Does the Epidermal Growth Factor Receptor Mediate Airway Epithelial Repair Following A Single Chlorine Exposure in Mice?

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ABBREVIATIONS

ADAM: a disintegrin and metalloprotease

ALI: acute lung injury

ARDS: Adult Respiratory Distress Syndrome

AREG: amphiregulin

AHR: airway hyperresponsiveness

ASM: airway smooth muscle

BAL: brochoalveolar lavage

BALF: bronchoalveolar lavage fluid

Cl_2: chlorine gas

CysLT: cysteinyl leukotrienes

LTD_4: cysteinyl leukotriene D_4

LTE_4: cysteinyl leukotriene E_4

DMTU: dimethylthiourea

EGF: epidermal growth factor

ELF: epithelial lining fluid

EGFR: epidermal growth factor receptor

FEV_1: Forced expiratory volume in 1 second

HB-EGF: heparin-binding growth factor

HGF: hepatocyte growth factor

LTC_4S: leukotriene-C_4 synthase

IIA: Irritant-induced asthma

MHC: major histocompatibility complex class II

MMP: matrix metalloproteinase

PCNA: proliferating cell nuclear antigen

RADS: Reactive Airway Dysfunction Syndrome

ROS: reactive oxygen species

TGF-α: transforming growth factor alpha

TGF-β: transforming growth factor beta

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

ABSTRACT

Chlorine gas exposure can occur through inadvertent mixing of household cleaning products, as a result of industrial accidents and intentionally in chemical warfare. Chlorine dissolves in the epithelial lung lining fluid to produce hypochlorous and hydrochloric acid, which cause oxidative stress. Oxidative stress has been shown to activate matrix metalloproteinases and to stimulate the synthesis of cysteinyl leukotrienes, which may, in turn, release ligands for the epidermal growth factor receptor (EGFR). The release of EGFR ligands and an increase in the IL-8 ortholog CXCL1 post chlorine in the mouse suggest the possible involvement of the EGFR in chlorine-induced airway injury. The hypothesis addressed in this thesis is that the EGFR mediates airway epithelial repair following chlorine exposure in mice. We studied the time-course of chlorine injury in the BALB/C mouse at 2, 6, 24 and 48 hours post nose-only exposure to 100ppm of Cl_2 for 5 minutes. The time course of changes involving the EGFR-EGFR ligand axis was studied at the transcriptional and protein levels using qPCR, Western blots and ELISAs to detect EGFR, heparin-binding EGF-like growth factor (HB-EGF) and amphiregulin (AREG) expression levels. The latter two proteins are ligands of the EGFR. Airway morphometry was used to quantify apoptotic cell numbers and proliferation visualized with TUNEL and PCNA immunostaining respectively. Airway inflammation was quantified with a total cell count and a differential cell count. Gefitinib, an EGFR inhibitor, was administered via oral gavage (50mg/kg) to assess the effect of inhibiting the EGFR pathway on chlorine injury, epithelial repair and airway mechanics (respiratory resistance and elastance) in response to methacholine challenge. Total EGFR protein was decreased following chlorine exposure starting at 2 hours

onwards with a gradual recovery by 24 hours while the transcription of EGFR was upregulated at 6h and 24h post exposure. Transcriptional levels of HB-EGF and AREG were not upregulated following chlorine exposure. Increased transcription of EGFR was prevented by the antioxidant DMTU (100mg/kg) given i.p. 1 hour prior to chlorine. HB-EGF ligand concentrations in the BALF increased with chlorine exposure and were also inhibited by DMTU, suggesting oxidative stress-dependent ligand release. Gefitinib inhibited airway hyperresponsiveness to methacholine relative to controls and diminished neutrophilia in the BALF at 6 hours post-chlorine exposure. The EGFR-EGFR ligand axis seems to be involved in the development of chlorine injury with a rapid increase in the receptor's ligand in BALF. Inhibitors of EGFR may reduce AHR as a consequence of inhibiting the neutrophilic inflammation, which may exacerbate the original injury caused by chlorine gas.

RÉSUMÉ

L'inhalation de vapeurs de chlore peut arriver à la suite d'un mélange accidentel de produits industriels, à la suite d'accidents du travail et intentionnellement pendant les attaques terroristes. Le chlore se dissout dans le liquide sur la surface des voies aériennes pour produire de l'acide hypochloreux et l'acide chlorhydrique, ce qui irrite le système respiratoire et provoque le stress oxydatif. Le stress oxydatif peut activer les métalloprotéinases matricielles et peut stimuler la synthèse des cystéinyl-leucotriènes, qui peuvent libérer des ligands pour le récepteur de facteurs de croissances épidermique (EGFR). La libération de ligands de l'EGFR dans les voies aériennes et l'augmentation de CXCL1, un orthologue du interleukine IL-8, suggèrent la possibilité de l'implication de l'EGFR dans la prolifération épithéliale observée suite à l'inhalation du chlore. L'hypothèse abordée dans cette thèse est que l'EGFR est impliqué dans la réparation de l'épithélium des voies aériennes des souris suite à une seule exposition au chlore. Nous avons étudié l'évolution temporelle de la réparation et régénération épithéliale chez la souris BALB/C à 2, 6, 24 et 48h après une exposition nasale à 100ppm de chlore pendant 5 minutes. L'axe du EGFR a été étudiée au niveau de la transcription du mARN, de la présence des protéines à l'aide du qPCR, Western Blot et ELISA pour détecter les niveaux d'expression du EGFR et les facteurs de croissance heparin-binding EGF-like growth factor (HB-EGF) et amphiréguline (AREG). Ces deux dernières protéines sont des ligands du EGFR. La morphométrie des voies aériennes a été employé pour quantifier le nombre de cellules apoptotiques et proliférâtes visualisées avec les techniques d'immunomarquages TUNEL et PCNA respectivement. L'inflammation des voies respiratoires a été quantifiée avec une numération cellulaire totale et différentielle.

Gefitinib, un inhibiteur du EGFR, a été administré à un dose de 50mg/kg par gavage oral pour évaluer l'effet de l'inhibition de la voie EGFR sur les dommages causes par l'inhalation du chlore, sur la réparation épithéliale et sur la mécanique des voies respiratoires (résistance et élastance respiratoire) suite à la métacholine. La protéine totale du EGFR a été diminué 2h après une exposition au chlore avec une reprise progressive jusqu'à 24h. La transcription de l'ARN du EGFR a augmenté à 6h et 24h à la suite de l'inhalation du chlore alors que la transcription du HB-EGF et AREG n'a pas augmentée d'une manière significative. Augmentation de la transcription du EGFR a été empêchée par la DMTU antioxydant (100mg/kg) i.p. propose 1h avant le chlore. Concentrations de ligand HB-EGF dans le BALF augmenté par l'exposition au chlore ont également été inhibées par DMTU, ce qui suggére un mécanisme de libération dépendante sur le oxydatif stress. Gefitinib (50mg/kg) annule la hyperréactivité bronchique à la métacholine et diminue les neutrophiles dans le BALF 6h suite à l'exposition au chlore. Le EGFR et ces protéines associées semble être impliquée dans les lésions des voies aériennes suite au chlore à cause d'une augmentation rapide du HB-EGF dans le BALF. Les inhibiteurs du EGFR peuvent réduire la prolifération grâce à l'inflammation plutôt des neutrophiles, qui peut aggraver les dommages initiales cause par le gaz de chlore.

INTRODUCTION AND LITERATURE REVIEW

1.1 Asthma Introduction

Asthma is a chronic inflammatory airway disease that affects 10-12% of the population in developed countries with increasing prevalence [1]. Asthmatics experience symptoms including but not limited to recurrent wheezing and coughing, chest tightness, dyspnea with signs such as variable and reversible airway obstruction, and laboratory findings such as airway remodeling and airway hyperresponsiveness (AHR) [1]. Chronic inflammation of the airways is typical of asthma and leads to the production and release of pro-inflammatory mediators such as chemokines, cytokines, adhesion molecules and recruitment of leukocytes and inflammatory cells [2]. A consequence of chronic inflammation includes extensive and permanent structural changes of tissue composition of the airways, which is a process termed airway remodeling.

Airway remodeling can affect multiple tissues within the airway and is associated with functional respiratory problems. This phenomenon can be summarized as including histological changes such as epithelial cell hyperplasia, goblet cell metaplasia, thickening of the sub-epithelial reticular basement membrane, increased angiogenesis, subepithelial collagen deposition, hypertrophy and hyperplasia of airway smooth muscle [3]. Increased ASM as a consequence of airway remodeling seems a likely candidate driving AHR [4, 5] although the contributions of the epithelium, connective tissue cells and inflammatory cells have not been ruled out. Clinically, increased severity of asthma has been associated with increased AHR and remodeling of the airways [6], highlighting the importance of the structure-function relationship in the normal airway.

