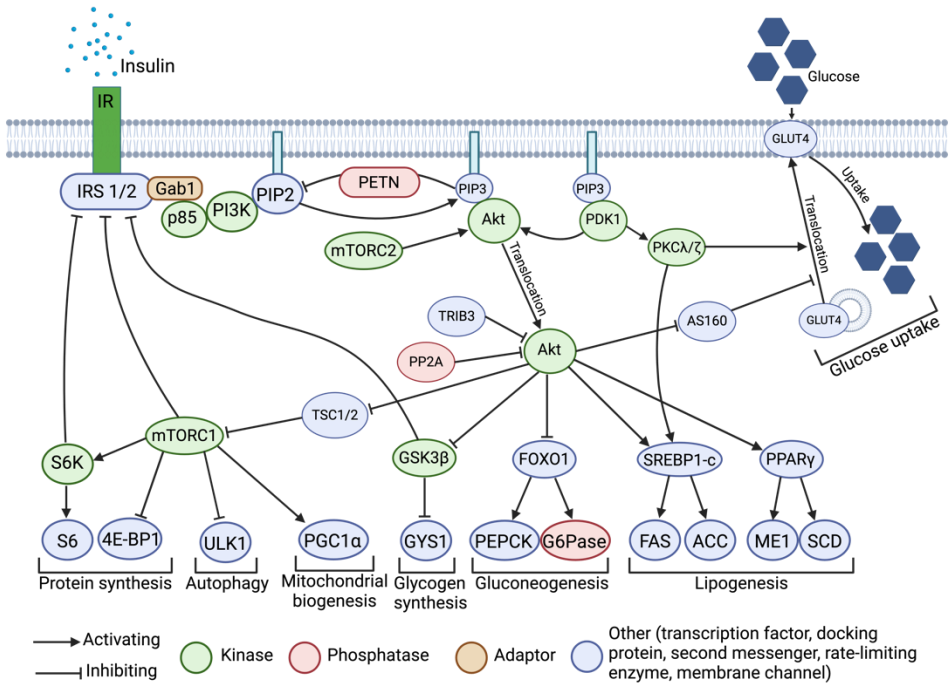


Illustration 3.1: Simplified drawing of insulin-induced PI3K/AKT signalling and its substrates in cellular metabolism. Abbreviations: ACACA, ACC, acetyl-CoA carboxylase; AKT, v-akt murine thymoma viral oncogene homologue 1; 4E-BP1, eIF4E-binding protein 1; FOXO1, forkhead box O1; GAB1, GRB2 associated binding protein 1; G6Pase, glucose-6-phosphatase; GSK3β, glycogen synthase kinase 3β; GYS1, glycogen synthase; INSR, IR, insulin receptor; IRS1/2, insulin receptor substrates 1/2; ME1, malic enzyme 1; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mTOR complex 2; p85, Phosphatidylinositol 3-kinase 85 kDa regulatory subunit alpha; PDPK1, PDK1, 3-phosphoinositide-dependent protein kinase-1; PGC1α, peroxisome proliferator-activated receptor gamma, coactivator 1α; PI3K, phosphoinositide-3-kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; PKC1, PEPCK, phosphoenolpyruvate carboxykinase 1; PKCλ/ζ, protein kinase Cλ/ζ; PPARγ, peroxisome proliferator-activated receptor g; PP2A, protein phosphatase 2A; RPS6, S6, ribosomal protein S6; SCD, stearoyl-CoA desaturase; S6K, ribosomal protein S6 kinase; SLC2A4, GLUT4, solute carrier family 2; SREBF1, SREBP1-c, sterol regulatory element binding transcription factor 1; TBC1D4, AS160, AKT substrate 160; TRIB3, tribbles homologue 3; TSC1/2, tuberous sclerosis complex 1/2; ULK1, unc-51-like kinase 1. Created with BioRender.com

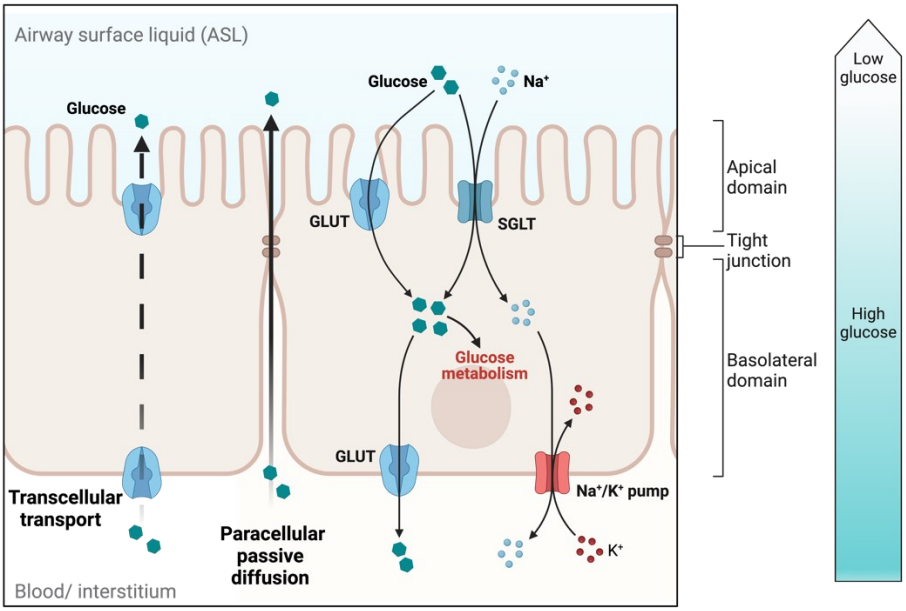


Illustration 3.2: Transcellular and paracellular glucose movement in airway epithelium. Glucose transport across AECs is determined by glucose gradient, epithelial permeability, and transepithelial ana paracellular transport. Created with BioRender.com

Methodology:

Cell culture:

Primary NHBE cells from five donors were either purchased from Lonza (Walkersville, MD) or grown from cells recovered from donors undergoing bronchoscopy for clinical indications with ethics approval from the University of Alberta Health Research Ethics Board (ID: Pro00099685). Cells were cultured in cell culture flasks or plates (Falcon, Corning, NY) that were pre-coated with 30 μg/ml bovine collagen type 1 (Life Technologies, Burlington, ON) in BEBM from Lonza. BEBM is serum-free medium that contains phenol red, glutamine, and 1.081 g/L glucose as based on LHC-9 medium [395].

NHBE cells were cultured in bronchial epithelial growth media (GM) consisting of BEBM supplemented with bovine pituitary extract, hydrocortisone, epidermal growth factor, epinephrine, insulin, transferrin, triiodothyronine, retinoic acid, gentamycin-amphotericin-B (all from Lonza) as well as 100 U/ml penicillin and 100 μg/ml streptomycin (both from Life Technologies). Cell cultures were incubated in Thermo Steri-Cult CO_2 cell incubators (ThermoFisher Scientific, Waltham, MA, USA) at 37℃ in 5% CO_2 and 90% humidity. Media was changed every other day. NHBE cells were passaged using 0.25 trypsin-EDTA to detach the cells followed by the addition of trypsin neutralizing solution (TNS) in 1:2 ratio. Cell suspension was then centrifuged at 300 g for 5 minutes and cell pellets were resuspended in GM and seeded in new coated flasks/plates at a density of 3500 cell/cm^2. Cells used for experiments were tested negative for mycoplasma using the MycoAlert Kit (Lonza).

WST-1 colorimetric assay:

The WST-1 reagent from Sigma (St. Louis, MO, USA) was used to assess cell proliferation according to the manufacturer instructions. WST-1 is a stable tetrazolium salt that is cleaved to a soluble formazan by the succinate-tetrazolium reductase system in the mitochondria respiratory chain that is active only in metabolically intact cells. The amount of formazan dye formed in this assay is directly related to the number of metabolically active cells.

NHBE cells were seeded in 96-well cell culture plates pre-coated with 30 μg/ml collagen in BEBM at seeding density of 3500 cell/well in 200 μl/well of medium. At the day of seeding, cells grown

in GM were activated with TNF 10 ng/ml or 100 ng/ml or remained in GM. Other cells were grown in BEBM that contained all supplements except insulin (all but no insulin – ABI) and activated with TNF or remained in ABI for 24 hours followed by addition of insulin 0.5 μg/ml for the rest of experiment period. WST-1 Proliferation assay was performed daily for 4 days using a separate plate for each day. WST-1 reagent was used at final concentration of 1:10 and incubated for 2 hours at 37℃. Absorbance was measured at 450 nm using plate reader Power Wave XS (BIO-TEK). Workflow drawing is available in (Illustration 3.3).

WST-1 assay was confirmed using cell count. Individual cell count for the cells used in WST-1 assay was performed using 0.4% trypan blue (Life Technologies). Plates used for WST-1 assay at days 1, 3, and 4 was further used for cell count. Media was removed gently through pipetting and replaced with 20 μl of trypsin for 5 minutes. Trypsin was neutralized with TNS, and cells were used for counting using hemocytometer (counting champer).

ECIS (Electrical Cell-substrate Impedance Sensing) assay:

NHBE cell attachment and resistance was measured using ECIS 1600R instrument (Applied Biophysics, NY, USA) as described previously [11,396]. ECIS is a non-invasive method to measure real-time cell behavior. The ECIS instrument is based on measuring changes in electric resistance generated by attachment and spreading of cells on plastic wells; the apparatus transmits weak and non-invasive alternating current (AC) signals through small gold-film electrodes deposited upon the bottom of these wells (Illustration 3.4). Real time data was stored to a connected personal computer. For our experiments we used 8 well, 1 electrode (8WIE) arrays that contain a single circular electrode (250 μm) in each well. This kind of arrays are suitable for multiple applications including barrier function and in situ electroporation and monitoring.

8W1E arrays were pre-coated with collagen 30 μg/ml overnight before being washed with PBS and filled with 400 μl/well of BM only. Each eight-well cell culture array got connected to the ECIS device in a lock-in amplifier with internal oscillator to switch between the different wells for electrode check. Baseline resistance was then measured to be compared later with measurements obtained from arrays covered with NHBE cell monolayers.

NHBE cells were cultured at 7.5×10^4 cells/well in 400 μl medium. Cells grown in GM were either activated with TNF 100 ng/ml or remained in GM. Other cells were grown in ABI and activated with TNF or remained in ABI for 24 hours followed by addition of insulin 0.5 μg/ml for the rest

of the experiment period. The selected frequency used to study cell attachment and resistance was set at 40 kHz. For electroporation / cell wound testing the frequency was set at 40 kHz and voltage pulses was at 5 V for 30 seconds, as used previously [11], followed by electrode check. Total time of assay was 48 hours. Resistance values are showed in ohm.

Cell lysis and protein extraction for western blotting:

NHBE cells were cultured in pre-coated multi-well plated in GM until 90% confluent. Medium was then replaced with BEBM supplemented with Penicillin-Streptomycin for overnight then stimulated with TNF 10 ng/ml with or without insulin 0.5 μg/ml. Cell lysates were prepared on ice using RIPA buffer mix containing 1:100 protease inhibitor cocktail (Sigma) and 1:200 2 mM sodium orthovanadate. Cells were then detached using a cell scraper. Lysates were then vortexed for a total time of 10 minutes on the highest speed and centrifuged at 4℃ for another 10 minutes at 16,000 g. A mix of electrophoresis blue loading buffer and 30X DTT reducing agent in 90:10 ratio was added to cell lysates before placing them on 100℃ hot plate for 5 minutes. Cell lysates were then stored at -80℃.

Lysates were separated on 12% PAGE Mini-Protein gels to detect IRS, p-IRS1 (Ser307) 180 kDa and Akt, p-Akt (Ser473) 60 kDa. Gels were wet transferred to nitrocellulose membranes at 400 mA for 60 minutes at 4℃. Primary antibodies for IRS-1, p-IRS1 (Ser307), Akt, p-Akt (Ser473) all from (Cell Signaling Technology) were used to detect the intended targets.

To detect COX, we used mouse monoclonal primary antibody anti-COX IV and loading reference mouse monoclonal anti-α-tubulin both from Abcam (Cambridge, MA, USA). NHBE cells were grown in GM until 90% confluent then medium replaced with BEBM supplemented with Penicillin-Streptomycin for overnight. Lysates were collected from cells activated with TNF 10 ng/ml or HDM 20 μg/ml or remained in BEBM with Penicillin-Streptomycin for 24 hours. Other cells were activated with TNF or HDM in BEBM supplemented with Penicillin-Streptomycin and insulin 0.5 μg/ml for 24 hours. Primary antibodies were detected via IRDye-conjugated goat anti-mouse IgG secondary antibody from LI-COR (Lincoln, NE, USA). The membranes were imaged and analyzed using Odyssey CLx Infrared Imager and Image Studio software (5.2.5) both from LI-COR.

Live-cell metabolic assay:

XF96 Cell Culture miniplates from Agilent Technologies (Santa Clara, CA, USA) were pre-coated with 30 μg/ml collagen in BMEM. NHBE cells were seeded at density of 40k cell/well in GM and incubated at room temperature under biosafety cabinet for 60 minutes to reduce ridge effect [397]. Cells then moved to a 37°C, 5% CO2 incubator and were incubated for 24 h when they formed confluent monolayers.

Cells were then washed X3 with warmed BEBM supplemented with Penicillin-Streptomycin 80 μl/well. Cells were activated for 4 or 24 hours with TNF 10 or 100 ng/ml, HDM 20 or 50 μg/ml, IL-13 20 ng/ml, or CE 5 μg/ml in BEBM supplemented with Penicillin-Streptomycin or in BEBM supplemented with Penicillin-Streptomycin and 0.5 μg/ml insulin in total volume 80 μl/well. Each condition was done in triplicate and incubated at 37 °C, 5% CO2.

At the day of assay, 60 μl/well of medium was removed and replaced with 200 μl of assay media (glucose-free Seahorse XF DMEM medium PH 7.4 containing 1 mM pyruvate, 2 mM glutamate; all from Agilent Technologies. The assay medium was prepared with addition of TNF, HDM, and CE stimulations, as prepared with the basal medium in activation. The cell culture miniplate was incubated for an additional hour in a 37 °C, non-CO_2 incubator for degassing and elimination of CO_2 to avoid interference with TCA cycle-produced CO_2. Immediately following the Seahorse XF Real-Time ATP Rate Assay (Agilent Technologies) was performed according to the manufacturer's instructions and as done previously [398].

A sensor cartridge was hydrated by loading 200 μl/well of sterile, tissue culture grade water into a utility plate. The sensor cartridge was then lowered into the utility plate and sensors were submerged into the water. Both of the utility plate and sensor cartridge were placed in a 37 °C, non-CO_2 incubator 24 hours before starting the assay. At the day of assay, the water in the utility plate was replaced with pre-warmed calibrant, at 37 °C, non-CO_2 incubator overnight, then both utility plate and sensor cartridge were placed in a 37 °C, non-CO_2 incubator for 1 hour prior to loading the injection ports of the sensor cartridge. Workflow is available (Illustration 3.5).

Injection solutions were then loaded into ports as followed: 20 μl of 60 mM glucose into port A, 20 μl of 15 mM oligomycin into port B, and 22 μl of 5 mM rotenone/antimycin A into port C, all from Agilent Technologies. Sensor cartridge and utility plate were loaded onto the Seahorse xFe96 Metabolic Analyzer (Agilent Technologies) for calibration. Utility plate was removed and replaced with the cell culture mini plate to start the assay. Basal measurements were taken and followed by full sequential dispensation of injection solutions to the cells in 18 minutes apart to allow

measurements after each injection: glucose (final concentration 5 mM), oligomycin (final concentration 1.25 μM), rotenone/antimycin A (final concentration 0.5 μM). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) kinetic data were collected and analyzed in the absence (basal measurements) or presence (induced measurements) of glucose. Rate of glycolytic ATP production (expressed in pmol ATP/min) is associated with the conversion of glucose to lactate in the glycolytic pathway, and mitochondrial ATP production rate (expressed in pmol ATP/min) is associated with oxidative phosphorylation in the mitochondria. Total ATP production rate is the sum of both glycolytic ATP and mitochondrial ATP production rates. Data was normalized to cell number seeding rate. Schematic drawing of ports during injection phase and measuring phase is available (Illustration 3.6).

ELISA:

Cell viability was evaluated 24 hours after stimulation using a 0.4% trypan blue (Life Technologies) exclusion test of cell viability [250]. In conditions we used insulin-free medium, we added all the supplements except insulin to BEBM to get all but no insulin (ABI) media.

DuoSet ELISA kit for human IL-8/CXCL8 was purchased from R&D systems (Minneapolis, MN, USA) and assay was performed according to the manufacturer's instructions. A flat-bottom 96 well microplate was coated overnight at room temperature with 100 μl/well of a 4 μg/ml solution of anti-human IL-8 capture antibody in PBS (Sigma). Then blocked for 1 hour at room temperature with 300 μl/well of 1% BSA (Sigma) in PBS. A seven point 2-fold serial dilution standard curve of recombinant human IL-8 was used with highest concentration of 2000 pg/ml. NHBE cell culture supernatants and standards were then added to the pre-coated microplate wells (100 μl/well) and incubated for 2 hours at room temperature. Detection antibody (20 ng/ml concentration in100 μl/well) was added and incubated for an additional 2 hours at room temperature. After each previous step the microplate was aspirated/wash three times with washing buffer 0.05% Tween 20 in PBS 400 μl/well with complete removal of liquid. The plate was then incubated for 20 minutes in the dark with 100 μl/well of Streptavidin-HRP followed by incubation with 100 μl/well of a 1:1 mix of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine) from R&D Systems for another 20 minutes in the dark. In house made stop solution (50 μl/well of 2N H2SO4 solution) was then used to stop the reaction. Optical density was measured immediately after with a Power

Wave XS Plate reader (BIO-TEK, Winooski, Vermont, USA) and a four-parameter logistic (4-PL) standard curve was generated by Graph Prism.