Measurement of elevated reactive oxygen species (ROS) in the airways of asthmatics suggests the involvement of oxidative stress in the pathogenesis of asthma [7]. In particular, levels of 8-isoprostane, which is a marker of oxidative stress, were elevated in asthmatic breath condensates compared to controls [8]. Endogenous ROS can be produced by the activation of the tumor necrosis factor receptor or activation of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), downstream of EGFR[9]. ROS can activate transcription factors such as nuclear factor-κB (NF-κB), hypoxia inducible factor-1 alpha, and the complex formed between forkhead box O (FOXO) and p300/CBP acetyltransferase [9]. The ROS-specific transcription of these genes, particularly downstream of NF-κB, will trigger a pro-inflammatory state. Exogenous sources of ROS from inhaled oxidants may also trigger inflammation through this pathway. Excess ROS can impair homeostatic mechanisms, irreversible cell injury and eventually cause oxidative stress. Markers of oxidative stress, including 8-isoprostane, and measurements of ROS are elevated in the breath condensates of asthmatics compared to controls [8] [10]. Excess ROS are produced physiologically by many phagocytes and may contribute to the pathogenesis and pathophysiology of asthma through induction of oxidative stress, which may activate epithelial cells of the bronchial tree. The consequences of oxidative stress are discussed in further detail below.

The adaptive and innate immune responses are crucial to the pathogenesis and subsequent pathology of asthma. A large proportion of asthmatics is atopic, defined by the presence of IgE specific for common aeroallergens, leading to allergic inflammation of the airways [2]. Inhaled allergens are phagocytosed by antigen-presenting cells such as dendritic cells, which present antigens in the context of major histocompatibility complex

II (MHC II) molecules to T cells specific to that particular antigen. Presentation of antigen leads to T cell activation and differentiation into primarily the Th2 subset, producing IL-4, IL-5 and IL-13. Th2 cells can then assist B cells to synthesize immunoglobulins specific to that allergen or may activate other T lymphocytes. Cytokine production by Th2 cells accounts for airway eosinophilia and IgE class switching [11]. Allergen-specific IgE associates with high affinity receptors on mast cells, which are present in several areas including the dermis of the skin and respiratory mucosa [12]. In response to subsequent exposure to allergen, cross-linking of IgE molecules will cause activation and degranulation of mast cells and release histamine, cytokines prostaglandins and cysteinyl leukotrienes, which produce the symptoms and signs seen in a type I IgE-mediated hypersensitivity reaction [12]. This early airway response occurs within minutes of exposure and generally resolved within an hour. It is not associated with the development of post-allergen airway hyperresponsiveness. However, a late-phase response has also been described occurring hours after allergen exposure[13]. Administration of MK-571, an antagonist of cysteinyl leukotrienes D_4 and E_4 (LTD_4 and LTE_4), abrogates increases in airway resistance during early and late responses in an ovalbumin-sensitized Brown Norway rats [14]. Cysteinyl-leukotriene antagonists administered to human subjects undergoing allergen challenge similarly abrogate changes in airway function [15]**.** Measurements of cytokine levels in ovalbumin-challenged rats reveal the immune system shifting to a mainly a Th2 response, which was eliminated by knocking out leukotriene-C_4 synthase (LTC_4S) [16]. In addition, LTC_4S null mice did not develop AHR subsequent to OVA challenge suggesting changes in airway function are cysteinyl leukotriene dependent [16]. Cysteinyl leukotriene receptor

antagonists such as montelukast are currently in use clinically as a prophylactic in a maintenance treatment of chronic asthma**.** Similarly to allergic asthma, asthmatic phenotypes have been described without allergic sensitization in response to inhalation of particular irritants, a subtype of asthma termed irritant-induced asthma [17].

1.2 Irritant-induced asthma

Irritant-induced asthma (IIA) is a non-immunologic asthmatic syndrome that can result from single or multiple dose exposures to irritants [17]. Exposure to a single, high dose of irritants can produce an acute form of asthma with persistent airway hyperresponsiveness and inflammation known as reactive airways dysfunction syndrome (RADS) [18]. Treatment of IIA does not differ from regular asthma therapy including beta-agonists (either short or long acting) as well as corticosteroids to reduce inflammation [19]. The pathogenesis of IIA is not well understood but the absence of a sensitization period or latency following irritant challenge suggests involvement of the innate immune system and/or other cells such as epithelial cells capable of producing and releasing inflammatory mediators.

Transient receptor potential ion channels TRPV1 and TRPA1, which respond to irritant chemicals and are expressed on epithelial cells, can exhibit altered function following irritant exposure, leading to potentiation of their responses and increased calcium influx into the cell [18]. A wide variety of receptors and channels that exhibit altered function due to chronic inflammation and increased oxidative stress may be responsible for the pathogenesis of irritant-induced asthma. Among chemical agents capable of producing IIA, chlorine is of particular concern since it is used in multiple industrial processes with workers at risk for potential exposure [17].

Chlorine, a highly reactive halogen first characterized by Swedish pharmacist Carl Wilhelm Scheele in 1772, has powerful oxidant properties and occurs naturally as a pale yellow-green gas [20]. Due to its reactive nature, chlorine gas is rarely free and is generally produced as a product of direct or indirect oxidation of oxygen [20]. The gas is produced in the US at a rate of 15 million tons per year and used in the manufacturing of chemical compounds, purification of drinking water and bleaching of textiles and paper [21]. Individuals may be exposed to chlorine either in the workplace during the production of these compounds, domestically with the use of cleaning products, chlorine-based bleaches and pool chemicals and as a chemical weapon during warfare or terrorist attacks [22].

1.3 Chlorine Gas Exposure to Humans

Acute exposure levels to chlorine gas are difficult to measure although exposure is typically to high concentrations, and has been associated with the development of acute lung injury (ALI) leading to adult respiratory distress syndrome (ARDS) largely due to its corrosive and oxidative capabilities [19]. Humans are capable of detecting chlorine gas at levels ranging from 0.2 to 3.5ppm depending on an individual's sense of smell and concentrations as low as 1-3ppm can irritate mucus membranes of the nasal passages and airways with 15ppm producing pulmonary symptoms such as pulmonary edema and airway constriction [21] [22]. Susceptible persons, including smokers and individuals with airway hyperresponsiveness, may be more responsive to chlorine gas inhalation and experience pulmonary dysfunction at chlorine 1 ppm, which is within acceptable limits of workplace exposure [22] [23].

Immediate typical symptoms include burning of mucous membranes leading to coughing and choking [24]. Chlorine may displace oxygen gas as it disperses leading to asphyxiation due to hypoxia. Delayed-onset non-cardiogenic pulmonary edema has been documented in chlorine-exposed patients, which may progress to ARDS, a form of hypoxemic lung failure [24]. Mechanical ventilation to ensure proper gas exchange with increasingly higher levels of oxygen has been typical for treatment of acute symptoms of chlorine exposure [24]. Chlorine gas is known to produce oxidative stress and symptoms following chlorine inhalation may be exacerbated as increasing percentages of oxygen gas are administered to the patient, further depleting their anti-oxidant defenses. Clinical presentations of patients have been documented following industrial or transportation incidents. Data compiled from patients treated for acute symptoms following chlorine gas in 2005 in South Carolina highlight a dose dependence of the exposure on the severity of clinical progression and the required medical intervention [24]. Effective medical care requires proper assessment of the severity of symptoms and the patient's type of exposure. Interestingly, once exposed persons survive the immediate event acute lung injury has a good prognosis [25].

Longer-term effects of acute IIA have been studied by assessing individuals, who had an established IIA diagnosis, years following irritant inhalation. Compared to mild asthmatics and healthy controls, IIA patients had increased basement membrane thickness, which correlated inversely with $PC_{20,}$ defined as the provocative concentration of methacholine causing a 20% fall in the forced expiratory volume in 1 second (FEV_1) [26]. This suggests an association between remodeling of tissues other than ASM on the responsiveness of the airways. Heightened responsiveness may be attributable to the

observation of a pro-inflammatory environment in the lungs with eosinophils and neutrophils in the airway subepithelium and lumen, which was significantly higher compared to mild asthmatics and healthy controls [26]. Many of the acute IIA patients had been exposed to chlorine gas. The establishment of a moderately severe asthmatic phenotype subsequent to irritant inhalation necessitates a thorough understanding of the mechanisms of chlorine gas injury in order to identify therapeutic targets.

1.4 Mechanisms of Chlorine Gas Injury

Chlorine gas is soluble in water and reacts quickly with the mucus membranes of the proximal airways of the lung, localizing injury initially to the larger conducting airways. Mathematical modeling of the respiratory airway tree demonstrated with a bolus inhalation method that the majority of nasally inhaled chlorine gas did not penetrate below the upper airways and that virtually none attained the respiratory spaces [27]. The chemical characteristics of chlorine such as ability to undergo hydration separate chlorine gas injury from other non-water soluble irritants such as ozone, which are capable of penetrating diffusely throughout the airway tree. Hydration of chlorine gas with water in the epithelial lining fluid (ELF) produces hydrochloric and hypochlorous acid as described by the equation $Cl_2 + H_2O \leftrightarrow HCl + HOCl$; $2HOCl \leftrightarrow 2HCl + O_2$. Hypochlorous acid is a strong oxidant normally produced by neutrophils, which convert hydrogen peroxide and chloride ion into hypochlorous acid, a reaction catalyzed by the enzyme myeloperoxidase, as part of the oxidative burst to eliminate pathogens. Measured concentrations of hypochlorous acid within the lung have reached 8mM during high levels of neutrophil activity [28].

The production of strong oxidants induces oxidative stress, which represents an imbalance between the presence of reactive oxygen species and the body's ability to cope with reactive intermediates. Under normal circumstances, cells can adapt to physiological increases in ROS without compromising cellular function. ROS are essential to normal cellular functioning and regulatory checkpoints exist to control their endogenous production such as through the NADPH oxidase (NOX) enzyme system [9]. The cell can cope with oxidative injury with antioxidant catabolic systems or buffering non-enzymatic reactions or by directly repairing reversibly damaged molecules [9].

Impairment of ROS production leaves individuals susceptible to recurrent infections since neutrophils require them for the oxidative burst [9]. This is the case in chronic granulomatous disease, which is characterized by NOX2 mutations and defective innate immunity. Recent evidence in zebrafish demonstrates a chemotactic role for ROS for phagocytes through autocrine or paracrine activation of the tyrosine kinase Lyn and other SRC family kinases [29]. Epithelial injury or infection will lead to the release of chemotactic factors such as IL-8, C5a, and LTB_4, which can trigger NOX-2 dependent H_2O_2 production [9]. Connexin channels that link epithelial cells can facilitate the intercellular propagation of ROS that quickly activates the epithelium [9].

Chlorine gas inhalation induces oxidative stress primarily in the apical surface of the epithelium seen using oxidative stress markers such as 3-nitrotyrosine [30]. It also recruits neutrophils, which may be driven by the release of epithelial cell derived mediators in response to oxidative stress [31]. As mentioned previously neutrophils can produce superoxide and hypochlorous acid through the myeloperoxidase enzyme system to kill pathogens [9]. However, it is plausible that the recruitment of inflammatory cells

such as neutrophils, which produce endogenous ROS, following the initiation of an inflammatory response may be contributing to airway injury. In particular, rapid depletion of glutathione levels following hypochlorous acid exposure will leave the bronchial epithelium susceptible to oxidative damage from endogenous ROS production [32]. Administration of intraperitoneal antioxidants such as dimethylthiourea (DMTU) immediately following chlorine exposure attenuates airway inflammation, AHR and oxidative stress in the mouse [31]. Chlorine gas rapidly reacts to produce chlorinated adducts and oxidized biomolecules. Injury attenuation with post-exposure administration of DMTU suggests that the injury triggered by the inhalational exposure continues even after the exposure has ended. This implies that the pathogenesis of chlorine-induced lung injury involves secondary mechanisms that are activated following the exposure to chlorine gas. Other candidates that have been hypothesized to drive injury include acute neutrophilia, which is seen shortly after chlorine exposure, and production of oxidants from the airway epithelium through the DUOX1 enzyme cascade [33]. Epithelial cells may also drive the recruitment of neutrophils through the release of chemotactic agents such as IL-8. Airway injury induced by hydrochloric acid in rabbits was attenuated using an anti-IL-8 antibody, suggesting injury is at least partially neutrophil-driven [34]. Therefore, the airway epithelium, the primary layer protecting the airway and responding to inhaled irritants and pathogens, is likely to be important in the pathogenesis of chlorine-induced lung injury. Chlorinated species such as chloramines can be formed as a consequence of chlorine reacting with biological molecules. The production of reactive molecules may trigger self-propagating reactions leading to an inflammatory response, which may exacerbate damage to the airway epithelium.

The benefit of antioxidant therapy following chlorine gas inhalation in attenuating lung injury [31] suggests the potential benefit of fortified baseline antioxidant defenses in preventing chlorine lung injury. Prophylactic intravenous administration of antioxidants in the rat 18 hours and 1 hour prior to chlorine inhalation was able to attenuate chlorine-related decreases in bronchoalveolar lavage (BAL) ascorbate levels, decreases in arterial oxygenation and increases in BAL protein levels [35]. The ability of antioxidants to reduce chlorine-induced lung injury both prior to and following gas exposure suggests that oxidants are an important part of the pathogenesis and pathology of the injury [31]. The airway epithelium is the first-line barrier between an agent and the body and therefore therapeutic interventions following accidental exposure would focus on reduction of oxidative stress to prevent increases in morbidity and mortality.

1.5 The Airway Epithelium

The airway epithelium in humans is organized as a pseudostratified layer with several cell types connected via tight junctions to form an impermeable barrier to the contents of the airway lumen. Unique characteristics of the airway such as high partial pressures of oxygen and constant exposure to inhaled allergens, irritants and pathogens have necessitated the development of an epithelial layer with adequate defense systems. In particular, antioxidant and immune defense mechanisms are well developed in the airway epithelium, which has a robust regenerative capacity. In order to achieve epithelial regeneration, a series of coordinated responses from other tissues such as airway smooth muscle and extracellular matrix is required. Epithelial wound repair and closure is discussed in further detail below.

The epithelial layer can respond to a variety of environmental insults through its capacity to counteract oxidants and by the release of growth factors. Antioxidant defense mechanisms include enzymes such as glutathione peroxidase, superoxide dismutase and catalase [31]. Other molecules such as glutathione (GSH), thiocyanates (SCN^-) and ascorbic acid also contribute to combating oxidative stress. In particular, glutathione, an antioxidant tripeptide, is stored in large quantities within epithelial cells and can be released in the presence of oxidative insults. Glutathione can cycle between a reduced form (GSH) and oxidized form (GSSG) with the assistance of glutathione peroxidase and glutathione reductase, respectively. In its reduced form (GSH), it can be conjugated to electrophilic sites that are present on particles in tobacco smoke, chlorine gas and carbon particles present in air pollution [36]. The enzymes responsible for this process are glutathione-s-transferase, with the pi subfamily form predominantly expressed in the cytosol of the lung epithelial cells [36]. Low oxidized glutathione levels are also maintained with additional antioxidant enzyme systems such as thioredoxins, thioredoxin reductases, peroxiredoxins and methionine sulphoxide reductases [9]. Other mechanisms to maintain a low concentration of oxidized glutathione include the extracellular export of oxidized glutathione [9]. Normally, GSH and SCN are also secreted into the epithelial lining fluid (ELF) through the CFTR channel and, therefore, mutations in this channel such as occur in cystic fibrosis can compromise an individual's ability to modulate both endogenous and exogenous oxidative stress [37].

Epithelial cells also trigger transcription of genes and synthesis of proteins as part of an antioxidant response to oxidative stress. The master regulator of the antioxidant response is the transcription factor nuclear factor erythroid-derived-2-like 2 (Nrf2), which

is activated under conditions of oxidative stress. Nrf2 is normal present tethered to a cytosolic protein Keap1 (Kelch-like ECH associated protein 1). However, oxidative stress can lead to the Keap1 releasing Nrf2, which has a nuclear localization signal [38]. Once in the nucleus, Nrf2 can bind the anti-oxidant response element (ARE) and trigger transcription of a plethora of antioxidant genes.

Growth factors such as transforming growth factor β (TGF-β) and chemokines including IL-8 are synthesized and released by epithelial cells in response to injury [39, 40]. In particular, the EGFR ligands heparin-binding EGF-like growth factor (HB-EGF), TGF-alpha and amphiregulin (AREG) may drive chemotaxis of inflammatory cells and activation of mitogenic pathways in these cells. Many ligands are present in an inactive pro-form and are released as active ligands following enzymatic cleavage by matrix metalloproteinases (MMPs), which can act on their receptors in an autocrine or paracrine manner [41]. Cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) are eicosanoids that are produced by eosinophils, macrophages, dendritic cells and mast cells from the precursor molecule arachidonic acid through the 5-LO/FLAP pathway. Epithelial cells have the capacity to produce cysteinyl leukotrienes (cysLTs) as well [42]. CysLTs can trigger bronchoconstriction, increase permeability of vessels and trigger inflammation by binding to their receptor cysLTR1, a G-protein coupled receptor. These inflammatory mediators are increased in the mouse following chlorine inhalation [43]. *In vitro* studies have shown that cysLTR1 may transactivate the EGFR through release of HB-EGF (see Figure 1) and trigger downstream CXCL8, an IL-8 homologue in the mouse [44]. Oxidative stress may transactivate the cysLTR1 pathway or directly activate the EGFR in an aberrant fashion [45]. The oxidative stress associated with chlorine gas inhalation, the

ability of cysLTs, which are increased following chlorine gas inhalation, to induce EGFR ligand release, suggests that the EGFR may be activated by chlorine during an acute exposure.