Glucose uptake:

Glucose Uptake-Glo Assay was used to measure glucose uptake in AECs as was shown previously [399]. This assay is nonradioactive, plate-based, homogeneous bioluminescent for measuring glucose uptake in mammalian cells based on the detection of 2-deoxyglucose-6-phosphate (2DG6P).

NHBE cells were seeded in pre-coated 96-well plate black/clear bottom with lid (Coaster). Cells were seeded in GM in 20k cell/ 200 μl/well density until they reached 80% confluency. Then medium was changed to either Pen/Strep basal medium with or without 5 μg/ml insulin and 10 ng/ml TNF for 24 hours. Cells were washed twice with warm PBS and incubated with 200 μl/well at 37°C for 15 minutes to deplete glucose. PBS was replaced with 1 mM of 2-deoxyglucose (2DG) with or without insulin in PBS 50 μl/well, shake briefly, and incubated 20 minutes at room temperature. Transferred 2DG across cell membrane is rabidly phosphorylated to 2DG6P. The enzymes that modify glucose-6-phosphate (G6P) cannot modify 2DG6P which accumulate inside the cells. An acid detergent solution, stop buffer, was added 25 μl/well to lyse the cells and terminate the glucose uptake. A high-PH neutralization buffer solution was added to neutralize the acid. A detection reagent containing glucose-6-phosphate dehydrogenase (G6PDH), $NADP^+$, reductase, Ultra-Glo Recombinant Luciferase and proluciferin substrate was added in 100 μl/well, shake briefly, and incubated for 45 minutes at room temperature. G6PDH oxidizes 2DG6P to 6-phosphodeoxygluconate and reduces $NADP^+$ to NADPH. The reductase uses NADPH to covert the proluciferin to luciferin, which is the used by the Ultra-Glo Recombinant Luciferase to produce a luminescent signal that is proportional to the concentration of 2DG6P. Workflow is available (Illustration 3.7).

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR):

Total RNA was isolated from NHBE cell lysates using TRIzol (Invitrogen Life Technologies). After activation cells were washed with PBS and all media removed. Then, 500 μl of TRIzol was added per well and incubated for 5 minutes at room temperature. Cell lysates were then transferred from cell culture plate to Eppendorf tubes. Chloroform (Sigma) was added (100 μl/tube) and the mixture was shaken vigorously for 15 seconds. Lysates were then incubated at room temperature

for 2 minutes before being centrifuged at 12,000 g for 15 minutes at 4 ℃. The upper clear phase of each lysate was transferred to a new tube and 250 μl of Isopropyl alcohol (Sigma) was added and mixed by inversion. Lysates were incubated for 10 minutes at room temperature and then centrifuged again at 12,000 g for 10 minutes at 4 ℃. Supernatants were decanted and 500 μl per tube of 75% Ethanol (Sigma) was added to wash RNA pellet. Tubes were then vortexed for few seconds and centrifuge at 7,500 g for 5 minutes to pellet RNA. Supernatants were decanted, and RNA pellets were air dried for 7-10 minutes before being resuspended with 30 μl/sample of UltraPure DNase/Rnase-free water (Invitrogen).

RNA concentration was measured by Nanodrop 2000c (ThermoScientific). For reverse transcription reaction, 1 μl of 100 mM of dNTPs (2'-deoxynucleoside 5'-triphosphate) from (Invitrogen, Carlsbad, CA) and 0.5 μg of Oligo(dT) 12-18 Primer (Invitrogen) was added to 0.5 μg of RNA in a total volume of 20 μl. The mixture was then heated at 65 ℃ for 5 minutes in PTC-100 machine (Programmable Thermal Controller, MJ Research, Inc) to hybridize the poly(A) tail of mRNA. A mixture of 4 μl of 5X First-Strand Buffer, 2 μl of 0.1 M DTT (dithiothreitol), which is a reducing agent that reduces disulfide bonds and inhibits Rnases activity, both from Invitrogen and 1 μl of Rnase OUT, which is a recombinant ribonucleases inhibitor from Invitrogen, was added to each sample prior to heating at 37 ^{o}C for 2 minutes. MMLV from Invitrogen was added in 200 U/sample. The samples were then heated at 37 ^{o}C for additional 50 minutes followed by 15 minutes heating at 70 ^{o}C. The product cDNA was used in TaqMan-PCR. PDH kinase-2 (PDK2) gene expression assay (cat # Hs00176865_m1), PDK4 gene expression assay (cat # Hs00176875_m1) and the housekeeping gene GAPDH gene expression assay (cat # Hs99999905_m1) (all from ThermoScientific) were used to measure gene expression.

Mitochondrial membrane potential:

Mitochondrial membrane potential was measured using MitoProbe tetramethylrhodamine methyl ester (TMRM) assay kit for flow cytometry (Invitrogen). NHBE cells were cultured in pre-coated multi-well plates until confluent. Cells grown in GM were activated with TNF 10 ng/ml or HDM 20 μg/ml or remained in GM for 4 or 24 hours. Media was then removed, and cells were detached using 0.25 mg/ml of trypsin-EDTA for 10 minutes. Trypsin-EDTA was neutralized with BM in 1:2 ratio and cell suspensions centrifuged at 300 g for 5 minutes. Supernatants were decanted and resuspended in 1 ml BEBM. Cell count was ~ $1.5x10^5$/ml in each sample. For each condition two

samples were used: test and positive control. Assay procedure was begun with preparation of positive controls for mitochondrial depolarization using carbonyl cyanide 3-chlorophenylhydrazone (CCCP). CCCP was added in 0.3 μl/vial for each positive control sample, samples were then incubated at 37℃ for 5 minutes. Mitochondrial membrane potential probe TMRM was used for every sample including the positive controls in 0.3 μl/vial. Samples were then incubated at 37℃ for 30 minutes. One extra vial of unstained cells was used to detect the background fluorescence. Samples were then centrifuged at 300 g for 5 minutes and washed with 1 ml of BM then re-centrifuged and decanted. Final volume of 250 μl/vial of BM was used for cell re-suspension. Samples were then examined immediately using LSR Fortessa-II instrument (BD Bioscience, Franklin Lakes, NJ, USA). Data were acquired using a 488-nm laser and a 575/26-nm emission filter. Results were analyzed using FlowJo software and presented as median of fluorescence intensity (MFI).

Statistical analysis:

Results are presented as "mean ± SEM" unless otherwise specified. Comparisons between three or more experimental conditions/groups were analyzed with one-way ANOVA followed by Tukey's multiple comparison test. Statistical analysis and comparison of two independent variables in WST-1 proliferation assay, ECIS, Real-Time ATP Rate Assay, and qPCR of PDK2 and PDK4 mRNA expression was done using two-way ANOVA followed by Tukey's multiple comparison test. $P < 0.05$ was considered significant and indicated with asterisks: * <0.05, ** < 0.01, *** < 0.001, and **** < 0.0001.

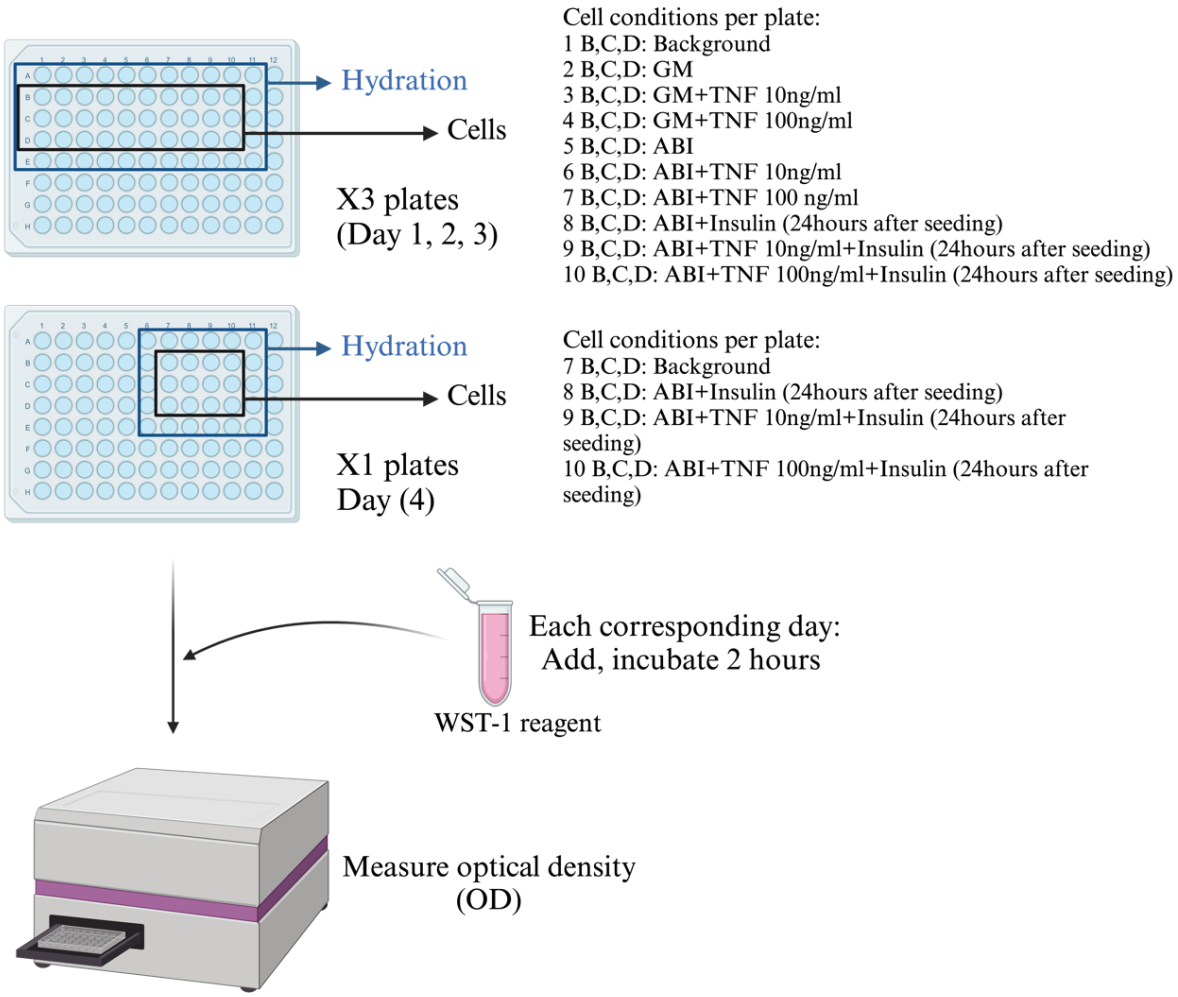


Illustration 3.3: Workflow of WST-1 proliferation assay. Created with BioRender.com

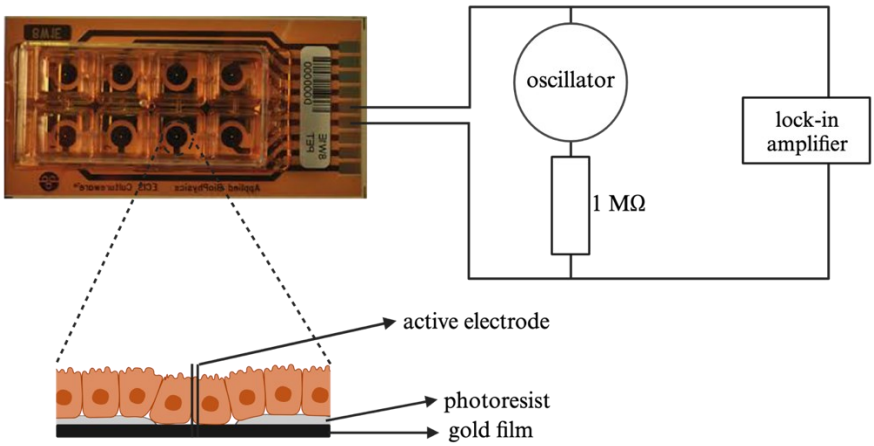


Illustration 3.4: Experimental setup for ECIS assay. Created with BioRender.com

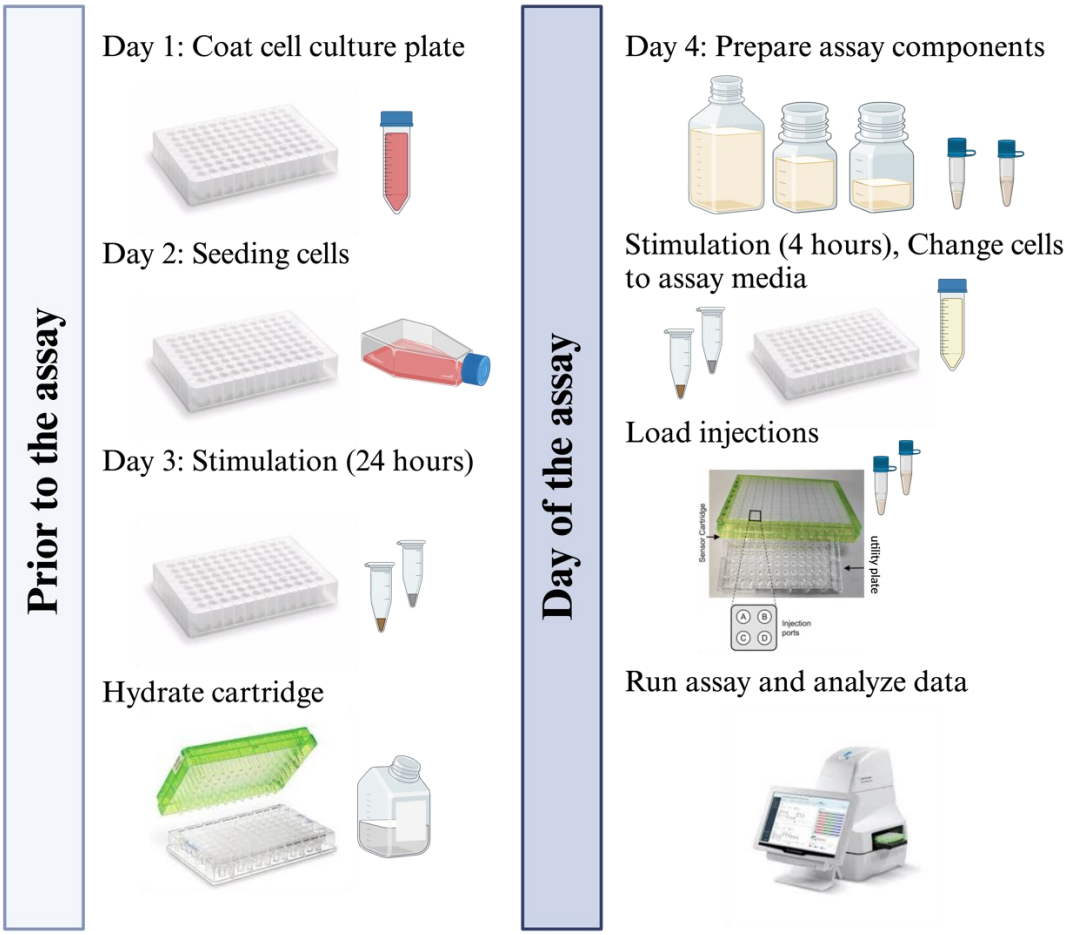


Illustration 3.5: Workflow for live cell metabolic assay. Created with BioRender.com

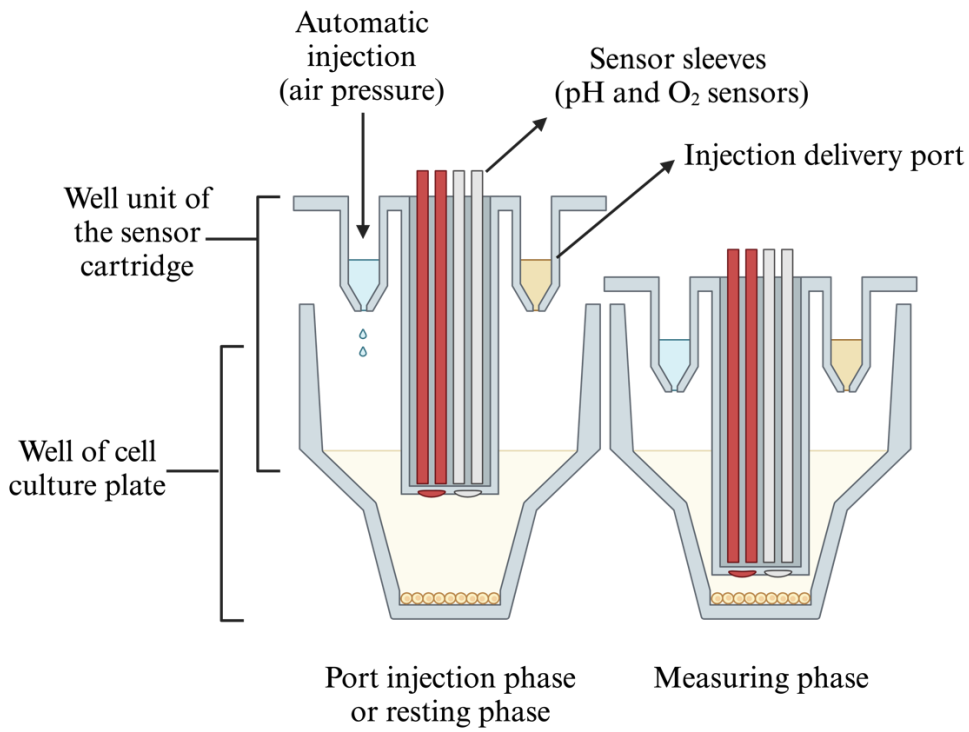


Illustration 3.6: Live cell metabolic assay during port injection phase and measuring phase. Created with BioRender.com