Chlorine gas exposure will elicit histopathological changes in the airways. The time course of these changes was studied using bronchial biopsies taken from a worker who developed RADS following high dose chlorine exposure in the workplace. Biopsies demonstrated rapid denudation of the epithelium, which was replaced with a layer of fibrinohemorrhagic exudate from the submucosa [46]. Desquamation of the epithelial layer persisted fifteen days post exposure although methacholine hyperresponsiveness was attenuated with a regimen of high-dose inhaled steroids [46]. Animal studies with daily administration of dexamethasone (300mg/kg/day) for a week prior to 1500ppm of chlorine gas for 5 minutes showed a reduction in methacholine responsiveness compared with vehicle controls [47]. The abrogation of morphological changes in the airway is seen with administration of dexamethasone to the rat. Glucocorticoids such as dexamethasone can attenuate inflammatory processes, suggesting their importance in airway remodeling.

Limited information is available on the histopathologic time course of RADS. Two case studies of workers exposed to either chlorine gas or isocyanate vapors have documented the histopathological and clinical time course of RADS subsequent to exposure. In the chlorine-exposed water-filtration plant worker, regeneration of basal epithelial cells was identified 60 days post exposure with complete restoration occurring by 150 days post exposure, at which time the inhaled steroid therapy was stopped [46]. Other characteristics that were visualized include thickening of basement membrane and lymphocyte and polymorphonuclear cell infiltration consisting of primarily neutrophils

[46]. Airway hyperresponsiveness occurred two months later with biopsies showing inflammatory cell infiltrate and epithelial regeneration, which was again treated with inhaled corticosteroids. The subsiding of symptoms and normalizing of pulmonary function tests with steroids along with the presence of inflammatory cell infiltrates during both episodes of airway hyperresponsiveness suggests these cells may be responsible for the partial reversibility of histological abnormalities in irritant-induced asthma. A 31-year-old machinist exposed to isocyanate vapors experienced a more severe epithelial damage with very few basal cells remaining with subepithelial airway edema and an infiltration of lymphocytes from a biopsy 45 days post-exposure [48]. Incomplete regeneration of the epithelium with persistent inflammatory cells in epithelium and connective tissue 98 days post-exposure despite returning to his normal state of health [48]. It seems as though reparative mechanisms require a certain level of intactness of the bronchial epithelial cells in order to re-populate the epithelium.

1.6 Epithelial Injury and Repair

Epithelial turnover of cells following injury follows the classic injury-wound healing paradigm. Injury to the airway epithelium will cause loss of epithelial cells as tight junctions and their associated proteins ZO1, occludin and claudins are damaged [49]. The basement membrane, composed of two parallel sheets of laminin and collagen held together by proteoglycans, is damaged in epithelial injury, requiring the formation of a temporary matrix to maintain epithelial barrier function [50].

Epithelial cells adjacent to the site of a wound will undergo cytoskeletal rearrangement and will dedifferentiate into a simple squamous epithelium to re-epithelialize the airway [51]. The dedifferentiation depends on the cell's ability to induce

cytoskeletal changes that break down cellular adhesions and junctions such as adherens junctions and hemidesmosomes. Tight junction proteins have been shown *in vitro* to be regulated by proinflammatory cytokines, suggesting a role in the repair process under inflammation [52]. Migration of squamous cells over the wound is likely dependent on several growth factors including EGF and Trefoil-Factor [53, 54]. Injury to airways with naphthalene has induced loss of a ciliated phenotype and development of a simple squamous epithelium [55]. Additionally, matrix metalloproteinases from the mesenchyme and epithelium are required to help dissolve intercellular and extracellular adhesions. Integrins will allow anchoring of these migrating cells and allow influx of fibroblasts through chemotactic and haptotactic mechanisms [56]. Both types of cells are needed for reconstituting the epithelium and basement membrane layers.

Regeneration of epithelial cell populations is mediated mainly through growth factors including primarily EGF, but also hepatocyte growth factor (HGF), fibroblast growth factor 7 (FGF-7) and transforming growth factor (TGF)-β. Proliferation markers have been identified in several mechanical and inhalation models 24-48h post injury, with the reconstitution of differentiated epithelial cell populations occurring after a period of maximal proliferative activity [55]. Following injury, the EGFR, which is normally predominantly located on the epithelial basolateral membrane, is trafficked to the apical surface, which will enhance receptor activation and trigger repair [57]. The EGFR and its ligands are discussed in detail below.

Fibroblasts and vascular smooth muscle cells can produce FGF-7, a mitogen for several epithelial cells including Clara and type II alveolar cells by binding to the receptor FGFR2 [58]. FGF-7 also increases matrix metalloproteinase 9 (MMP-9) activity.

MMP-9 can enhance the migration of cells and is present in increased concentrations in human ARDS bronchoalveolar lavage fluid [59, 60]. Intraperitoneal or intratracheal administration of FGF-7 in animal models of lung injury can mitigate damage [59]. Bronchial epithelial cells and fibroblasts also synthesize HGF, which has mitogenic and migratory effects on alveolar type II cells [59]. It is believed to work synergistically with FGF-7 as both growth factors are released concurrently and are involved in type II alveolar cell DNA synthesis [61].

TGF-β can be activated following injury through proteolytic and nonproteolytic mechanisms [62]**.** Among its many functions, TGF-β can promote epithelial-mesenchymal transition, increase synthesis of matrix factors by epithelial cells and fibroblasts, enhance the maturation and proliferation of fibroblasts and induce the differentiation of epithelial cells [63]. Excessive TGF-β can result in the formation of fibrotic lesions in the airway.

In a chlorine-induced model of bronchiolitis obliterans (BO), airway fibrotic lesions arose in areas where high-dose chlorine injury (350ppm for 30 minutes) had eliminated the epithelial basal cell populations leading to persistent epithelial denudation and severe respiratory distress [64]. Low-dose chlorine exposure (250ppm for 30 minutes) led to epithelial sloughing of necrotic cells and spreading of basal cells to form a simple squamous epithelium. Normal morphology of the epithelium is restored within 14 days [64]. Epithelial repair mechanisms seem to require at least partial survival of epithelial basal cell populations in order to resolve injury and restore epithelial integrity. In the BO animal model, epithelial cell injury along with depletion of progenitor basal cell populations was required to develop fibrotic lesions. Epithelial injury by itself was

not sufficient to generate persistent pathologic changes, although recurrent injuries may lead to goblet cell metaplasia through the EGFR pathway. Mucus cell metaplasia is a sign of abnormal airway repair that may remain or undergo apoptosis to return to normal epithelial architecture [65]. Normally, in response to injury, necrotic or apoptotic epithelial cells slough off and the basal cells flatten to compensate for epithelial denudation and maintain epithelial integrity. Basal epithelial cells are progenitor cells that self-renew and can repopulate the epithelial layer during instances of injury [49]. A lack of viable basal cells following injury may initiate fibrotic responses that lead to airway remodeling.

The time course of chlorine-induced airway injury has been previously studied in the mouse. Immunohistochemical techniques specific for apoptotic and proliferative cell markers have demonstrated that cellular apoptosis persists for 24h following chlorine exposure [66], despite rapid elimination of chlorine gas from the airways following exposure [27]. Chlorine gas may form chlorine adducts, and reactive oxygen species, which react rapidly with surrounding airway tissues [30]. In addition, the generation of a proliferative signal does not occur immediately after termination of epithelial apoptosis, but rather 24h later at 48h post chlorine exposure. Therefore, it is plausible to hypothesize that the endogenous inflammatory response of the mouse may be in part responsible for the extended resolution of the injury. Neutrophils, lymphocytes or macrophages may play an important role in resolution of injury in the bronchial epithelium.

1.7 Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR) is part of a family of ErbB tyrosine kinase receptors involved in cellular proliferation, survival and metastasis of tumors. Experimental evidence has shown this receptor's involvement in remodeling of airways in an allergic model of asthma [67]. Airway epithelial EGFR levels are elevated in asthmatics suggesting a role for the receptor in pathologically modifying the airway architecture [68]. Several ligands can activate the receptor such as epidermal growth factor (EGF), TGF-α, amphiregulin (AREG), betacellulin, epiregulin and heparin-binding EGF-like growth factor (HB-EGF). EGFR activation triggers downstream signaling through the phosphatidyl inositol 3' kinase/Akt and Ras-Raf pathways [69]. The classical pathway of receptor activation involves release of soluble ligand from its pro-form, which then binds the receptor and triggers dimerization following a conformational change. Dimerization then triggers autophosphorylation of the receptor through its tyrosine kinase domains, which expose docking sites for downstream adaptor proteins SHC, GRB2 and SOS.

Experiments in animal models of allergic asthma have demonstrated the importance of EGFR activation for ASM growth and epithelial remodeling**.** T cells, as part of the late phase response, may drive airway remodeling through the EGFR pathway. Activated T cells have previously been shown to trigger ASM proliferation through cell contact [70]. *In vitro* studies have demonstrated an EGFR-dependent ASM proliferation driven by CD4+ T cells [71]. The mechanism may lie in that the activated T cell increases expression of EGFR ligands such as TGF-α, AREG and HB-EGF [72]. Th17 cells are also particularly important in asthma development and airway remodeling by

exerting effects downstream of EGFR. Adoptive transfer of Th17 cells to sensitized C57BL/6 mice that were repeatedly challenged with ovalbumin highlighted that over-expression of HB-EGF was needed for Th17 dependent airway remodeling [73]. The remodeling processes of the ASM were ablated in the mice using a monoclonal anti-IL17, anti HB-EGF and by inhibiting the EGFR with a specific tyrosine kinase inhibitor [73].

In vitro studies of normal bronchial epithelial cells have demonstrated that cysteinyl leukotrienes can transactivate the EGFR by inducing HB-EGF release [44]. In particular, cysteinyl leukotrienes are also capable of upregulating HB-EGF expression in the epithelium of sensitized Brown Norway rats [67]. The EGFR can be upregulated by TNF-α stimulation and with repeated ovalbumin challenges [74]. Repeated ovalbumin challenges in the BN rat also increased HB-EGF expression [67]. Cysteinyl leukotriene antagonists may also block ASM growth through inhibition of HB-EGF release [67]. The remodeling processes seem to involve the release of cysteinyl leukotrienes, which are also increased in the airway lumen following chlorine gas exposure [43]. The evidence implicating the EGFR-EGFR ligand axis in asthmatic remodeling processes suggest that animal models of irritant-induced asthma may undergo airway remodeling through similar pathways.

The effect of oxidative stress on the epithelium is dependent on the duration and magnitude of the injury and can result in activation of reparative or apoptotic mechanisms. Using hydrogen peroxide as an initiator of oxidative stress, NIH3T3 cells increased Akt activation in an EGFR-dependent manner following stimulation [69]. Oxidative stress is also capable of activating the EGFR independently of ligand in an aberrant fashion [45]. The pro-survival signal associated with Akt may be protective

against oxidative stress-induced apoptosis, suggesting an importance for the EGFR in partially maintaining epithelial integrity and function during cellular stress. In chlorine-induced injury, EGFR activation may represent a line of protection along with antioxidants present in the epithelial lining fluid.

However, in instances where epithelial apoptosis does occur due to significant injury, the EGFR may also play an important role in the repair process. The EGFR and its role in bronchial epithelial repair has been the subject of extensive experimentation. Kim et al suggested a role for EGFR in repair by demonstrating an EGF-driven cell migration and wound closure in confluent airway epithelial layers [54]. Further experimentation highlighted that within 18 hours epithelial repair occurred independently of proliferation [75]. It is plausible to hypothesize that the EGFR may initially drive epithelial repair through wound contraction and inhibition of epithelial apoptosis and, later on, promote restoration of normal epithelial integrity through proliferative mechanisms.

The time course of chlorine gas injury has been documented in the mouse with epithelial cell apoptosis until 24h post exposure, followed by a proliferative signal beginning at 48h post exposure [66]. Rapid elimination of chlorine through its reaction with biological molecules does not support that it is solely driving injury [27]**.** It seems likely that endogenous responses subsequent to chlorine gas may be largely driving epithelial cell injury.

Cigarette smoke extract, which induces oxidative stress, and EGF can experimentally induce the release of interleukin-8, a potent chemoattractant, from bronchial epithelial cells *in vitro* [76, 77]. The increase of pro-inflammatory cytokines in the airways following oxidative stress may exacerbate airway inflammation. Similarly,

the oxidative stress induced by chlorine gas is known to increase the release of IL-8 ortholog KC in the mouse through the EGFR [30]. Therefore, chlorine gas inhalation in the mouse may in fact drive the release of EGFR ligands, which initiate an inflammatory response to injury and recruit neutrophils. Neutrophils are able to generate endogenous ROS and synthesize proteolytic enzymes that will damage the bronchial epithelium. Further investigation is needed to determine whether there is a synergistic role of chlorine gas and neutrophilia in acute chlorine gas injury.

Experiments on bronchial epithelial cells *in vitro* following multiple challenges with ovalbumin demonstrated a cysteinyl leukotriene-dependent remodeling of both airway epithelium and ASM through the EGFR axis [67]. The importance of the EGFR in epithelial remodeling following chlorine injury has not been elucidated although chlorine gas increases cysteinyl leukotriene release in the mouse 24h post-exposure [43]. The transactivation of the EGFR via the cysteinyl leukotriene receptor 1 and matrix metalloproteinase release of EGFR ligand has been reviewed [78].

Paradoxically, it may be that the EGFR is simultaneously exacerbating injury through the recruitment of neutrophils while providing a pro-survival signal. Proliferative signals may also be triggered following wound contraction and attenuation of oxidative stress. It is therefore plausible to hypothesize that EGFR may be driving epithelial proliferation following chlorine gas, which begins only 48 hours post-exposure. The EGFR may be driving an inflammatory response that could be the primary cause of airway injury following an acute chlorine gas exposure. Inhibition of this receptor with specific inhibitors such as gefitinib could conceivably attenuate chlorine-induced lung injury by reducing the subsequent inflammatory response.

Several growth factors and mediators are responsible for normal epithelial repair and regeneration; however, the individual contribution of these factors to airway epithelial repair and function in irritant-induced asthma is not well understood. In particular, the EGFR has been studied in the airways of allergic asthmatics but its role in irritant-induced asthma is unclear. We hypothesize that the EGFR contributes to epithelial repair and regeneration following a single chlorine exposure. The objectives of this study are to study the time courses of changes in epithelial apoptosis and proliferation following chlorine exposure while assessing the effect of chlorine directly on the EGFR and the release of its ligands HB-EGF and AREG in the airway lumen. The effects of EGFR inhibitors prior to chlorine exposure will demonstrate the growth factor receptor's contribution to bronchial airway epithelial repair and regeneration. The effects of EGFR inhibition on airway hyperresponsiveness and inflammation will be studied as well. The study will provide further insight into the physiological basis of epithelial repair mechanisms, which may drive the development of irritant induced asthma.

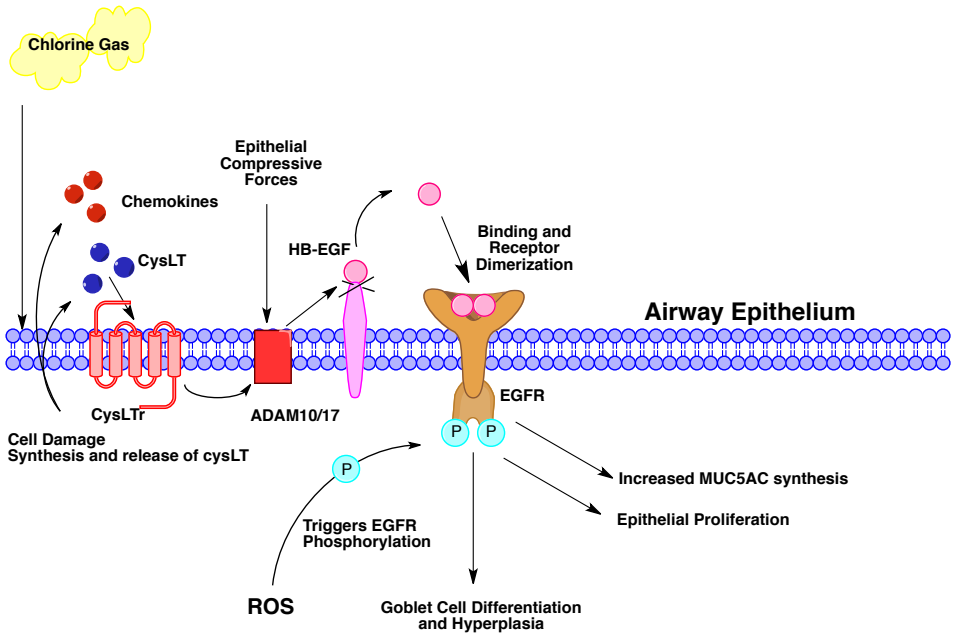


Figure 1: Proposed mechanism of cysteinyl leukotriene receptor 1 (CysLTR1) activation of the EGFR axis mediated by exposure of the airway epithelium to chlorine gas [78].

EGFR can be transactivated by CysLTR1 following cysteinyl leukotriene released either from the damaged epithelial cells or adjacent inflammatory cells. Reactive oxygen species (ROS) can also activate the EGFR independently of receptor ligand. Activation of ADAMs and/or epithelial compressive forces due to bronchoconstriction can lead to the release of heparin-binding epidermal growth factor-like growth factor (HB-EGF), which will bind and activate the EGFR. MMP inhibitors can inhibit the sheddase activity of the ADAMs and, therefore, prevent release of EGFR ligands.