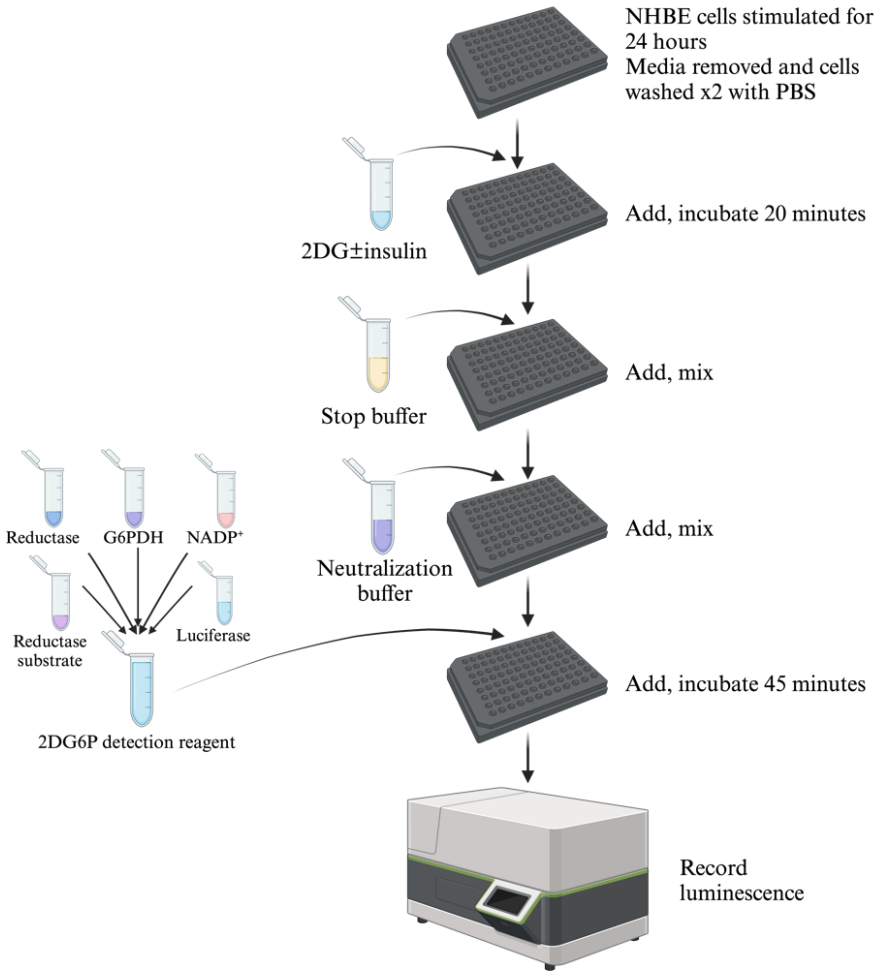


Illustration 3.7: Workflow for Glucose-Uptake Glo assay. Created with BioRender.com

Results:

1. *TNF reduced insulin-induced AECs proliferation:*

AECs from healthy donors that were cultured in the absence of insulin exhibited gene expression closely approximated to gene expression of asthmatic AECs [286]. These data suggest that insulin resistance, represented as insulin deprivation [286], causes structural abnormalities in AECs. To study the effects of insulin resistance in the context of inflammation, we activated NHBE cells with TNF, which is known to induce insulin resistance in other cell types [159,272,400], and studied the effects of TNF on insulin-induced AECs activation. We hypothesized that exposure of AECs to TNF induces insulin resistance, and consequently reduces epithelial proliferation, epithelial cell adhesion and barrier function, and delays cell recovery after injury.

NHBE cells were cultured in different conditions and proliferation was measured with a WST-1 colorimetric assay. Cells cultured in GM proliferated over 48 hours and presence of TNF 10 ng/ml (Figure 3.1 A-B) or 100 ng/ml (Figure 3.1 C-D) inhibited cell proliferation; in the absence of insulin, ABI, cells showed decreased proliferation and addition of TNF had a further inhibitory effect. Cells deprived of insulin for 24 hours exhibited low proliferation, but they started proliferating vigorously when insulin was added to these cells at the 24 hour time point; this increased proliferation was also inhibited in the presence of TNF 10 ng/ml (Figure 3.1 A-B) or TNF 100 ng/ml (Figure 3.1 C-D).

The WST-1 assay is based on the cleavage of tetrazolium salt in live cells, an effect that requires mitochondrial succinate-tetrazolium-reductase in metabolically active cells. This means that the decrease we saw in the WST-1 proliferation assay in the above results may indicate reduced numbers of epithelial cells (reduced proliferation) or less metabolically active cells. To validate that the first is the case in our experiment we also measured live cell numbers at the end of each experiment using trypan blue. Similarly to the data presented in Figure 1, a trend of reduced cell count was detected in cells cultured in GM in the presence of TNF, though it did not reach statistical significance, compared to cells in GM. Cell count of cells grown in ABI was further reduced by TNF 10 ng/ml (Figure 3.2 A-B) or TNF 100 ng/ml (Figure 3.2 C-D), and addition of insulin to cells grown in ABI at the 24 hour time point increased the cell count. These data are suggesting that TNF reduced AECs cell count and prevented the insulin effect on AECs proliferation, indicating that TNF may reduce insulin sensitivity of normal airway epithelial cells.

2. *TNF impaired insulin-induced AECs barrier function, and recovery after injury:*

Insulin resistance can disrupt epithelium permeability, junctional integrity, and wound healing [295,352,353]. To study whether the same is true for AECs, we used ECIS electrode arrays to evaluate AEC monolayer barrier function and recovery after injury. NHBE cells in GM were treated with TNF 100 ng/ml and monitored for 48 hours (Figure 3.3 A). TNF reduced the resistance of NHBE cells in GM in the first 24 hours (Figure 3.3 B-C). At the 24 hour time point, injury was induced through electroporation and TNF prevented the epithelial recovery after electroporation (Figure 3.3 D-E). These data are suggesting that insulin improved AECs barrier function and recovery after injury, and TNF reduced insulin action, impaired AECs barrier function, and prevented recovery after injury. TNF may have increased AECs permeability through a mechanism involving decreased epithelial junctional function, as has been shown [401–404].

To examine the effect of insulin on epithelial resistance, NHBE cells were cultured in ABI or ABI with TNF 100 ng/ml for 24 hours before the addition of insulin (Figure 3.4 A). NHBE cells cultured in ABI and TNF showed a trend toward declining resistance compared to cells cultured in ABI (Figure 3.4 B-C), though that result did not reach statistical significance. Addition of insulin the second day to cells in ABI improved cell resistance compared to ABI (Figure 3.4 D-E). In cells treated with TNF for the first 24 hours, addition of insulin showed a trend toward improving cell resistance over the TNF only; however, that was still lower than ABI with addition of insulin (Figure 3.4 D-E).

3. *TNF induced pIRS-1 (Ser^{307}) and decreased insulin-induced pAkt (Ser^{473}) in AECs:*

TNF can induce insulin resistance through a mechanism that involves reduced binding of IR to IRS-1 due to phosphorylation of IRS-1 at Ser^{307} (IRS-1-Ser^{307}) [370,372]; however, this effect has not been shown in epithelial cells. Phosphorylation of IRS-1 at Ser^{307} and reduced binding of IR to IRS-1 was also shown to reduce insulin-induced Akt activation [272]. Insulin-induced cell proliferation is mediated through activation of PI3K/Akt and MAPK pathways [404–406].

We hypothesized that TNF-induced insulin resistance in AECs and reduction of proliferation and barrier function is through a mechanism involving reduction of IR and Akt activation. NHBE cells were stimulated with TNF 10 ng/ml, and cell lysates were used to detect IRS-1 and pIRS1 (Ser^{307}). We showed that stimulation of NHBE cells with TNF for 60 minutes increased pIRS1-(Ser^{307}) (Figure 3.5 A-B). Increased pIRS1 (Ser^{307}) would predict reduced Akt activation. We tested Akt activation by detecting pAkt (Ser^{473}). Similarly, stimulation of NHBE cells with insulin induced Akt phosphorylation pAkt (Ser^{473}) and that was decreased in the presence of TNF (Figure 3.6 A-B). Our results are suggesting that TNF inhibited IR binding to IRS-1 and reduced insulin action in AECs.

4. TNF reduced insulin-induced use of glucose for glycolytic ATP production in AECs:

We have shown above that TNF prevented insulin effects in AECs. One of the consequences of blocking insulin action is alteration of cellular metabolic state [407]. It was shown that increased epithelial permeability can be due to depletion of glycolytic [408] or mitochondrial ATP [409]. We previously showed that TNF reduced insulin sensitivity and reduced epithelial barrier function in NHBE cells (Figure 3.3). Based on that we examined the effect of TNF on AECs ATP production. We hypothesized that exposure of AECs to TNF will initiate metabolic changes that may approximate the metabolic reprogramming detected in airway inflammatory diseases [183,410–413].

Measuring ATP production rate allows for the detection of cellular bioenergetic changes in both the cytosolic glycolytic pathway and in mitochondrial OXPHOS [414]. ATP production rate was measured using Seahorse real-time ATP rate assay. NHBE cells were cultured in GM then stimulated for either 4 or 24 hours before measuring ATP production rate. To evaluate the effect of insulin in AECs metabolic changes, we examined ATP production in the presence and absence of insulin.

TNF 10 ng/ml or 100 ng/ml did not induce changes in total ATP (Figure 3.7 E), mitochondrial ATP (Figure 3.7 F), or glycolytic ATP (Figure 3.7 G) production rates following 4 hours of stimulation in the presence or absence of insulin. We also showed that insulin increased glucose utilization for glycolytic ATP production in 4 hours (Figure 3.7 G) and 24 hours (Figure 3.8 G) which is reflected in extracellular acidification rate (ECAR) in (Figure 3.7 C-D) and (Figure 3.8 C-D). Both doses of TNF reduced insulin-induced use of glucose for glycolytic ATP production (Figure 3.8 C, D and

G) with no significant changes in total (Figure 3.8 E) or mitochondrial ATP (Figure 3.8 F) production rates following 24 hours of stimulation. These data suggest that TNF reduced insulin action in AECs and thereby decreased insulin-induced glycolytic ATP production.

5. *HDM modulated ATP production rate in AECs:*

A study showed that enhanced glycolysis is required for IL-1 induced proinflammatory changes in an HDM model of allergic asthma [284], suggesting that enhanced glycolysis is essential for allergen-induced proinflammatory changes in allergic asthma. Based on that we hypothesized that exposure of AECs to allergens like HDM or CE will induce metabolic shifts that may approximate metabolic reprogramming detected in allergic airway inflammatory diseases [183,410–413]. NHBE cells were cultured in GM then stimulated with HDM or CE for either 4 or 24 hours, then ATP production rates were evaluated. To evaluate the effect of insulin in AECs metabolic changes, we examined ATP production in the presence and absence of insulin. Stimulation of NHBE cells with HDM 50 μg/ml for 4 hours reduced total ATP production (Figure 3.9 C) and insulin-induced glycolytic ATP production (Figure 3.9 E).

These changes were more evident in 24 hours of stimulation with HDM and very likely multifactorial. HDM 20 μg/ml reduced the total (Figure 3.10 C), and insulin-induced glycolytic ATP production rates (Figure 3.10 E) with no change in mitochondrial ATP production (Figure 3.10 D). Conversely, HDM 50 μg/ml showed increased oxygen consumption and consequently increased mitochondrial ATP production (Figure 3.10 D), reduced glucose utilization for glycolytic ATP production in the presence or absence of insulin (Figure 3.10 E) and reduced total ATP (Figure 3.10 C). ECAR and OCR are shown separately (Figure 3.10 A and B). CE did not show changes in AECs ATP production following 4 or 24 hours stimulation (Figure 3.9 C-E and 3.10 C-E).

6. *HDM but not TNF reduced NHBE cells viability:*

Cell viability was tested using trypan blue for NHBE cells treated with TNF or HDM. Viability of cells treated with TNF for 4 and 24 hours or HDM 4 hours did not change compared to resting cells. HDM 50 μg/ml treated cells for 24 hours showed decreased viability by 19% (Figure 3.11) compared to resting cells, similar to previous data showed that exposure of bronchial epithelial cells to high dose of HDM for at least 20 hours increased cell death [415]. Decreased cellular viability

might contribute to the observed modulation of ATP production in HDM treated cells showed previously in (Figure 3.10 C-E).

7. TNF and HDM activated AECs and increased IL-8 release:

It was shown previously that TNF [416] and HDM [417] enhanced NFκB activity and increased IL-8 expression in epithelial cells. To validate our NHBE cells activation, we measured IL-8 release in cells stimulated with TNF or HDM for 24 hours. We showed that TNF and HDM increased IL-8 release from NHBE cells compared to resting cells (Figure 3.12).

8. Insulin induced glucose uptake in AECs:

To test whether the reduction of insulin-induced glycolytic ATP by TNF is due to reduced glucose uptake, NHBE cells were stimulated with TNF in presence or absence of insulin. Glucose Uptake-Glo Assay was then used to measure glucose uptake using 2DG. Insulin increased glucose uptake in AECs in 24 hours compared to no-insulin control, and TNF did not change insulin-induced glucose uptake (Figure 3.13). Our data suggest that reduction of glucose uptake is very likely not the mechanism that TNF used to reduce insulin-induced glycolytic ATP production and another indirect mechanism is involved.

9. Insulin regulation of PDK2 and PDK4 mRNA in AECs:

PDH regulates glucose metabolism and converts pyruvate, the final product of glycolysis, to acetyl-CoA [418–420]. That increases the influx of acetyl-CoA from glycolysis into the TCA cycle in the mitochondria [418–420]. PDH is feedback inhibited by its products and is inactivated by phosphorylation of its alpha-subunit of E1, one of the three components of the PDH complex. Four different PDK are known [420,421]. It was also shown that starvation upregulated and insulin downregulated PDK2 and PDK4 mRNA [422]. We aimed to measure the PDK2 and 4 mRNA levels in AECs in response to insulin and test weather that will be modulated by TNF or HDM. We showed that PDK2 and PDK4 mRNA expression were increased in starved cells and decreased in cells cultured in insulin in 4 and 24 hours. A trend of higher expression of PDK2 mRNA was detected in cells stimulated with TNF and HDM plus insulin compared to insulin stimulated cells by 4 hours (Figure 3.14 A); although that difference was not statistically significant presumably

due to small sample size. However, expression of PDK2 mRNA in cells stimulated with TNF and HDM plus insulin was reduced to the level of PDK2 mRNA in insulin stimulated cells by 24 hours. No changes were detected in PDK4 mRNA expression in insulin stimulated cells compared to TNF and HDM (Figure 3.14 B). These data suggest that early changes in PDK2 mRNA expression may modulate the later changes in insulin-induced glycolytic ATP production we detected in cells stimulated with TNF and HDM.

10. TNF and HDM induced changes in mitochondrial membrane potential (ΔΨMMP):

Proton pumps generated MMP which is required for sustained mitochondrial ATP production [423]. Changes in mitochondrial ATP production rate was detected with TNF and HDM treated AECs. We tested whether changes in mitochondrial ATP production rate is associated with changes in MMP. MitoProbe TMRM was used in AECs stimulated with TNF or HDM for 4 or 24 hours. In 24 hours, TNF caused mitochondrial depolarization and decreased MMP (Figure 3.15 A). Conversely, HDM caused mitochondrial hyperpolarization and increased MMP (Figure 3.15 B). Both were compared to the resting cells and the positive control CCCP. That is matching the time point of changes in mitochondrial ATP production rate. Mitochondrial depolarization is suggesting reduced cellular ATP and increased ratio of ADP/ATP [424]. However, hyperpolarization leads to ATP depletion and could happen due to lack of ADP, inhibition of ATPase, or decreased expression of mitochondrial uncoupling proteins (UCPs) [425,426].

Changes in mitochondrial function and MMP could also be altered by fission/fusion or damage. To test whether changes in mitochondrial mass is involved in the MMP changes we detected, we evaluated mitochondrial mass in AECs using western blot to detect mitochondrial content of COX IV as done previously [427]. COX is the terminal complex (complex IV) of the electron transfer chain (ETC) in the mitochondria that is expressed at a consistence high level. No changes in COX IV were detected in stimulated cells compared to resting (Figure 3.16).

Figures:

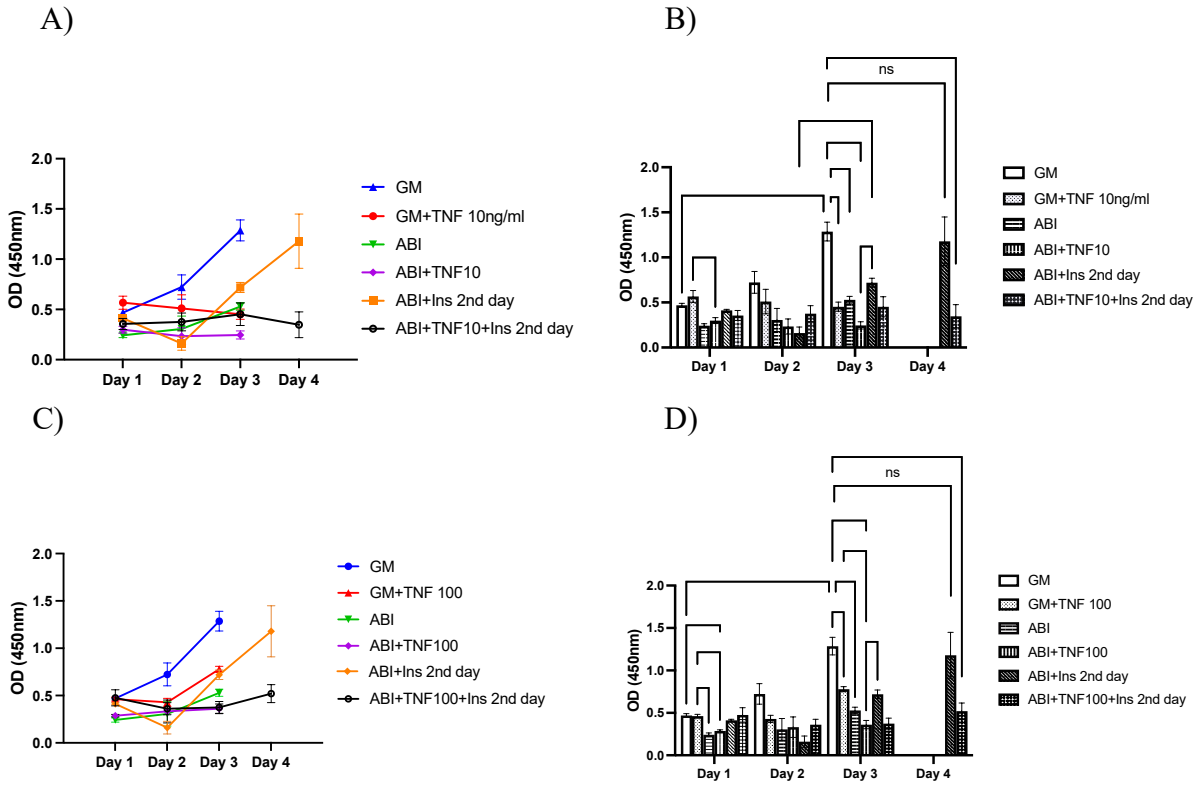


Figure 3.1: Effect of TNF on insulin-induced NHBE cell proliferation (average optical density of WST-1 assay 450 nm). TNF 10 ng/ml (A and B) and 100 ng/ml (C and D) data is presented to allow visualization of changes in proliferation rate over 4 days for every condition (A and C) and as individual bar graph for every condition (B and D). Each condition was done in triplicate in every experiment. Values are presented as mean ± SEM, n=3 (triplicate in each experiment), statistical analysis done using two-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

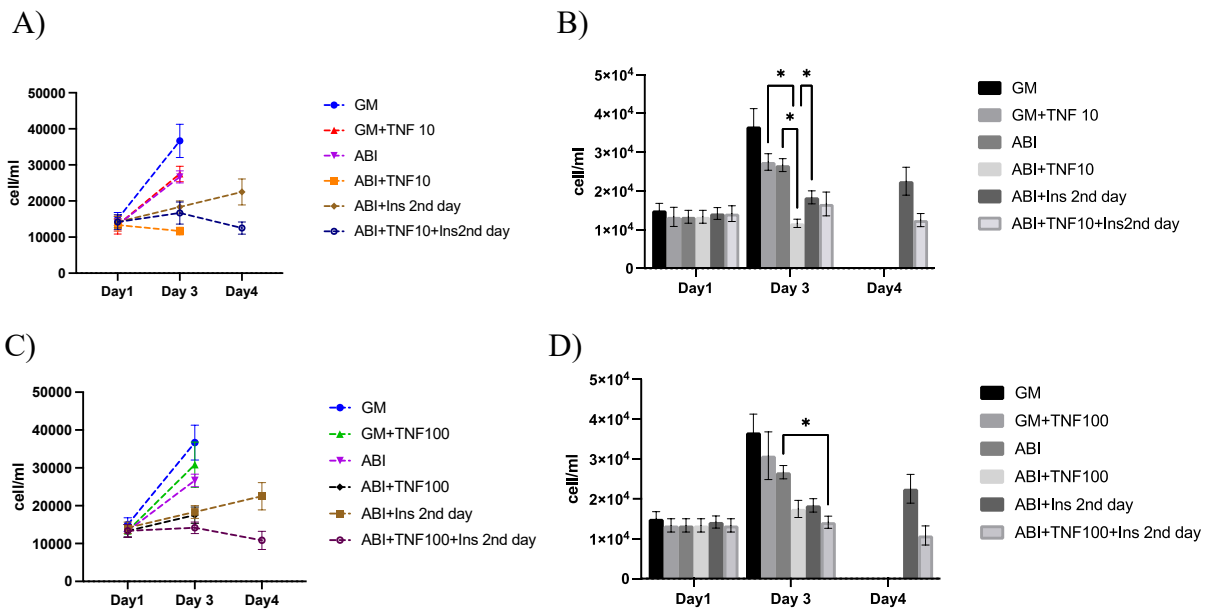


Figure 3.2: Effect of TNF on insulin-induced NHBE cell count. TNF 10 ng/ml (A and B) and 100 ng/ml (C and D) data is presented to allow visualization of changes in cell count rate over 4 days for every condition (A and C) and as individual bar graph for every condition (B and D). Each condition was done in triplicate in every experiment. Values are presented as mean ± SEM, n=3 (triplicate in each experiment), statistical analysis done using two-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

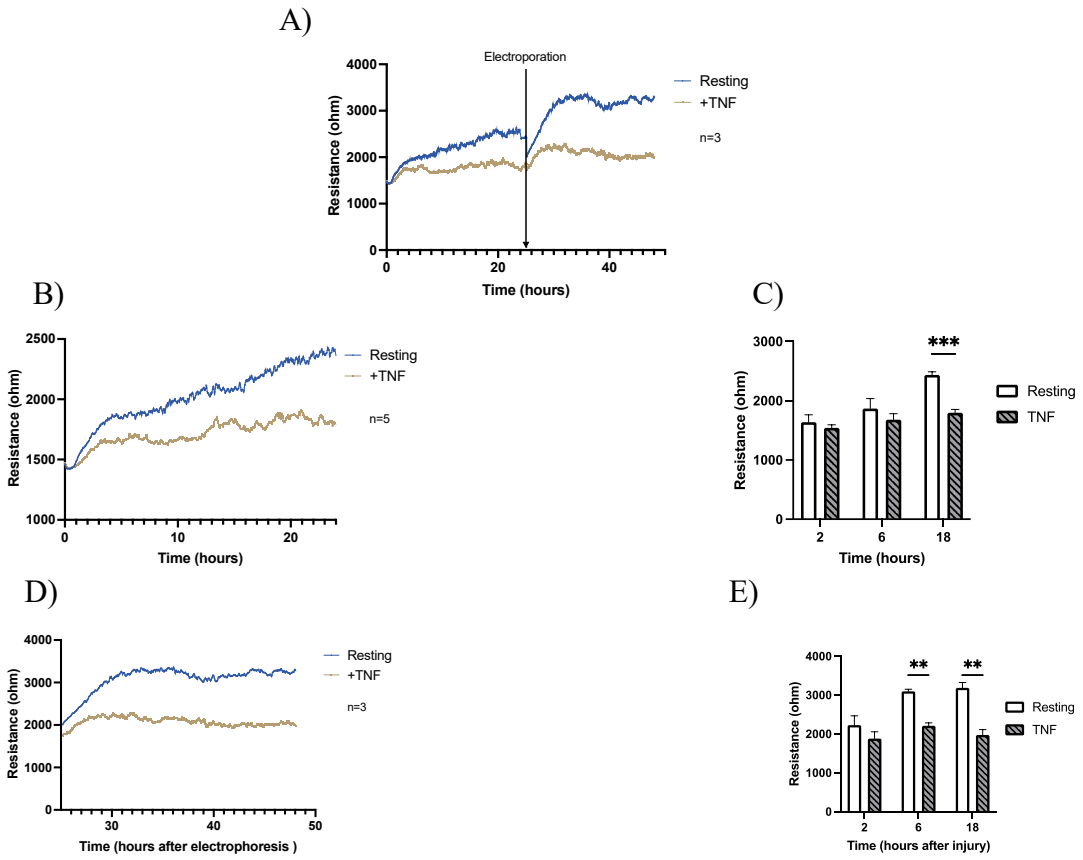


Figure 3.3: Effect of TNF 100 ng/ml on NHBE cells barrier function and recovery after injury. A) Resistance was monitored immediately after seeding over a time course of 48 hours, n=3. Resistance monitored for B) the first 24 hours resistance of cell adhesion (before electroporation), n=5 and D) the 24 hours after electroporation, n=3. Values are presented as mean only.

Resistance in bar graph to compare barrier function of resting vs. TNF treated cells for 2-, 6-, and 18-hours C) resistance of cell adhesion, n=5 and D) after electroporation, n=3. Values are presented as mean ± SEM. Statistical analysis done using two-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

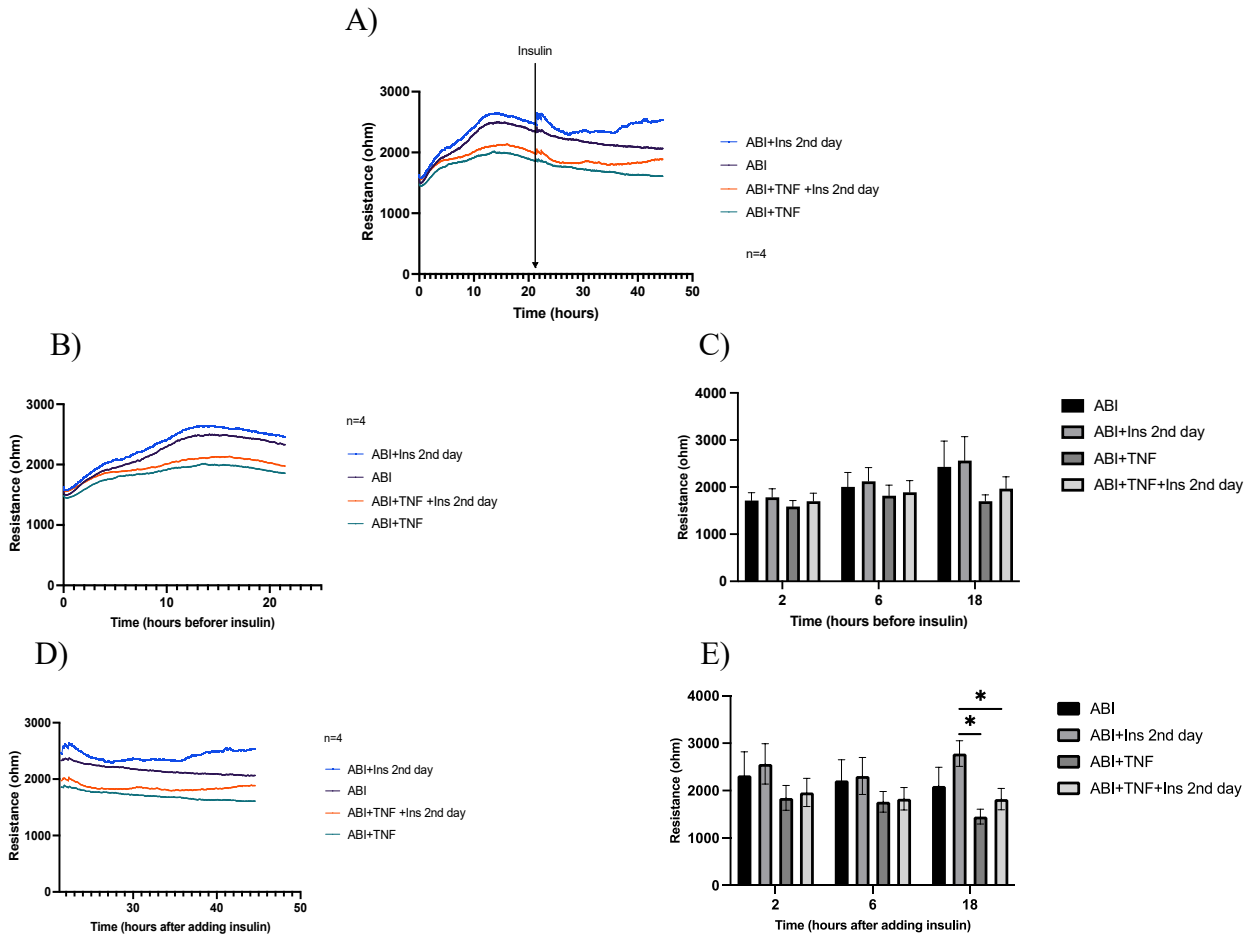


Figure 3.4: Effect of TNF 100 ng/ml on insulin-induced barrier function and recovery after injury of NHBE cells. A) Resistance was monitored immediately after seeding over a time course of 48 hours, n=4. Resistance monitored for B) the first 24 hours (resistance of cell adhesion) and D) the 24 hours after addition of insulin. Values are presented as mean only, n=4.

Resistance in bar graph to compare barrier function for 2-, 6-, and 18-hours C) resistance of cell adhesion and D) after addition of insulin. Values are presented as mean ± SEM, n=4. Statistical analysis done using two-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

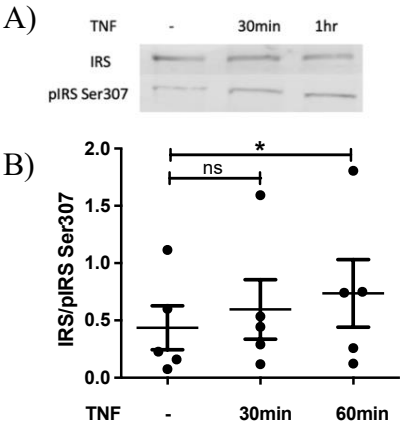
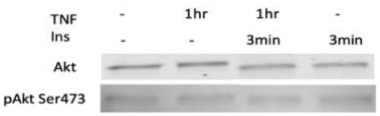


Figure 3.5: Increased pIRS1 (Ser^{307}) in NHBE cells treated with TNF for 30 and 60 minutes. A) Western blot of IRS (top) and pIRS1 (Ser^{307}) (bottom) 180 kDa. B) Densitometric analysis of protein abundance evaluated as ratio of signal optical density pIRS1 (Ser^{307}) / IRS. Values are presented as mean ± SEM, n=3. Statistical analysis done using one-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

A)



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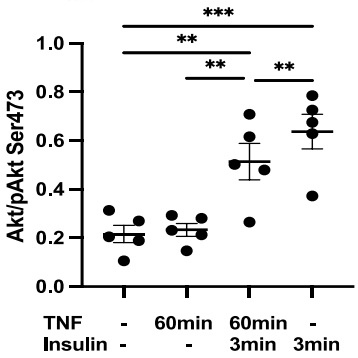

Figure 3.6: Reduced insulin-induced pAkt (Ser^{473}) in NHBE cells treated with TNF for 60 minutes followed by insulin for 3 minutes. A) Western blot of Akt (top) and pAkt (Ser^{473}) (bottom) 60 kDa. B) Densitometric analysis of protein abundance evaluated as ratio of signal optical density pAkt (Ser^{473}) / Akt. Values are presented as mean ± SEM, n=3. Statistical analysis done using one-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

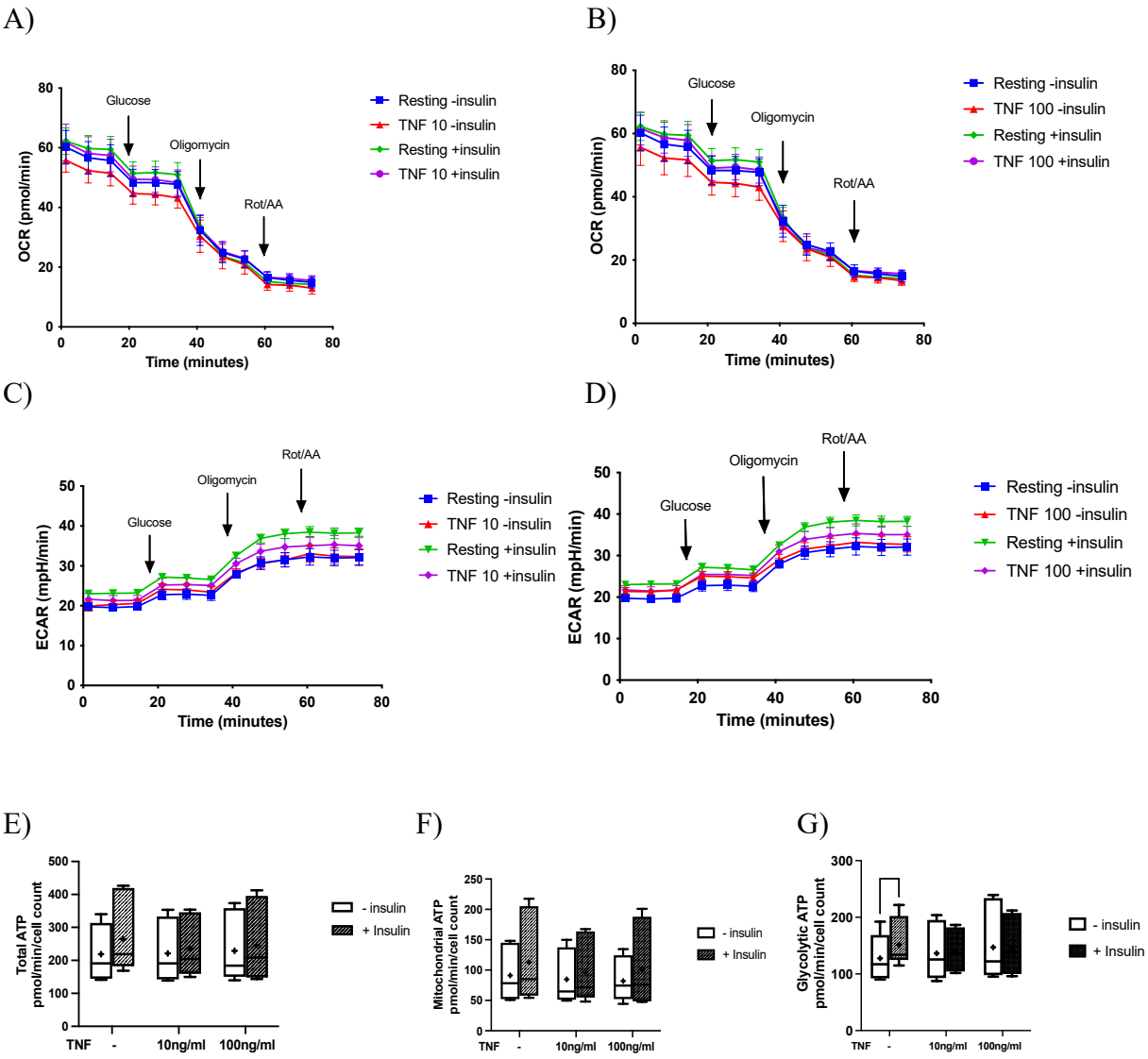

Figure 3.7: Measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in NHBE cells stimulated with TNF 10 ng/ml (A and C) and 100 ng/ml (B and D) for 4 hours with and without insulin using Seahorse live-cell metabolic assay platform. Glucose, oligomycin, and rotenone + antimycin A (Rot/AA) were injected at the indicated times. Glucose-induced: E) Total ATP, F) Mitochondrial ATP, and G) Glycolytic ATP production rate in NHBE cells stimulated with TNF for 4 hours. Values are presented as: mean '+', minimum to maximum (whisker), and the median (line inside the bar), n=3 (triplicate in each experiment). Statistical analysis done using two-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