METHODS AND MATERIALS

Chlorine gas exposure

Eight-week-old BALB/C mice (18-22g) from Charles River Laboratories (Wilmington, MA, USA) were housed in an animal facility at McGill University. All protocols used throughout this study were approved by the Animal Care Committee of McGill University and animals were handled according to the guidelines of the Canadian Council for Animal Care. 48 mice were exposed to 100ppm chlorine gas diluted in room air or to room air only for five minutes using the Dynacalibrator and a nose-only exposure chamber. Consistent delivery of this concentration of chlorine gas was done based on manufacturer established gas diffusion values and through adjusting the flow of air through the chamber. The concentration of chlorine gas used was selected to limit injury to the epithelial layers of the airways.

Bronchoalveolar lavage, lung histology and morphometry

Mice were euthanized with an injection of xylazine hydrochloride (8mg/kg) followed by an overdose of sodium pentobarbital (30mg/kg). Microforceps and surgical scissors were used to expose the trachea and, using an 18-gauge cannula, the mice were tracheostomized and bronchoalveolar lavage (BAL) was collected using a 0.1% bovine serum albumin solution in phosphate buffered saline. The BAL fluid was collected and spun in a microcentrifuge for 5 minutes at 3000rpm. The supernatant was kept for further experiments while the cell pellet was resuspended for total cell and differential cell counts using Trypan blue and DiffQuick staining solutions respectively. The left lung was fixed with 1 milliliter of 10% buffered formalin for a period of 24 hours. Following

fixation, the left lung was sliced into three sections (hilar, medio-sagittal, peripheral regions) along with the proximal-distal axis. The lung slices were placed in a tissue processor and processed through solutions of formalin, ethanol, xylene and paraffin. The processed tissue was then embedded in paraffin, cut with a microtome into 5-micrometer thick sections and placed on microscope slides for immunohistochemistry. The right lung was kept for protein and mRNA measurements and was perfused and stored in RNA later solution for 24 hours before use. RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) was used subsequently to extract RNA.

Reserve Transcription and quantitative PCR

The isolated mRNA was transcribed into cDNA using the Super-Script II^{TM} reverse transcriptase (Invitrogen, Burlington, ON, Canada) and Oligo(dT) primers. SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the Applied Biosystems Step One Plus Real-Time PCR System was used for quantitative reverse transcriptase-PCR. Primers were developed using Roche primer design software for ErbB1 (EGFR), HB-EGF and AREG. ErbB1 forward; GACCTTCACATCCTGCCAGT, reverse; GCATGGAGGTCAGTCCAGTT. HB-EGF forward; GACCCATGCCTCAGGAAATA, reverse; AGAGTCAGCCCATGACACCT. AREG forward; GACTCACAGCGAGGATGACA, reverse; CTGTGATAACGATGCCGATG. The PCR cycle was divided into a holding stage (95°C for 10 min), a cycling stage (45 cycles at 95°C for 15s, 60°C for 30s, 72°C for 25s) and a melting curve stage (95°C for 15s, 60°C for 45s, 95°C for 30s). Data analysis was performed using StepOne software to obtain the RQ with S9 used as an endogenous control.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunostain

Slides were heated in a 60°C oven for 30 min and passed through baths of xylene, 100% ethanol, 95% ethanol, 70% ethanol and TBS to deparaffinize tissue sections. Approximately, 60µl Proteinase K diluted to 20µg/ml to each section for 15 minutes at room temperature. Hydrogen peroxide was used to quench the reaction and then washed twice. Equilibration buffer was applied (75µl per section) and then TdT enzyme was applied to each section left to incubate in a humidified chamber at 37°C for 1 hour. Stopping buffer was then applied followed by the secondary substrate anti-digoxigenin conjugate for 30 minutes at room temperature. Color was developed using peroxidase substrate for 3-6 minutes at room temperature following by a methyl green counterstain.

Proliferating cell nuclear antigen (PCNA) immunostain

Tissue sections were treated as above to deparaffinize them. Antigen unmasking was done with 0.15M sodium citrate pH 6 solution in a pressure cooker for 8 minutes at 100°C. The membranes were then permeabilized using 0.2% Triton X-100, blocked with protein blocking solution and then incubated with primary antibody overnight. Secondary antibody was applied after three washes of TBS for an hour at room temperature in a humidified chamber. To visualize the stain, streptavidin was added for 45 minutes, following by Vector-Red alkaline phosphatase substrate solution for 15 minutes. Slides were counterstained with methyl green and mounted with cover slips. Positive nuclei were quantified and averaged for 6 airways per section with a total of six sections being evaluated under blinded conditions and normalized to the length of the basement membrane.

Quantification of proliferation and apoptosis

Immunostained sections were visualized by light microscopy (Leica Microsystems, Richmond Hill, ON, Canada) and six airways were analyzed using pre-set calibration settings at 10x magnification. The number of immunoreactive positive nuclei was counted and corrected for the length of basement membrane that was also measured using commercial software (Media Cybernetics, MD, USA). The average of the normalized positive nuclei/mm of basement membrane was taken from the six airways and counted for n = 1. This process was repeated for a group of six animals.

Inhibitor administration

Gefitinib (CAS 184475-35-2, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 100mg/ml. A diluted concentration of gefitinib and 1% DMSO was administered to the mice via oral gavage (50mg/kg) at a concentration of 20mg/ml in a 500µl 1% carboxymethylcellulose ddH_2O solution 1 hour prior to Cl_2 gas exposure. Control mice were similarly administered the vehicle.

Methacholine responsiveness

Mice were anesthetized with an injection of xylazine hydrochloride (8mg/kg) followed by sodium pentobarbital (30mg/kg). Microforceps and surgical scissors were used to expose the trachea and, using an 18-gauge cannula, the mice were tracheostomized and attached to the small animal ventilator. A full total lung capacity maneuver of 30 cm H_2O was performed prior to each set of doses to ensure an adequate seal for total resistance and elastance measurements using the single compartment model of respiratory system mechanics. The animal was paralyzed with an intraperitoneal injection of pancuronium

bromide (0.2 mg/kg i.p.) and was ventilated at a frequency of 150 breaths per minute with a positive end expiratory pressure of 3cm H_2O. Methacholine was administered using an in-line nebulizer (Aeroneb Lab, standard mist model, Aerogen Ltd, Ireland) at progressively doubling concentrations from 6.25mg/ml to 50mg/ml for 10 seconds, synchronized with inspiration. A coefficient of determination of greater than 0.85 was applied to determine the acceptability of the model fit to data.

Western Blot

Proximal (parahilar) right lung tissue was homogenized with a tissue processor for 3 minutes in protein extraction buffer 50mM TrisHCl (pH 8), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS. The lysate was centrifuged at 13500 RPM for 30 minutes. Protein supernatant was collected and protein content was quantified using a Bradford Assay (BioRad, Hercules, CA, USA). 20µg of protein diluted in dH_2O and loading dye was boiled for 6 minutes with 30µl of each sample being loaded into the separating gel. The gel was run for 1 hour at 120V and then transferred to a PVDF membrane (BioRad, Hercules, CA, USA) where it was blocked for 1 hour with 2% BSA in Tris-buffered saline (TBS) at room temperature. The membranes were incubated overnight with total-EGFR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GADPH (EMD Millipore, Germany) primary antibodies that were diluted 1:500 in TBS containing Tween-20 (TBS-T) at a temperature of 4°C. Swine anti-rabbit antibodies diluted 1:5000 in TBS-T were applied to the membranes for one hour at room temperature. Western Blots were visualized using the ECL Plus Western Blot detection system (BioRad, Hercules, CA, USA).

Statistical Analysis

One-way analysis of variance was used to determine the effect of time on the dependent variables. The Newman-Keuls test was used to test for significance of differences among multiple means. A $p < 0.05$ level was set for significance.

RESULTS

Chlorine gas increases EGFR mRNA expression in parahilar mouse lung tissue

We exposed BALB/C mice to 100ppm of chlorine gas for 5 minutes and assessed EGFR expression in the proximal lung tissue. The expression of EGFR mRNA, measured by RT-qPCR, was increased at 2h and 24h chlorine exposure relative to air-exposed controls (Figure 2). Although increases in expression at 6h post chlorine exposure were not significantly different from controls, there were significant increases in EGFR expression between 6h and 24h post-chlorine exposures (Figure 2). BALB/C mice treated with an intraperitoneal injection of DMTU had reduced expression although not statistically significant ($n = 1$). We also assessed temporal expression of EGFR protein levels in proximal lung tissue following chlorine gas exposure. Total EGFR expression was detected using a monoclonal antibody and also quantified by normalizing samples to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading control. Two predominant isoforms of EGFR were detected using Western Blot with the greater molecular weight band of 170kD used for quantification analysis. Visually, a slight decrease in EGFR protein expression is seen at 2h following chlorine exposure compared to air-exposed controls (Figure 3a). Protein levels returned to air-exposed animal levels at the later time points of 6h and 24h (Figure 3a). Intraperitoneal administration of DMTU 1h prior to chlorine exposure did not significantly alter total EGFR protein levels (Figure 3b). Comparative densitometry analysis of EGFR bands normalized to their corresponding GADPH controls (Figure 3c) did not demonstrate significant changes in protein levels throughout at any time points.