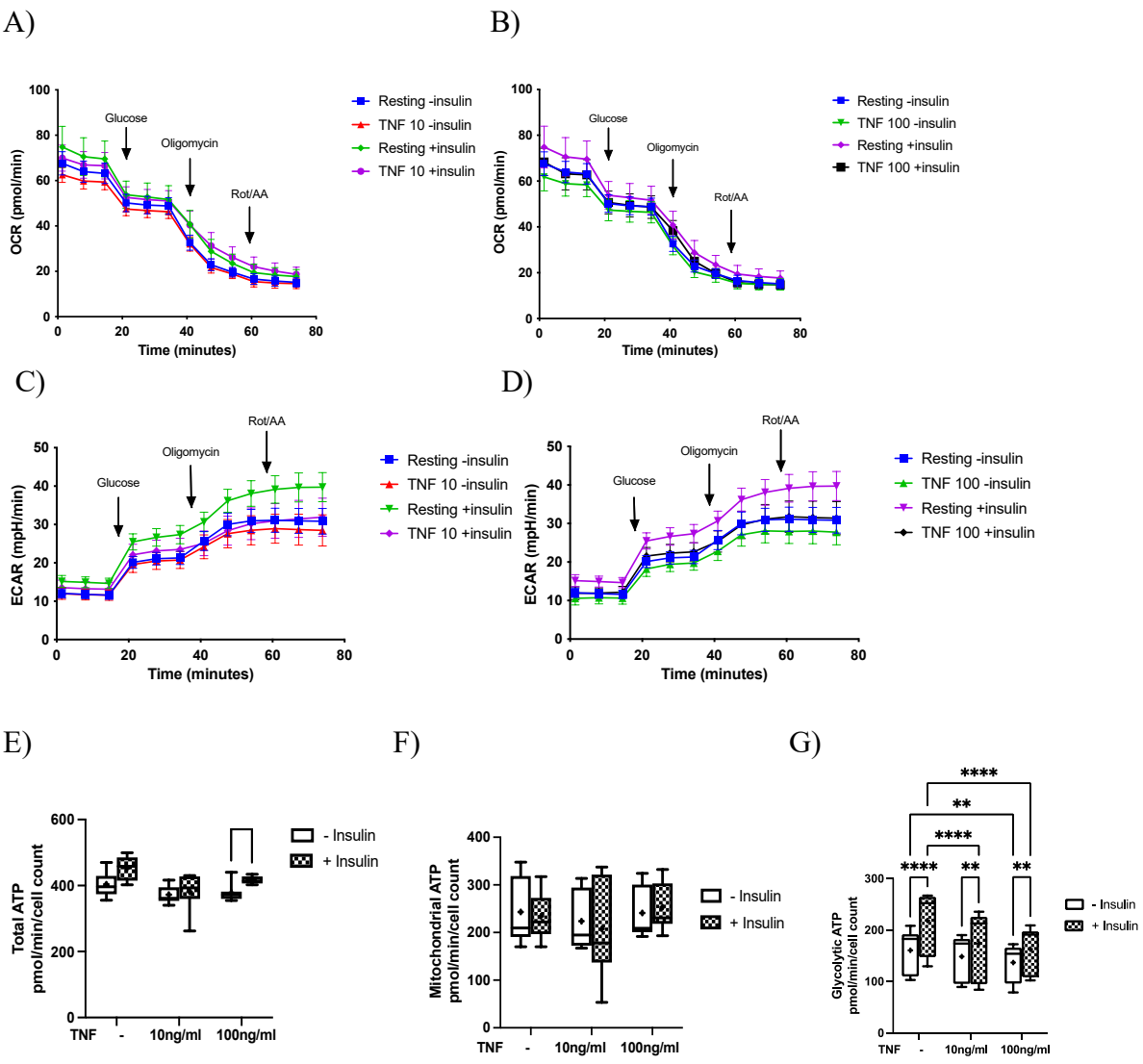

Figure 3.8: Measurements of OCR and ECAR in NHBE cells stimulated with TNF 10 ng/ml (A and C) and 100 ng/ml (B and D) for 24 hours with and without insulin using Seahorse live-cell metabolic assay platform. Glucose, oligomycin, and Rot/AA were injected at the indicated times. Glucose-induced: E) Total ATP, F) Mitochondrial ATP, and G) Glycolytic ATP production rate in NHBE cells stimulated with TNF for 24 hours. Values are presented as: mean '+', minimum to maximum (whisker), and the median (line inside the bar), n=3 (triplicate in each experiment). Statistical analysis done using two-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

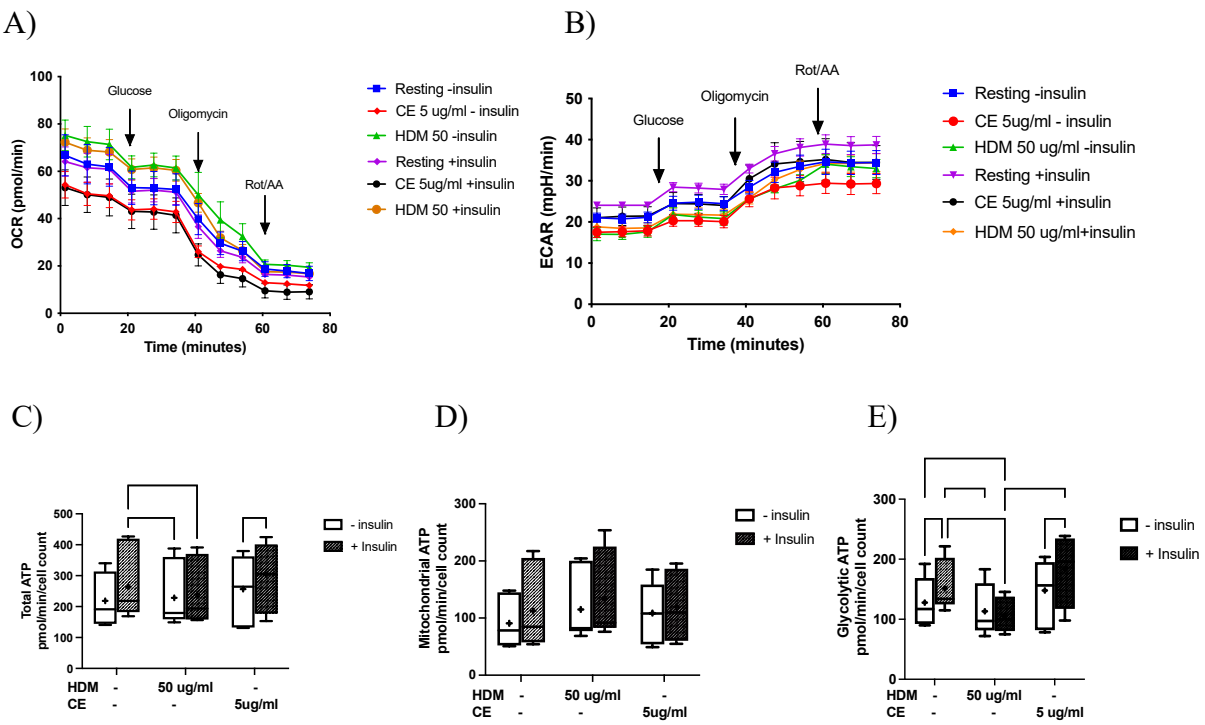

Figure 3.9: Measurements of (A) OCR and (B) ECAR in NHBE cells stimulated with HDM 50 μg/ml and CE 5 μg/ml for 4 hours with and without insulin using Seahorse live-cell metabolic assay platform. Glucose, oligomycin, and Rot/AA were injected at the indicated times. Glucose-induced: C) Total ATP, D) Mitochondrial ATP, and E) Glycolytic ATP production rate in NHBE cells stimulated with HDM and CE for 4 hours. Values are presented as: mean '+', minimum to maximum (whisker), and the median (line inside the bar), n=3 (triplicate in each experiment). Statistical analysis done using two-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

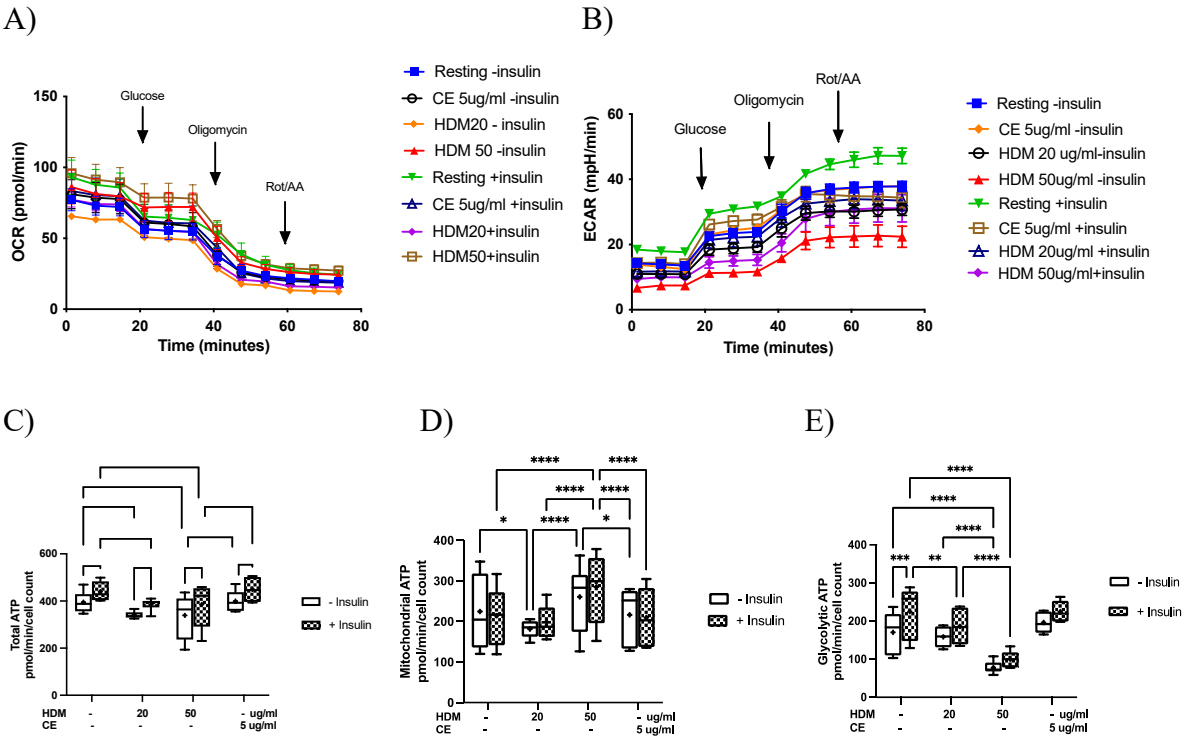

Figure 3.10: Measurements of (A) OCR and (B) ECAR in NHBE cells stimulated with HDM 20, 50 μg/ml and CE 5 μg/ml for 24 hours with and without insulin using Seahorse live-cell metabolic assay platform. Glucose, oligomycin, and Rot/AA were injected at the indicated times. Glucose-induced: C) Total ATP, D) Mitochondrial ATP, and E) Glycolytic ATP production rate in NHBE cells stimulated with HDM and CE for 24 hours. Values are presented as: mean '+', minimum to maximum (whisker), and the median (line inside the bar), n=3 (triplicate in each experiment). Statistical analysis done using two-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *p <0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

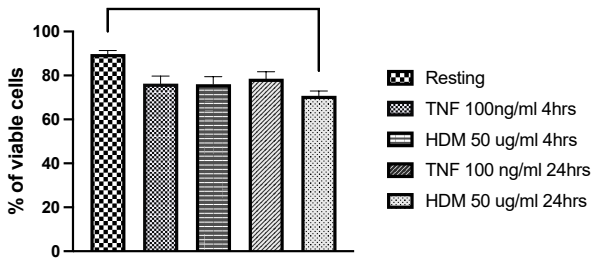

Figure 3.11: Viability of NHBE cells stimulated for 4 or 24 hours with TNF 100 ng/ml or HDM 50 μg/ml. Values are presented as mean ± SEM of % of viable cells, n=4. Statistical analysis done using one-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *p <0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

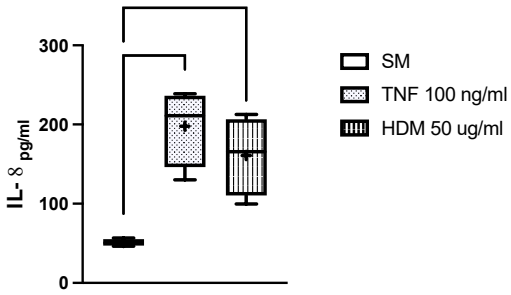

Figure 3.12: IL-8 protein release from NHBE cells activated for 24 hours with TNF 100 ng/ml and HDM 50 μg/ml. Values are presented as: mean '+', minimum to maximum (whisker), and the median (line inside the bar), n=4 (duplicate in each experiment). Statistical analysis done using one-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

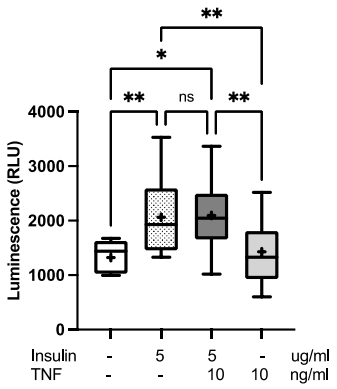

Figure 3.13: Insulin-induced glucose uptake in NHBE cells stimulated for 24 hours with TNF 10 ng/ml with or without insulin. Values are presented as: mean '+', minimum to maximum (whisker), and the median (line inside the bar), n=6 (duplicate in each experiment). Statistical analysis done using one-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

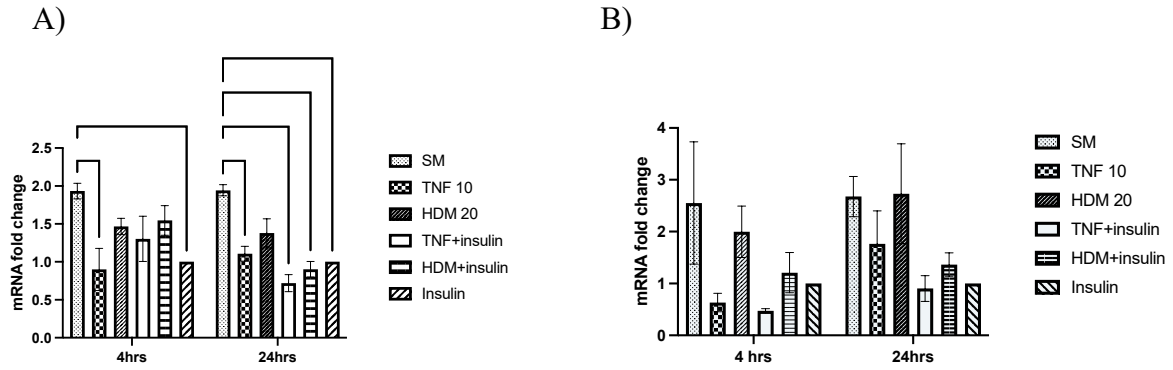

Figure 3.14: Regulation of A) PDK2 and B) PDK4 mRNA expression in NHBE cells stimulated for 4 and 24 hours with TNF 10 ng/ml or HDM 20 μg/ml with or without insulin. Values are presented as mean ± SEM, n=3. Statistical analysis was done using two-way ANOVA followed by Tukey's multiple comparison test, ns = not significant *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

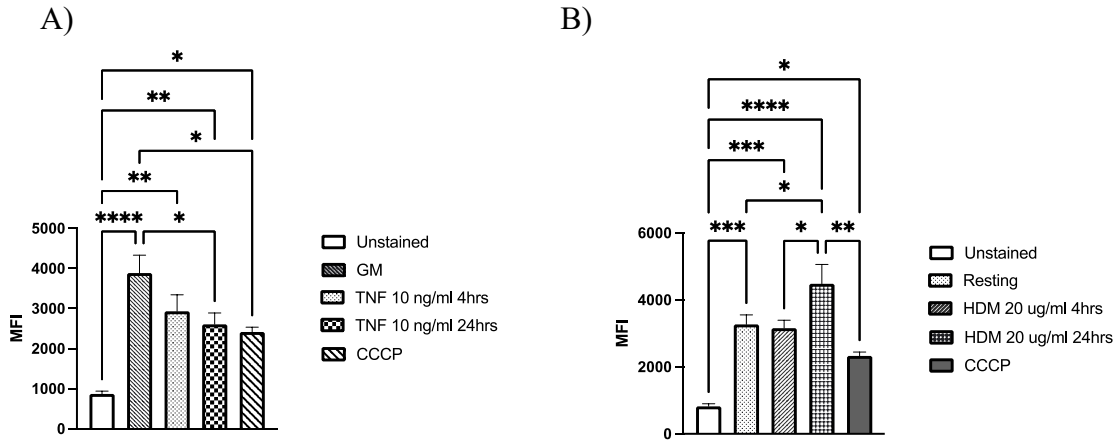

Figure 3.15: MMP flow cytometry analysis of NHBE cells stimulated for 4 or 24 hours with A) TNF 10 ng/ml, n=4 and B) HDM 20 μg/ml, n=3. Values are presented as MFI ± SEM. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison test, ns = not significant **p <0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

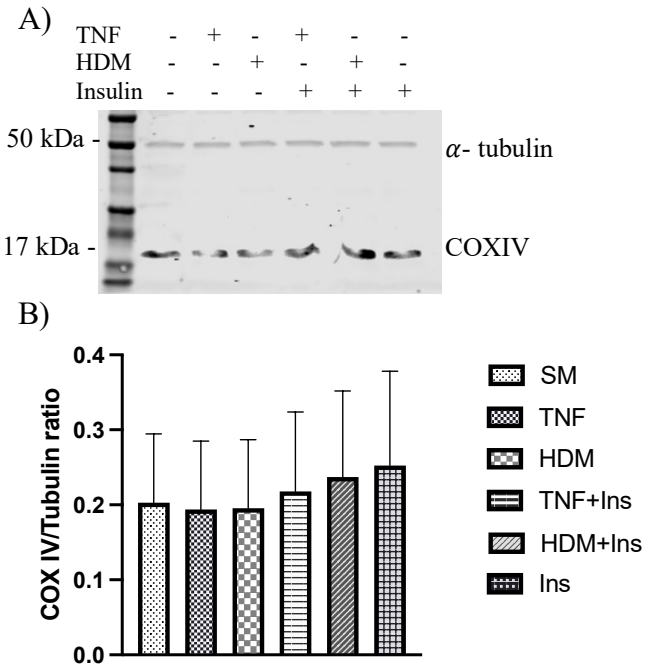

Figure 3.16: Mitochondrial content of COX IV in NHBE cells treated for 24 hours with TNF 10 ng/ml or HDM 20 μg/ml in the presence or absence of insulin. A) Western blot of α- tubulin 50 kDa (top) and COXIV 17 kDa (bottom). B) Densitometric analysis of protein abundance evaluated as ratio of signal optical density COX IV / α- tubulin. Values are presented as mean ± SEM, n=3. Statistical analysis done using one-way ANOVA followed by Tukey's multiple comparison test.

Discussion:

In this project we hypothesized that exposure of AECs to allergens and proinflammatory mediators induce insulin resistance and alter epithelial barrier function and metabolic shifts. Due to lack of experimental models to study AECs metabolic shifts *in vitro*, we used a simple model of unpolarized primary normal human bronchial epithelial cells to examine the effect of allergens and proinflammatory mediators on insulin-induced effects. We believe that metabolic changes in normal cells in response to inflammation is critical to understand the metabolic changes seen in airway inflammatory diseases.

We first showed that TNF reduced insulin-induced AECs proliferation. Our results suggest that TNF prevented insulin-induced AECs proliferation. However, our results of the proliferation assay was not completely matching the absolute cell count. Considering that WST-1 assay requires metabolically active mitochondrial succinate-tetrazolium-reductase to cleave tetrazolium salt, we may conclude that TNF reduced insulin-induced proliferation is very likely due to reduced metabolic activity of AECs in the presence of TNF rather than reduction of cell count. Using a different assay that does not rely of metabolic activity to measure proliferation may show more precise data and draw accurate conclusion.

Our data also showed that TNF reduced insulin-induced AECs resistance and recovery after injury which is suggesting impaired epithelial tight junctions and increased permeability. That is very likely associated with reduced epithelial regeneration. It was shown previously that reduced proliferation and impaired barrier function was due to downregulation of PI3K/Akt pathway activation [295,353,354]. We then tested insulin-induced PI3K/Akt activation in TNF treated cells in presence or absence of insulin. Addition of insulin to TNF treated cells showed reduced insulin-induced pAkt (Ser^{473}) compared to insulin treated cells which indicated reduced insulin action in AECs. Studies showed that TNF reduced insulin action through increased pIRS (Ser^{307}) which reduce IR to IRS-1 binding and insulin activation of PI3K/Akt pathway [370,372]. Our results showed that AECs treated with TNF increased pIRS-1-Ser^{307}, which is very likely the reason for reduced Akt activity and insulin resistance in AECs.

Insulin resistance due to increased pIRS-1-Ser^{307} and downregulation of PI3K/Akt activity were linked to altered glucose metabolism [407] which predict altered glycolytic energy production. Depletion of glycolytic or mitochondrial energy production was also associated with reduced

epithelial permeability [408,428]. According to these observations we predicted that TNF would modulate AEC ATP production. We showed that chronic TNF stimulation reduced insulin-induced use of glucose for glycolytic ATP production. Our results indicate that TNF is shifting AECs metabolism away from glycolysis without affecting total and mitochondrial ATP production. These data may suggest activation of alternative pathway to maintain cellular ATP production rate, very likely toward use of free pyruvate derivatives and free fatty acid metabolism. Reduced use of glucose for glycolysis ATP production may result in reduced production of pyruvate. It was shown that knockdown of mitochondrial pyruvate carrier (MPC), which facilitate pyruvate uptake into the mitochondria, in primary human skeletal muscle and lung epithelial cells did not change ATP-linked mitochondrial respiration [429]. Use of ^{13}C-labelled tracers also showed metabolic shifts away from glucose into dependence on other substrates like amino acid and fatty acid oxidation for energy production and biosynthetic metabolism [429]. MPC knockdown did not change glucose and glutamine uptake neither lactate nor glutamate production [429]. These changes were associated with increased levels of glutamine intermediates which suggest more dependence on glutamine metabolism [429]. In glutaminolysis, glutamine is metabolized into pyruvate in the mitochondria via malic enzymes and that facilitate generation of acetyl-CoA through pyruvate dehydrogenase [429]. On the other hand, glutamine conversion into lipogenic acetyl-CoA was significantly increased [429]. Increased level of TCA cycle metabolites downstream of citrate, which is derived from the combination of acetyl-CoA and oxaloacetate was also detected [429]. MPC inhibition was also shown to increase oxidation of branched-chain amino acids, valine, leucine, and isoleucine, which enriched TCA cycle [429]. Interestingly, ATP-associated respiration was not affected unless pyruvate transport, and glutamine and fatty acid oxidation were all inhibited [429]. These data are indicating that disruption of glycolysis-generated pyruvate would potentiate increased oxidation of glutamine, amino acids, and fatty acids to maintain metabolites flux through the TCA cycle.

We also showed that reduction of glucose use for ATP production was not associated with reduction of glucose uptake in TNF treated cells. We showed that insulin increased glucose uptake in NHBE cells which did not change in the presence of TNF. This conflict involves two parts to explain. First, TNF was shown to reduce glucose uptake through inhibition of GLUT4 translocation in other studies [430]; GLUT4 is insulin sensitive glucose transporter and inhibition of insulin-induced PI3K/Akt activation arrests GLUT4 translocation [431,432]. However, GLUT4 expression is uncertain and not shown in AECs. On the other hand, GLUT12 was proposed as an insulin sensitive

transporter; GLUT12 transcripts and protein were detected in human ciliated AECs [317,325–327] and other types of epithelial cells like ovarian and intestinal epithelial cells [338,339]. Internalized GLUT12 was translocated to cell membrane in response to insulin in skeletal muscle [340]. Based on these studies our data are suggesting that NHBE cells are expressing intracellular insulin sensitive GLUT12 that is translocated to the plasma membrane in response to insulin stimulation and resulted in increased glucose uptake and that was not prevented by TNF.

Second, we showed that TNF disrupted insulin-induced Akt phosphorylation but did not prevent insulin-induced glucose uptake. Interestingly, similar observation was shown in a previous study. It was shown that adipocytes activation with platelet-derived growth factor (PDGF) decreased insulin stimulated tyrosine phosphorylation of IRS-1 and subsequently reduced IRS-1 association with PI3K without affecting insulin-stimulated GLUT4 translocation and glucose uptake [433]. Additionally, insulin-induced activation of Akt and other kinases downstream of PI3K were not affected which may explain non inhibited GLUT4 translocation. This study provided evidence that the function of IRS-1 and PI3K is dissociated from stimulation of insulin-sensitive GLUT4 translocation and glucose uptake, and similar scenario could take place with GLUT12 since glucose uptake via both GLUT4 and GLUT12 is insulin sensitive. Our data are suggesting that glucose uptake in NHBE cells is independent of Akt activation and other pathways might be involved. Similar old theory was established and indicated that additional signaling pathways are required for insulin stimulated glucose uptake [434].

Taken together, our data are also indicating that glucose uptake detected in our NHBE cells is likely mediated through GLUT12. In adipocytes, insulin through Akt and TNF through AMPK activation increased glucose uptake by induced GLUT12 translocation to cell membrane [435]. In brief, adipocytes stimulation with TNF induced GLUT12 translocation to the plasma membrane and increased glucose uptake through AMPK activation [435]. AMPK inhibitor reduced TNF-induced AMPK phosphorylation, glucose uptake, and prevented GLU12 expression in the plasma membrane [435]. TNF activation of AMPK pathway in adipocytes was shown in other studies [436,437]. These data are suggesting that TNF can induce glucose uptake through AMPK independently of insulin-induced Akt activation which explain the unaffected glucose uptake in presence of TNF. Thus, TNF inhibition of glucose use for glycolytic ATP production is very likely involving another indirect mechanism without affecting glucose uptake. TNF might reduce transcription/expression or activity of some glycolytic enzymes. Further studies are required to identify that mechanism.

We also showed that exposure of NHBE cells to HDM can modulate cellular ATP production differently than TNF. HDM treated NHBE cells showed reduced total and insulin-induced glycolytic ATP both after acute, 4 hours, and chronic, 24 hours, stimulation, indicating that HDM reduced glucose use for glycolytic ATP and reduced total ATP production. Chronic stimulation with HDM increased mitochondrial ATP production, reduced insulin-induced glycolytic and total ATP. Increased oxygen consumption and mitochondrial ATP production failed to compensate for the excessive reduction in glycolytic ATP production and did not elevate total ATP production very likely due to reduced cell viability, leading to reduction in total ATP production. There is no previous study showed the effect of HDM on epithelial ATP production.

HDM is known to regulate airway allergic inflammation via activation of TLR4 in AECs [438]. Free fatty acids activation of TLR4 increased TNF and IL-6 expression and induced insulin resistance in adipocytes [277,439,440]. It was predicted that increased TNF and IL-6 are contributed to free fatty acids induced insulin resistance. It was shown later that free fatty acids do not bind directly to TLR4 but act through an endogenous ligand of TLR4 called fetuin-A [441,442]. Mutated TLR4 or fetuin-A prevented insulin resistance [441]. Fetuin-A is a liver produced glycoprotein which make it unlikely to be involved in the mechanism used by HDM to reduce insulin-induced glycolytic ATP in AECs. However, that does not rule out TLR4 and TLR-4-induced TNF and IL-6 to be routs for insulin resistance in AECs which require further testing. Another mechanism that could be involved in HDM reduced insulin-induced use of glucose and modulation of ATP production in our NHBE cells is oxidative stress. HDM was shown to increase mitochondrial oxidative stress through ROS production, and inducible nitric oxide (iNOS) gene expression in human bronchial epithelial cells [415]. Increased ROS generation was shown to disrupt Akt activation and induce insulin resistance in multiple studies [379–382].

One study that examined the effect of HDM sensitization and challenge *in vivo* and in cultured nasal epithelial cells from asthmatic patients, showed that HDM increased glycolysis rate and lactate dehydrogenase A production in an IL-1-dependent manner [284]. It is possible that the metabolic changes we detected in HDM treated cells is mediated through IL-1. However, that was not examined our NHBE cells.

Since we detected changes in insulin-induced glucose metabolism in TNF and HDM treated AECs, we aimed to examine the effect of TNF and HDM on the regulation of PDH. PDH is a rate-limiting enzyme for the pyruvate entry to TCA cycle and conversion to acetyl-CoA [422]. PDH is inactivated

by phosphorylation through PDK 2 and PDK4; mRNA of both kinases were shown to be regulated by starvation and insulin stimulation [422]. Insulin downregulation of PDK2 and 4 reduces phosphorylation and increases the activation of PDH, which trigger increased metabolism of pyruvate to acetyl-CoA. We showed that PDK2 and PDK4 mRNA expression were increased in cells deprived of growth factors and decreased in cells cultured in insulin for both 4 and 24 hours. TNF and HDM in presence of insulin caused a trend of high expression of PDK2 mRNA compared to insulin; that is a trend for preventing insulin-induced downregulation of PDK2 mRNA. Since PDH is a rate-limiting enzyme, it is possible that the increased expression of PDK2 mRNA at 4 hours might cause persistence increase, up to 24 hours, of PDK2, inhibit PDH activity, and reduce glycolysis in TNF and HDM treated cells.

Reduced PDH activity decreased the acetyl-CoA influx from glycolysis to TCA in the mitochondria [418–420] which may impact mitochondrial function. We examined the changes in mitochondrial membrane potential as an indicator for mitochondrial changes. Chronic stimulation with TNF resulted in loss of MMP and mitochondrial depolarization. Mitochondrial depolarization could occur for different reasons including calcium overload that induce opening in mitochondrial membrane and loss of potential, oxidative stress and ROS generation, and increasing expression of uncoupling proteins [424]. Interestingly reduced MMP in TNF treated cells did not affect mitochondrial or total ATP production. It was shown that mitochondrial depolarization due to CCCP treatment for 5 minutes did not affect cellular ATP content but showed increased ADP/ATP ratio in smooth muscle cells [424]. The increased ADP/ATP ratio could be due to depleted ATP reserves, energy depletion, or increased cellular demand for energy which indicates the need for enhanced ATP production or could be due to compromised mitochondrial ATP synthase which leads to accumulation of ADP. Either of these scenarios could be the reason for unchanged mitochondrial and total ATP in TNF treated NHBE cells in our experiments. In our experiments we have not done further testing to investigate the reason for mitochondrial depolarization in TNF treated AECs.

On the other hand, HDM showed opposite measures for MMP. Chronic stimulation of NHBE with HDM showed hyperpolarization of MMP. Mitochondrial hyperpolarization was detected in pulmonary smooth muscle cells from pulmonary hypertension and vascular remodeling patients [443]. Hyperpolarization was due to downregulation of uncoupling proteins and that was accompanied with increased superoxide release and oxidative stress [443]. It was also suggested that

increased MMP is due to decreased respiration, and that is caused by inhibition of mitochondrial membrane channels [443]. Other mechanisms for mitochondrial hyperpolarization were shown in another study [425], in which ATPase complex uses the energy of ATP hydrolysis to pump the protons to the mitochondrial intermembrane space which result in increased MMP of peripheral blood lymphocytes from lupus erythematosus. That was also associated with increased reactive oxygen species, increased ADP/ATP ratio, decreased cellular ATP, and apoptosis of lymphocytes [443]. Based on these previous studies, it is possible that HDM induced mitochondrial hyperpolarization caused the reduction of mitochondrial respiration and total ATP production and may contribute for the reduced cell viability. However, further study is required to know the mechanism behind these mitochondrial changes and how that contribute to mitochondrial changes seen in allergic asthma.

In conclusion, our data showed that proinflammatory environment of the airways and exposure of AECs to TNF or HDM might reduce insulin-mediated biological effects in AECs through insulin resistance. Reduced epithelial proliferation may lead to loss of epithelial differentiation, increased inflammation, impact epithelial continuity, and disrupt airway epithelium regeneration after damage [444]. Increased epithelial permeability was shown to increase airways hyperreactivity [445], airway hyperresponsiveness, and airway inflammation [446]. Increased permeability can also promote paracellular glucose movement to ASL which make airways more susceptible for infection. TNF and HDM reduced glucose use for glycolytic ATP production which would impact AECs bioenergetics, biological processes, and viability [447,448]. Most of these changes have been detected in airway inflammatory diseases and been linked to dysregulation of insulin action. Further investigation of metabolic changes in advanced models like differentiated AECs from healthy and asthmatic donors, and *in vivo* models of asthma will bring more insight into the role of insulin resistance in the pathogenesis of airway inflammatory diseases and provide a new class of metabolic therapeutic strategies.