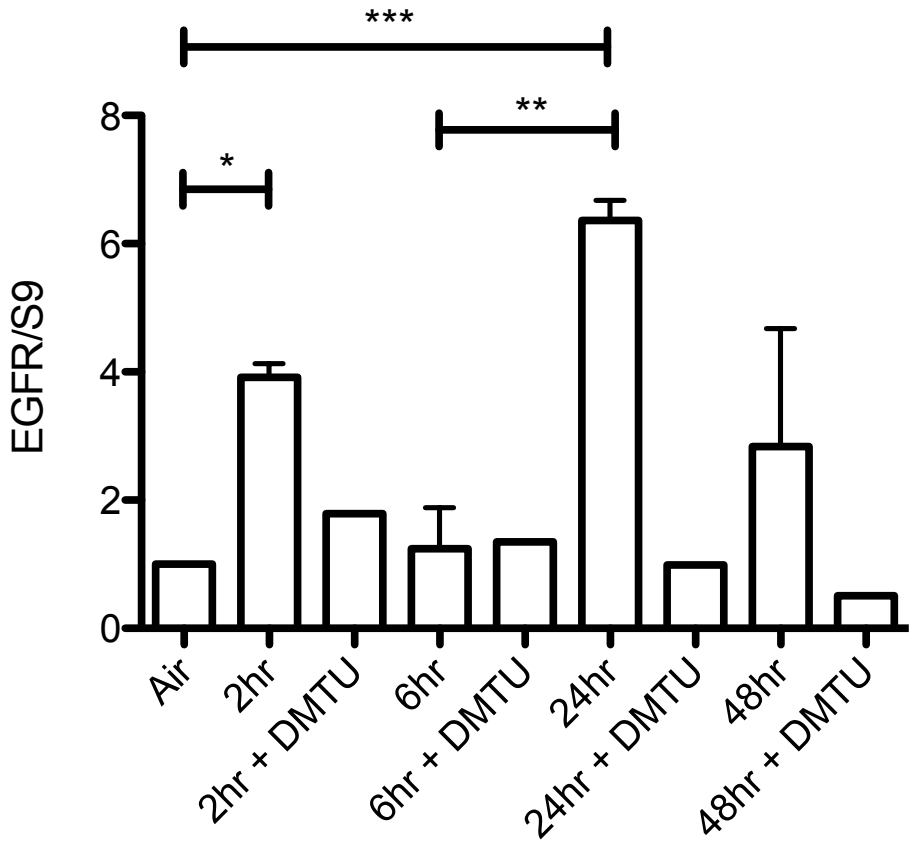
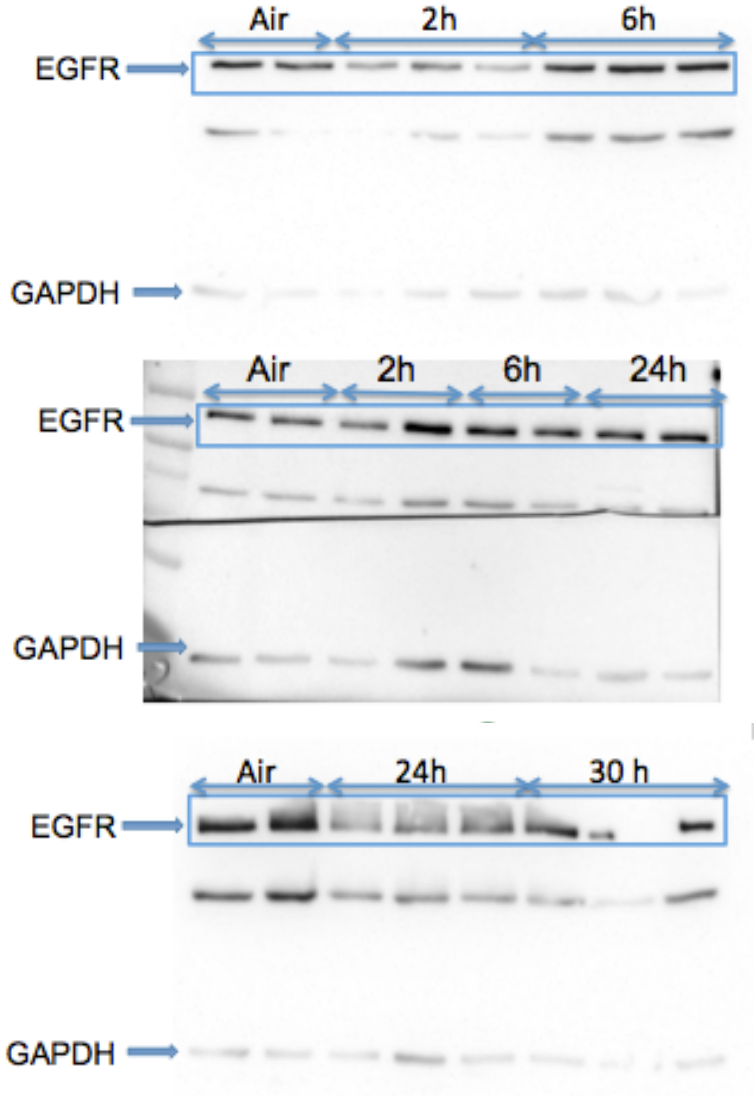


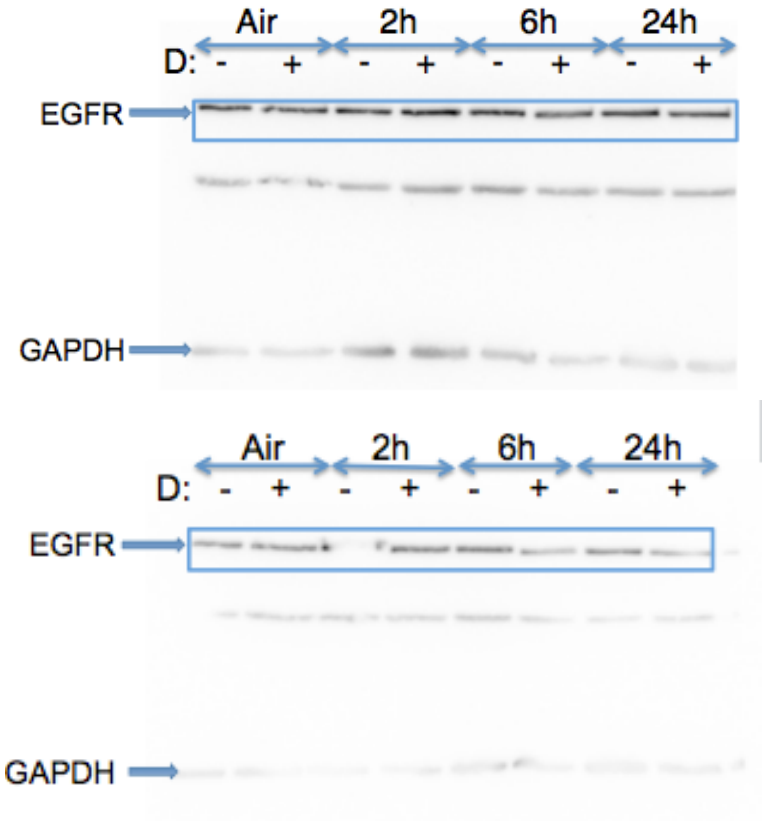
Figure 2: Chlorine gas increases EGFR mRNA expression in parahilar mouse lung tissue.

BALB/C mice were exposed to 100ppm of chlorine gas for 5 minutes. Lung tissue was harvested at 2, 6, 24 and 48h post chlorine exposure to assess EGFR mRNA transcription in the proximal lung regions. Mice were either treated with the antioxidant DMTU (n = 1) 1h prior to chlorine gas exposure or were untreated prior to exposure (n = 6). The expression of EGFR mRNA was measured by RT-qPCR using the S9 gene as our endogenous control. All statistical differences between groups were analyzed with a one-way ANOVA. *$p<0.05$, **$p<0.05$, ***$p<0.05$ n = 6 per group.