Limitations:

- We used unpolarized NHBE cells in our assays throughout this project which may not represent the changes in polarized epithelial cells.
- We did not examine our hypothesis or measured the metabolic changes in asthmatic cells.
- WST- proliferation assay that we used rely on metabolic activity to detect reduced proliferation, which may not reflect accurate readout of proliferation in case the stimulus reduced cellular metabolic activity rather that reduction in cell number.
- The method we used to examine glucose uptake did not involve time or dose dependent assays neither for TNF stimulation nor 2DG. Additionally, we did not verify that glucose uptake in our experiments is mediated only through GLUTs.
- We did not examine lactate metabolism which may help to explain the changes in glycolysis and shifts toward anaerobic glycolysis.
- PDK2 and PDK4 regulates PDH activity. We only examined the mRNA expression of PDK2 and PDK4 which may not reflect the accurate protein level and activity of both. Besides, did not examine the direct activation of PDH or the PDH products like pyruvate and acetyl-CoA as PDK activity can also be regulated by feedback of PDH products.
- Mitochondrial changes were only assessed through MMP in this project. Changes in mitochondrial biogenesis like population, morphology, fission and fusion would add more information.

Future directions:

Various metabolomics-based studies have established metabolomic analysis using breath, plasma, BAL, or urine metabolites to help identifying biomarkers of asthma severity and differentiate between asthmatics and COPD patients [449–451]. These altered biomarkers included, but not limited to, glucose, glutamine, arginine, fatty acids, amino acids, and energy metabolites [449–451]. These findings showed that use of metabolite biomarkers can be a useful tool for clinical diagnosis and may provide new methodology to create of asthma endotypes based on metabolite signatures.

Studying metabolic changes and insulin resistance in normal AECs may add to this field. We believe that airways inflammation induces insulin resistance. Subsequently, intervention with insulin resistance at early stages of airway inflammation may help to reverse metabolic changes, maintain epithelium integrity, and homeostasis of subepithelial tissues; maybe through neutralization of proinflammatory mediators like TNF and IL-6. However, there are still unanswered questions. We predict that insulin resistance has a key role in AECs metabolic shifts. We still do not know how the reduction of insulin-induced glycolysis affects other metabolic pathways in AECs. We also did not evaluate mitochondrial function and changes. Examination of enzymatic activity of the key enzymes like HKs, PDH, and LDH that regulate metabolic pathways will help our understanding of the altered metabolic pathways in AECs. Targeted live cell metabolic assays for glycolysis and mitochondrial function can help understanding the capacity and alterations of insulin-induced glycolysis and mitochondria in AECs respectively. Mitochondrial imaging would answer many questions regarding mitochondrial biogenesis. Translation of our observations in vivo model of airway inflammation and neutralization of TNF will prove if anti-TNF would preserve epithelial barrier function, reverse metabolic changes, and inflammatory responses.

We believe that exploring metabolic changes in normal AECs in response to inflammation provides new tools to predict and diagnose airway inflammatory diseases especially in subjects with metabolic disorders, and those with high risk to develop asthma. It will also contribute to create new methodology/parameters for new signatures of asthma endotypes. These approaches will help to avoid severe complications and improve life quality of patients.

Chapter 4:
Discussion

Epithelium barrier function depends on the integrity of the adjacent cells and their adhesion through tight junctions [68,289,452]. Airway epithelium dysfunction is a key driver in asthma pathogenesis and exacerbations [39]. Exposure of airway epithelium to inhaled allergens contain active proteases disrupt tight junctions, increase permeability, and facilitate invasion of these irritants through airway epithelium [453–455]. The recognition of these antigens by AECs and immune cells leads to release of epithelial cytokines, attraction of adaptive immune cells and release of their proinflammatory mediators [456,457]. Increased expression of type-2 and fibrogenic cytokines such as TNF, IL-1β, IL-6, IL-13, IL-4 and chemokines aggravate the epithelial damage and reduce expression of junctional proteins like E-cadherin, occludin, and ZO-1 [458–461]. These inflammatory responses lead to airway hyperreactivity and excessive inflammation in asthmatics.

In this project we showed that exposure of AECs to CE serine proteases inhibited IL-13-induced CCL26 and may interfere with the proinflammatory effects of IL-13. These changes suggest profound inflammatory responses and increased expression of proinflammatory mediators. Chronic exposure of AECs to HDM or TNF increased epithelial permeability, altered energetic phenotype, induced insulin resistance, and disrupted MMP. These changes may augment epithelial damage and induce metabolic shifts.

4.1. Remodeling in airway inflammatory diseases:

Epithelial damage in airway inflammatory diseases involve EMT and remodeling that is characterized by lose of epithelia polarization, adhesiveness, and attachment to basal membrane which enhances epithelial migration and mesenchymal features [462,463]. In EMT epithelial cells gradually lose their epithelial characteristics and function and transform to mesenchymal (fibroblast cell) phenotype [464,465]. In some cases, EMT is critical for embryonic, neural, and heart development [464,465]. Besides, EMT is also an essential pathological feature in cancer metastasis and resistance of treatment [465]. Loss of cell-cell junctions due to downregulation of junctional components is an early sign of EMT [464,466]. Expression of E-cadherin and tight junction proteins are reduced in asthmatics [39]. Interestingly, expression of tight junction protein may modulate immune responses. Expression of E-cadherin has a role in maturation of dendritic cells and the subsequent T cell responses [467]. E-cadherin-stimulated dendritic cells failed to induce immunostimulatory cytokines and showed a regulatory T cell response as opposed to inflammatory induced maturation [467] suggesting a regulatory role of E-cadherin in adaptive immune response.

Additionally, EMT involves thickening of the basement membrane accompanied with subepithelial fibrosis, and hypertrophy of smooth muscle [468]. Fibroblasts and myofibroblasts serve as producers of growth factors like TGF-β and increase expression of extracellular matrix (ECM) proteins like collagen and proteoglycans in the airways [57,469]. These changes have a key role in fibrosis and fibroblast-myofibroblast differentiation [470].

4.1.1 Role of allergens and airway inflammation in airway epithelium remodeling:

In this project we showed that CE serine proteases prevented IL-13-induced CCL26 expression in AECs [471] . This inhibitory effect is very likely due to degradation of IL-13. Clearance of apoptotic eosinophils in allergic inflammation requires CCL26-activated $CX_3CR_1^+$ alveolar macrophages [472]. It was shown that CCL26 binding to CX_3CR_1 receptor mediates secretion of C1q, a complement component, from $CX_3CR_1^+$ alveolar macrophages and facilitate clearance of eosinophilic inflammation [472]. Depletion of $CX_3CR_1^+$ in animal model of allergic lung inflammation delayed the clearance of tissue eosinophilia compared to control [472]. Similar findings were shown upon depletion of CCL26 in AECs or using CCL26 neutralizing antibody [472]. Inhibition of IL-13-induced CCL26 suggest delayed eosinophil clearance and may trigger epithelial remodeling through eosinophil-derived proteins and other pathways [473].

Airway remodeling was also shown to be mediated by aeroallergen-derived proteases which contribute to EMT through epithelial injury and disruption of epithelial tight junctions [453,455,474–477]. Human bronchial epithelial cells treated with HDM purified allergen Der p 1 showed disruption of ZO-1 and occludin. HDM Der P 1 cleaved extracellular domain of occludin and claudin-1 [454]. This cleavage was also associated with cleavage of ZO-1 which is intracellular protein suggesting that Der P 1 cleavage of extracellular tight junctions could lead to disassembly of ZO-1 [454]. Similar scenario was shown in human bronchial epithelial cells stimulated with CE and showed significant reduction of monolayer resistance [478]. Effect of CE on resistance was inhibited by heat inactivation of CE suggesting that reduction of epithelial resistance by CE is protease dependent [478]. Other studies showed that CE can disrupt epithelium by another mechanism involving expression of matrix metalloproteinases (MMPs) [479,480]. AECs treated with CE showed reduced occludin and ZO-1 proteins and trans-epithelial electrical resistance (TEER) [480]. However, cells pretreated with MMP inhibitor or transfected with MMP1 siRNA maintained occludin and ZO-1 expression [480].

CE-induced MMP1 was mediated through TLR2 and ERK/MAPK pathway [480]. Other MMPs were also reported to cause epithelial damage and digest tight junction proteins [55,56,481]. These data are indicating that proteases are contributing to epithelial damage and remodeling very likely through degradation of tight junction proteins.

4.1.2. Epithelial remolding-metabolic changes crosstalk:

Other studies have shown that insulin regulates expression of tight junctions and cellular proliferation in different tissues [482–485], but it is not known whether insulin resistance is a mechanism involved in disruption of epithelial permeability and proliferation. Our data showed that reduced epithelial barrier function could be related to insulin resistance. Chronic exposure of AECs to TNF reduced insulin-induced barrier function and recovery after injury. TNF was shown previously to increase permeability and decrease expression of tight junctions [486,487] through a mechanism involves activation of myosin light chain kinase (MLCK) through a pathway involved NF-κB p50/p65 binding and activation of MLCK promoter. However, the exact signaling pathway is unclear [488,489]. Our observation is very likely linked to downregulation of PI3K/ Akt pathway. Inhibition of PI3K/Akt in human lens epithelial cells reduced the capacity of cells for spreading and adherence [490]. Additionally, insulin resistance due to hyperinsulinemia and hyperglycemia showed disruption of adherence junctional proteins in endothelial cells [491]. These data are indicating that reduced insulin sensitivity is very likely involved in the disruption of airway epithelium integrity.

Regulation of EMT is also mediated through multiple transcription factors like Snail, Slug, ZEB-1, ZEB-2, Tcf/LEF, Twist, and β-catenin [464,466,492,493]. It was shown that some of these transcription factors induce metabolic reprogramming in cancer cells [494,495]. Downregulation of E-cadherin was associated with elevated activity of Snail [494]. Besides, Snail regulation of a downstream promoter was also shown to increase glucose uptake, generation of glycolytic intermediated, reduce oxygen consumption, and ROS production [494]. Similarly, Twist regulates expression of energy metabolism related genes and induces alterations of energetic phenotype [495]. Increased expression of glucose metabolism related genes and proteins like glucose-6-phosphate dehydrogenase (G6PD) and lactate dehydrogenase A (LDHA) were detected in Twist transfected cells compared to control [495]. Consequently, increased glucose uptake and lactate production, and reduced mitochondrial mass were observed in Twist transfected cells compared to vector [495]. These alterations were reversed

by loss of Twist expression [495]. Twist was shown to induce these energy metabolic changes through activation of β1-integrin, which is a cell surface receptor that binds to ECM proteins, and downstream PI3K/Akt pathway [495]. These data are supporting the crosstalk between EMT pathways and metabolic reprogramming.

4.2. The cytokines of asthma:

4.2.1. Type-2 cytokines:

Asthma features like eosinophilia, increased antigen-specific IgE, AHR, and airway remodeling were shown to be related to increased type-2 cytokines IL-5, IL-13, and IL-4; these changes were abolished in knockout animals for type-2 cytokines [496–498]. Later it was shown that inhibition of IL-13/IL-4 during mice sensitization phase to ovalbumin resulted in reduced level of airway eosinophilia, CD4+ T cells, allergen-specific IgE, IL-5 in BAL. However, after established allergy inhibition of IL-4/IL-13 resulted in minor reduction of all measured allergic parameters. These data are suggesting slight efficacy of IL-4 and IL-13 inhibition in established allergy like the case of asthma. Notably, some literature showed a high tendency of some asthma-mediated mediators including type-2 cytokines for degradation by proteases [499–501]. Mast cell released serine proteases upon degranulation degraded endogenously produced and exogenously added IL-13 [499,500]. It was also predicted that this mechanism may involve other mediators as well. Another study examined the effect of mast cells tryptases, the most abundant granule-derived serine proteases from mast cells, on Th2-promoting cytokines showed highly selective cleavage of multiple mediators [501]. This study used recombinant human and mouse mast cell tryptase and showed that human tryptase effectively cleaved TSLP, IL-21, IGF-1, IGF-2, and fibroblast growth factor-1 (FGF-1), besides degradation of some chemokines including CCL11 (eotaxin-1) and minor N or C terminal trimming of others [501]. In addition, mouse tryptase showed efficient cleavage of several mouse cytokines including IL-13, IL-9, IL-21 besides chemokines like CCL11 and C or N terminal trimming of others [501]. In our project we showed that CE efficiently degraded recombinant human IL-13 which was very likely the mechanism prevented IL-13-induced effects in AECs [471]. On the other hand, mouse mast cell chymase was shown to degrade epithelial cytokine IL-33 [502,503]. In HDM-induced airway inflammation, deficient mice for mast cell chymase showed airway hyperreactivity, eosinophilia, and elevated serum IgE compared to wild type [502]. These data suggest

a regulatory role of the mast cell serine proteases through degradation of proinflammatory mediators to protect airways, limit inflammatory responses, and prevent accumulation of excessive proinflammatory mediators. It may also propose protective effect toward tolerance against inhaled irritants. Meanwhile, CE-mediated degradation of IL-13 and inhibition of CCL26 expression suggest another possible scenario in addition to decreased levels of eosinophil chemotactic factors in the airways. Since CCL26 expression was shown to activate alveolar macrophages to clear apoptotic eosinophils [472], it is suggesting that lack of CCL26 may result in prolonged inflammatory responses. Our data may also suggest that epithelium sensitization and production of IgE are more critical in airway allergic inflammation, and eosinophil migration is likely more dependent on IgE pathway. IgE-mediated eosinophil migration was shown to be influenced by prostaglandin D2 (PGD2) that binds to chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) that is expressed by eosinophils [504]. IgE-dependent activation of mast cells is the main source of PGD2 [505] and potent antagonism of CRTH2 showed to block eosinophil and basophils migration [506].

4.2.2. Neutralization of epithelial cytokines and/or type-2 cytokines:

Degradation of IL-13 and other mediators like IL-33 is drawing attention to the efficacy of neutralization of asthma cytokines. As it is possibly that the degradation would have similar biological significance to neutralization and may help us to understand the consequences of degradation. Neutralization of epithelial cytokines and/or type-2 cytokines propose reduced expression of alarmins and limited activation of Th2 cells in airway inflammation. Targeting the airway alarmins with biological therapies have showed that anti-TSLP, Tezepelumab, which prevents interaction of TSLP with its receptors has promising outcomes and reached furthest clinical development [507]. In inhaled allergen challenge, mild allergic asthma patients receiving Tezepelumab showed smaller reductions of post-allergen challenge FEV1 compared to placebo that was also accompanied with significant reduction of blood and sputum eosinophils pre- and post-allergen challenge [508]. Similar outcomes were detected in patients with uncontrolled asthma [509]. Some approaches for anti-IL-33 are in progress but not yet published in peer reviewed journals [507]. No clinical studies were done to test anti-IL-25 [507].

Some approaches have been established to neutralize IL-13 using anti-IL-13 monoclonal antibodies. Lebrikizumab and Tralokinumab are the most widely investigated agents to neutralize IL-13; however, both did not improve quality of life of asthma exacerbations significantly in phase III studies [510]. Other approaches were developed to block IL-13 activity using kinase inhibitors, bispecific antibodies, or peptide vaccines are still in infancy and would need to reach clinical development [510]. We learn from these data that targeting pathways that are downstream in immune response cascade like that mediated by type-2 cytokines is very likely partially efficient and leave some of the upstream responses like that associated with alarmins active which may abrogate exacerbations. Alternatively, suppression of alarmins can trigger the early events of immune responses and consequently suppress type-2 mediated responses which make this approach more promising.

4.3. Inflammation and metabolic changes:

Chronic inflammatory state and increased expression of proinflammatory mediators have been associated with insulin resistance in metabolic disorders [145,360,511,512]. We hypothesized that chronic inflammation in airway inflammatory diseases might be involved in the metabolic changes detected in asthma. Increased expression of inflammatory cytokines TNF, IL-6, and IL-1β were shown to induce insulin resistance and metabolic adaptation in multiple inflammatory diseases like rheumatoid arthritis [513], systemic lupus erythematosus [514,515], T2DM [511,516], and metabolic syndrome [517]. The dysregulation of metabolic pathways is highly relevant in asthma [187,518,519]. We showed that TNF and HDM reduced insulin-induced use of glucose to produce glycolytic ATP. That might indicate AECs dependence on other metabolic derivatives to maintain cellular ATP supply and/or activation of anaerobic glycolysis and production of lactate.

In vivo study revealed that metabolites in BAL from ovalbumin sensitized and challenged mice showed alterations in energy, amino acids, and lipid metabolism [187]. Concentrations of mannose, galactose, and arabinose carbohydrates were significantly reduced in experimental model of ovalbumin-induced allergic asthma compared to naïve mice [187]. Correlation analyses were positive between increased lactate and malate versus macrophages and eosinophils, suggesting active anaerobic glycolysis and mitochondrial rely on malate to support TCA cycle and maintain ATP production. A positive correlation between reduced galactose, mannose, and arabinose versus eosinophils and neutrophils suggects increased consumption of these surge metabolites either for

glycolysis-mediated energy production or due to anaerobic glycolysis [187]. Unfortunately, correlation with lung epithelium was not tested. Additionally, alteration in lipid metabolism was detected with decreases in phosphatidylcholines, diglycerides, triglycerides, cholesterol, and increased choline level [187] supporting relay on lipid metabolism as shown from bronchial epithelium previously [41]. Similar alteration in metabolomics have been also shown in murine model of HDM-induced asthma [285]. Depletion of carbohydrates and elevation of energy metabolites were detected HDM-induced asthma compared to naïve model [285]. That is suggesting increased use of carbohydrates for energy production in HDM-induced asthma in contrary to what we showed in normal AECs.

In our project we showed that chronic exposure of normal AECs to TNF or HDM reduced insulin-induced glucose use for glycolytic ATP production and altered AECs energetic phenotype. That might be related to the metabolic shift toward anaerobic glycolysis and lactate production. Increased levels of lactate have been shown in asthmatics [520], patients with cystic fibrosis [521], and lung inflammation [522] which is supporting impaired glycolysis and metabolic shifts toward lactate production. On the other hand, depletion of lipid metabolites may also indicate metabolic shift toward fatty acids oxidation. These findings are supported by another study used transcriptome analysis and showed reduced expression of OXPHOS related genes in patients with severe asthma and upregulated of fatty acid related genes [41]. Collectively, our data are suggesting like others that modulation of epithelium metabolism in asthma is very likely shift away from aerobic glycolysis toward anaerobic glycolysis which increase lactate production and deplete pyruvate production. These changes would push the mitochondria to use TCA derivatives to support OXPHOS to keep up the epithelial cell demand for energy.

4.3.1. Metabolic shifts influence mitochondrial changes:

Alterations in energy and fuel supply influence mitochondrial dynamics and biogenesis [523,524]. Adults with obesity and/or T2DM showed reduced mitochondrial enzymatic activity and content besides reduced FAO in insulin resistance muscle cells [523–525]. Skeletal muscle NADH:O_2 oxidoreductase activity was decreased in obese compared to lean volunteers [525]. NADH:O_2 oxidoreductase activity was positively correlated with insulin sensitivity and negatively related to obesity [525]. Morphological changes in skeletal muscle samples also showed smaller mitochondria in obese and T2DM compared to lean volunteers [525]. Reduced expression of several nuclear

respiratory factor-1 (NRF-1) dependent genes which are encoding oxidative metabolism and mitochondrial function enzymes were shown to be associated with insulin resistance and T2DM [526]. These data are suggesting impaired capacity of mitochondrial function and OXPHOS in insulin resistance.

Mitochondrial changes were also detected as a part of the metabolic changes in asthmatics. Increased oxidative stress [527–529] , mitochondria morphological changes, and reduced OXPHOS [41,530] were detected in airway inflammatory diseases [531]. In OVA-induced experimental asthma, airway epithelial mitochondria showed less dense matrix, loss of cristae, ballooning morphology, and that was associated with reduced total ATP production and reduced COX1 protein activity; compared to untreated control which showed tightly packed granules and well-characterized cristae, normal ATP production, and active COX1 [530]. These data are suggesting that mitochondrial changes in vivo might be complex and need multiple parameters to be evaluated precisely.

To assess mitochondrial changes in our project, we measured MMP and mitochondrial content of COX IV in response to acute and chronic inflammation. Significant changes were detected in chronic exposure and showed MMP hypopolarization in presence to TNF and hyperpolarization in presence of HDM. TNF or HDM did not induce significant changes in COX IV compared to resting cells. It was shown previously that low-grade chronic exposure to TNF induced mitochondrial changes in adipocytes [532]. Mitochondria were smaller and condensed and some looked hollow with absent of cristae; these changes were associated with increased level of ROS and elevated fission and fusion proteins in TNF-induced insulin resistance in adipocytes compared to control [532]. TNF has also reduced MMP, similar to our data, and production of intracellular total ATP [532]. TNF treated human airway smooth muscle cells showed increased mitochondrial volume density and mitochondrial DNA copy number compared to untreated cells with no data regarding MMP [531]. No published data indicated HDM-induced mitochondrial dysfunction. Although, these data are supporting our observations that TNF-induced insulin resistance is accompanied with loss of mitochondrial membrane potential, the MMP parameter is not enough to evaluate mitochondrial dysfunction and further examination of mitochondria enzymatic and morphological changes would be essential to indicate mitochondrial dysfunction.

4.4. Insulin resistance and subsequent immune changes:

Insulin resistance was shown to modulate immune responses [158,361,511,533,534]. Insulin was shown to regulate PAR-2 expression in normal AECs [535]. AECs grown in absence of insulin showed increased PAR-2 expression through downregulation of PI3K/Akt pathway [535]. These data are indicating a key role of insulin resistance in the regulation of airway inflammation and exacerbation of protease-mediated activation of AECs.

It was also shown that metabolic pathways are critical for some immune cell responses. An effective immune response depends on successful uptake and presentation of antigens by dendritic cells. Dendritic cells which have a key role in initiation, progression, and activation of adaptive immune responses were shown to be manipulated via metabolic reprogramming [536]. During inflammation, dendritic cell activation, expression of cytokines, and activation of T cells depend on energy supply [536]. Glycolytic burst and stimulated synthesis of fatty acids mediated through Akt pathway is a known event during inflammation and dendritic cell activation with TLR ligands, particularly TLR4 [536,537]. Increased glycolysis and fatty acid synthesis to meet the demand for energy during dendritic cell activation support the antigen presentation, cytokines synthesis, and secretion [538–540]. Suggesting that insulin resistance or depletion of carbohydrates [41,187,285] may limit the dendritic maturation and cell activation and contribute to dysfunctional immune effector pathways.

Decline in airway pH is a common observation in asthmatics [541–543]. It was shown that breath condensate from asthmatics was associated with decreased pH, increased nitric oxide concentration, and accelerated eosinophil necrosis [541]. Acidic pH was also shown to disrupt normal ciliary beating, increase mucous viscosity, and damage epithelial cell membrane [544–546]. Recent metabolomic findings showed increased levels of lactate in asthmatics [187,285], which could be relevant to the acidic pH in airway inflammation. Elevated levels of lactate and acidic pH could be partially counted for altered metabolism and decreased use of glucose for glycolytic ATP production. Collectively, these data suggest that insulin resistance-mediated airway acidity may disrupt epithelial function as innate barrier, exacerbate epithelial activation, and modulate immune cell responses like limitation of dendritic cell activation, and enhanced eosinophil necrosis.

Conclusion:

Our findings showed that airway epithelium inflammation and exposure to allergens may modulate Th2 responses and lead to profound inflammatory responses which may induce insulin resistance and disrupt mitochondrial function. Accordingly, it is favoring epithelium remodeling, damage, and modulation of immune effector pathways. Intervention with inflammatory and metabolic pathways may provide new therapeutic targets that prevent exacerbation of inflammatory responses and metabolic changes.

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Appendix:

1. IL-6 induced insulin resistance:

Suppressor of cytokine signaling (SOCS) proteins are SRC homology 2 (SH2) domain containing proteins that regulate innate and adaptive immune cell responses to cytokines through ubiquitin-mediated degradation of signaling complexes [547–549]. These proteins can also inhibit JAK tyrosine kinase activity directly through kinase inhibitory regions [547] and regulate insulin signaling [550]. Chronic exposure of adipocytes to IL-6 altered insulin signaling through increased expression of COCS3, downregulation of insulin receptor and IRS-1 proteins expression, and reduced insulin-induced AKT phosphorylation [551]. Inhibition of IL-6-induced SOCS3 and reversed IL-6-induced insulin resistance [551]. Similar findings were shown in hepatocytes and skeletal muscle cells [552–554]. We hypothesized that asthma cytokine IL-6 induces insulin resistance in AECs and alter epithelial metabolic functions. To examine our hypothesis, NHBE cells were stimulated with IL-6 (4 or 100 ng/ml) for 1 or 2.5 hours. IL-6-induced SOCS3 mRNA was detected in cells treated with 100 ng/ml for 1hour in a pilot experiment (Figure A.1). To detect IL-6-induced SOCS3 protein, NHBE cells were stimulated with IL-6 (4, 20, or 100 ng/ml) for 20 minutes, 1, or 2.5 hour. A pilot experiment showed increased SOCS3 at 20 minutes and 1 hour of IL-6 stimulation with 4 or 100 ng/ml respectively (Figure A.2). More work is required to confirm these observations and examine the contribution of IL-6-induced SOCS3 to insulin resistance in AECs.

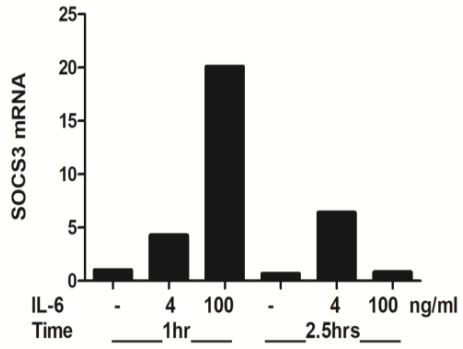


Figure A.1: IL-6 induced SOCS3 mRNA in NHBE cells treated with IL-6 (4, or 100) ng/ml for 20 minutes or 1 hour, n=1

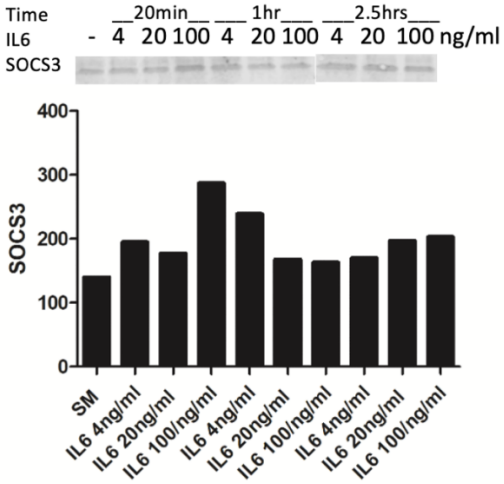


Figure A.2: SOCS3 signal intensity from western blot. IL-6 induced SOCS3 protein in NHBE cells treated with IL-6 (4, 20, or 100 ng/ml) for 20 minutes 1, or 2.5 hours, n=1

2. Role of PAR-2 activation in AECs TSLP expression:

This work was done in collaboration with my colleagues Yahya and Nadia for a hypothesis generating approach. Increased TSLP mRNA in the lungs of mice after administration of PAR-2 AP for 3 consecutive days was observed in unpublished data. Epithelium activation by inhaled irritants leads to the release of epithelial cytokines or alarmins that includes IL-25, IL-33, and TSLP [40]. Alarmins activate DCs which prime Th2 cell responses and inhibit Th1 priming cytokine IL-12 in airway inflammation [40]. TSLP is epithelium cytokine localized in epithelial cells, bronchial smooth muscle cells, fibroblasts, mast cells, and dendritic cells [555,556]. TSLP expression is prompted by exposure to multiple environmental triggers involving proteases, pathogens, and endogenous triggers like IgE, TNF, IL-1β, IL-4, and IL-13 which create a positive feedback loop for Th2 related responses [555,556]. There are two isoforms of human TSLP: short TSLP (shTSLP) and long TSLP (lTSLP); homeostatic shTSLP was shown to be associated with anti-inflammatory responses whereas lTSLP with inflammatory responses [557]. Use of human anti-TSLP monoclonal antibody that binds to TSLP and prevents receptor interaction in mild asthmatics attenuated most of allergen-induced asthmatic responses [508]. Anti-TSLP attenuated allergen-induced bronchoconstriction and blood and sputum eosinophils before and after allergen challenge with season pollens [508] suggesting a key role of TSLP in allergic asthma. We hypothesized that AECs activation by proteases or TLRs agonist will have a synergistic effect on PAR-2-induced TSLP expression.

Methodology:

The animal work was done by Yahya, Intranasal administration of PAR-2AP for 3 consecutive days then lungs were harvested. I processed the lung tissues and extracted total RNA to do qRT-PCR. Then I examined TSLP expression in AECs as the following: BEAS-2B or asthmatic huma primary bronchial epithelial (AHBE) cells were cultured in coated multi-well plates as described previously [471]. The cells were then stimulated in GM with CE or polyinosinic:polycytidylic acid (poly I:C), a synthetic double strand RNA TLR3 agonist, alone or in combination of either PAR-2AP or PAR-2CP for 4 or 8 hours. Cells were then harvested, and total RNA was isolated to perform reverse transcription and q-PCR assay as described [471].

Results:

Administration of PAR-2 AP increased mouse TSLP (mTSLP) mRNA expression in mice lung tissue and that was alleviated in PAR-2 knockout mice (Figure A3). In BEAS-2 B cells, ploy I:C showed a trend to upregulate lTSLP mRNA in BEAS-2B cells at 8 hours (Figure A4 B). PAR-2AP showed a synergistic effect to upregulate poly I:C mediated upregulation of lTSLP. CE did not upregulate lTSLP mRNA. A pilot experiment with AHBE showed a trend of excessive upregulation of lTSLP mRNA at 3 and 6 hours which did not change in presence of PAR-AP (Figure A5 A-B).

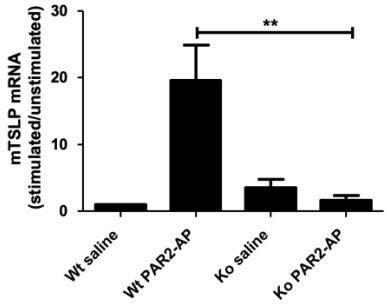


Figure A3: PAR-2AP increased mTSLP mRNA in lung tissues of wild type mice compared to PAR-2 knockout, n=3 in each group. Results are shown as mean ± SEM. Analyzed with one way ANOVA multiple comparisons, **$p \leq .01$.

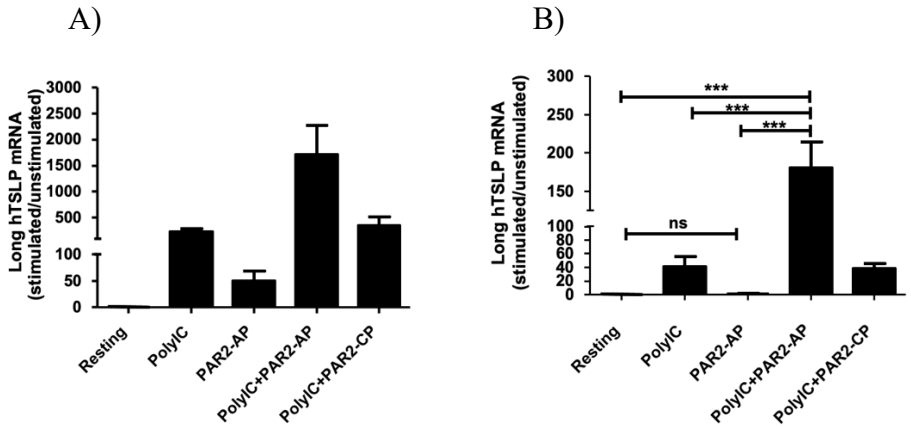
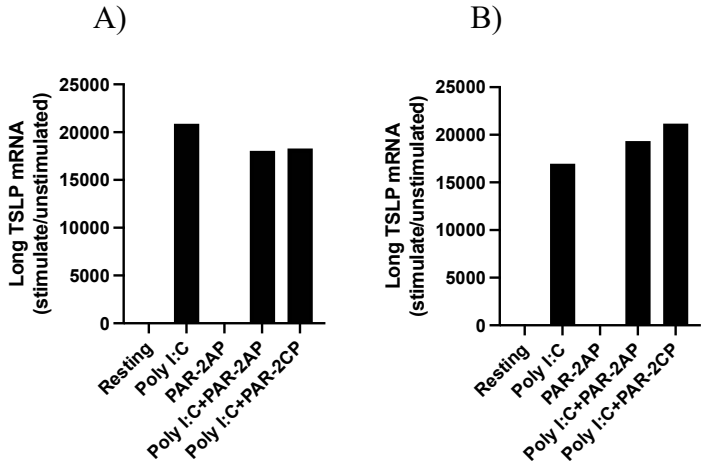


Figure A4: PAR-2AP synergistic effect to upregulate poly I:C mediated upregulation of lTSLP mRNA. Resting BEAS-2B cells in GM were stimulated with poly I:C, PAR-AP, a combination of both, or poly I:C with PAR-2CP for A) 4 hours, n=2 or B) 8 hours, n=3. Results are shown as mean ± SEM. Analyzed with one way ANOVA multiple comparisons, ns = non-significant, *p ≤ .05, **p ≤ .01, ***p ≤ .001.



Figur A5: Resting AHBE cells in GM were stimulated with poly I:C, PAR-AP, a combination of both, or poly I:C with PAR-2CP for A) 3 hours, n=1 or B) 6 hours, n=1.

3. Monocyte transmigration in response to factors present in the airways of patients with asthma:

This project aims to study the role of monocytes and monocytes-mediated accumulation of other inflammatory cells in the airways of asthmatics. We hypothesized that exposure to allergens stimulate monocyte migration into the airways and through airway epithelial cells where monocytes mediate accumulation of other inflammatory cells through release of chemotactic factors. Similar observations were reported previously [558–560]; however, the mechanism of migration through AECs is still poorly understood. This study in performed and monitored by our group post doc: Nami Shrestha.

Methodology:

Monocyte-chemotactic factors in sputum supernatants from mild asthmatics at baseline or 24 hours post allergen challenge were measure by ELISA. NHBE cells were cultures on upside down fibronectin pre-coated cell culture inserts for 2 hours to allow cell attachment. Inserts were then placed right back into the plate with GM at the basolateral side and incubated in cell culture incubator overnight. Transepithelial electrical resistance (TEER) was measured to examine epithelial monolayer integrity. Cells were used only if resistance is > 400 ohm. Isolated peripheral blood mononuclear cells from healthy donors were incubated with monocyte panel markers to aid with identification of monocytes by flow cytometry. The isolated labelled mononuclear cells were then added in the upper chamber of trans wells and diluted sputum supernatants taken from asthmatics at baseline or post challenge were added to the bottom chamber of the plate. The plate then incubated to allow migration. Flow cytometry used to examine the fluid at the bottom chamber to identify any migrated pre-labelled cells. Through this study I assisted Nami by establishing the cell culture process for several months and stimulation of a pilot experiment.

Results:

Preliminary data showed that the post-challenge diluted sputum supernatant induced monocyte trans-epithelial migration, migration of monocytes through epithelial monolayers from the top chamber to the bottom chamber, compared to the baseline sputum supernatant.

Acknowledgment:

I have been acknowledged to participate in generating some data for a project supervised by Dr. Lisa Cameron form UWO. I processed 32 samples for qRT-PCR, but unfortunately these data did not make it to the publication. My efforts have been recognized and acknowledged in the publication: https://doi.org/10.1165/AJRCMB.14.5.8624247.