A) Total EGFR Western Blot time course following Cl_2 exposure



B) Total EGFR Western Blot with and without DMTU administration



C) Pooled Western Blot Densitometry Analysis

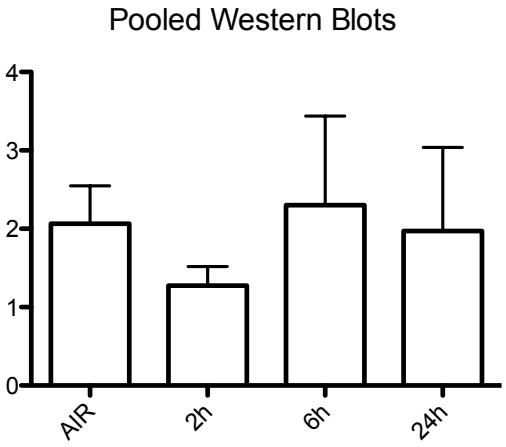


Figure 3: Chlorine gas does not affect EGFR expression in mouse parahilar lung tissue.

(a) Temporal expression of total EGFR protein was assessed at 2, 6 and 24h following chlorine gas exposure. (b) Temporal expression of total EGFR protein was assessed at the above mentioned time points either with or without intrapertioneal dimethylthiourea (DMTU) administration 1h prior to exposure. Total EGFR was detected using a monoclonal antibody and expression was reported by normalizing densitometry-quantified samples to their respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading controls. (c) Comparative densitometry analysis of EGFR bands normalized to their corresponding GADPH controls (n = 4 per group).

Oxidative stress increases the release of HB-EGF in Balb/c mouse airways following chlorine gas exposure

The effects of oxidative stress were also studied through intraperitoneal administration of antioxidant DMTU (100mg/kg) 1 hour prior to chlorine exposure. Using an enzyme-linked immunosorbant assay (ELISA) specific for murine HB-EGF, the temporal expression of EGFR ligand HB-EGF was measured at 2, 6, 24 and 48h post chlorine exposure. We observed increases in HB-EGF following chlorine exposure relative to air-exposed BALB/C mice at each time point (Figure 4). The increase in BAL HB-EGF was greatest at 6h, and decreased gradually until 48h where it was still greater than air-exposed animals (Figure 4). The attenuation of these increases in HB-EGF following exposure with intraperitoneal DMTU administration 1h prior to chlorine exposure at each time point (Figure 4) suggests an oxidative stress dependent mechanism underlying HB-EGF release into the airway lumen.

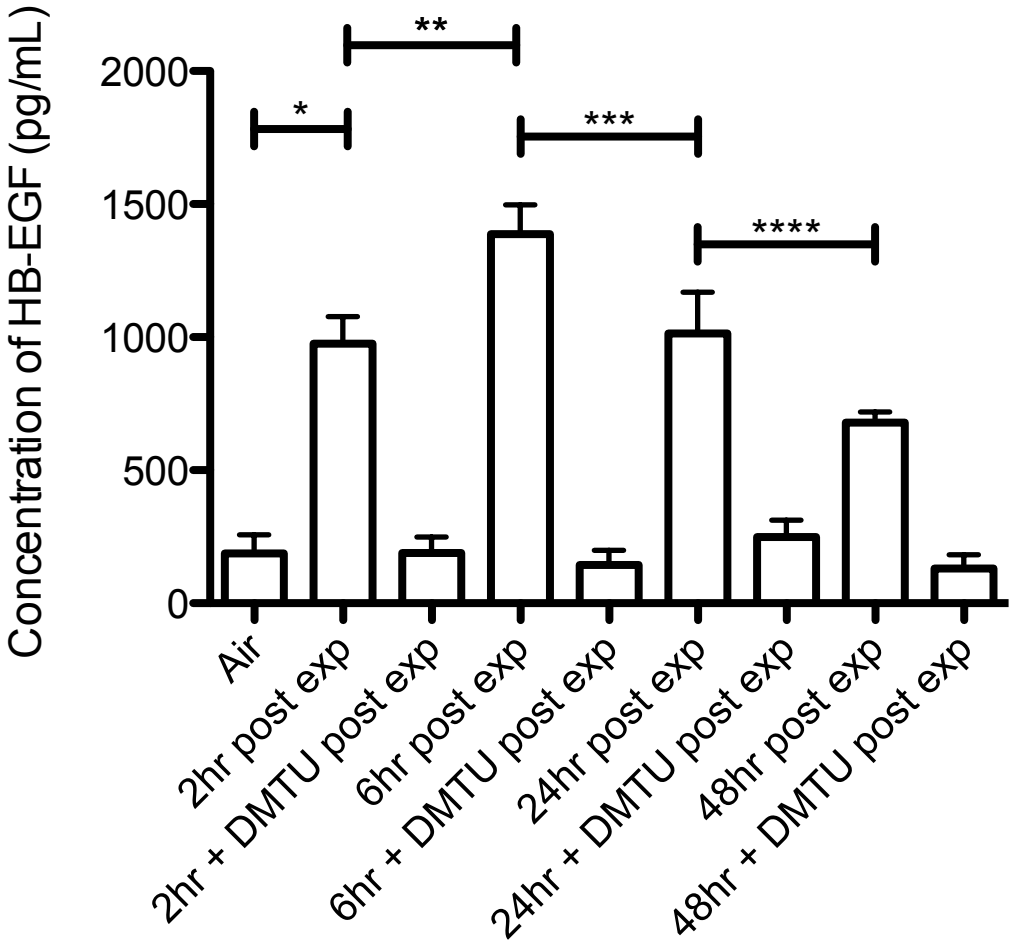


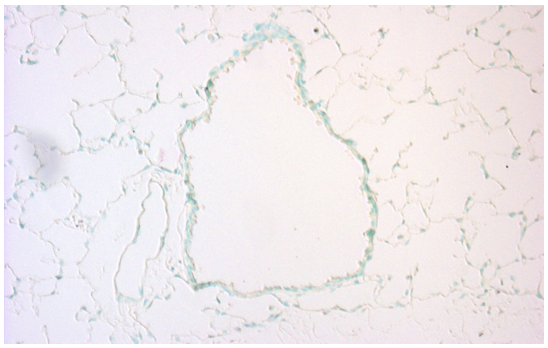
Figure 4: Oxidative stress increases the release of HB-EGF in Balb/c mouse airways following chlorine gas exposure.

BALB/C mice were exposed to 100ppm of chlorine gas for 5 minutes. A bronchoalveolar lavage (BAL) was performed at 2, 6, 24 and 48h post chlorine exposure to quantify HB-EGF ligands released into the airway lumen. Mice were either treated with the antioxidant DMTU (100mg/kg) 1 hour prior to chlorine gas exposure or were untreated prior to exposure. HB-EGF was measured using an enzyme-linked immunosorbant assay (ELISA) specific for murine HB-EGF. All statistical differences between groups were analyzed with a one-way ANOVA. *p <0.05, **p < 0.05, ***p < 0.05 n = 5 per group.

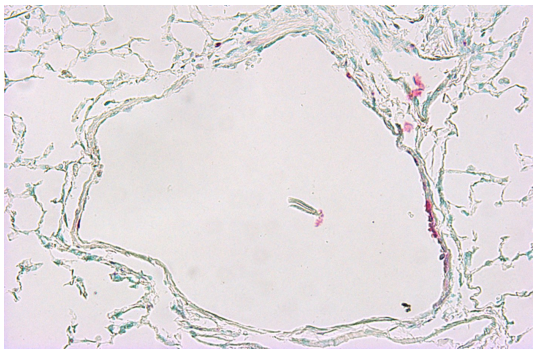
Bronchial epithelial proliferation and apoptosis occurs following chlorine exposure with proliferation being partially inhibited with prior gefitinib administration

The role of the EGFR in proliferative and apoptotic responses subsequent to chlorine gas exposure to mice was studied. We assessed the temporal course of bronchial epithelial proliferation and apoptosis in BALB/C mice. Proliferating cell nuclear antigen (PCNA) was used as a marker of cell proliferation using immunohistochemical staining techniques on 5μm paraffin-embedded sections of airways of the mouse. Nuclei that were immunoreactive for PCNA were counted in 6 airways per section and normalized to the perimeter of the basement membrane that was measured using commercial software. While there were negligible numbers of immunoreactive nuclei in air exposed animals, there were significantly increased numbers of immunoreactive nuclei in the bronchial epithelium of BALB/C mice at 48h post chlorine exposure, consistent with previously published results (Figure 5). This increase in immunoreactive nuclei was attenuated at 48h post chlorine exposure if gefinitib (50mg/kg) was given by oral gavage one hour prior to exposure (Figure 5). Apoptotic load was quantified using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunostaining on 5μm paraffin-embedded sections of airways of the mouse. Quantification of immunoreactive nuclei was performed similarly to the PCNA immunostaining quantification. Chlorine exposure did induce epithelial apoptosis in BALB/C at 6h post chlorine exposure relative to controls (Figure 6). Gefitinib (50mg/kg) administration 1h prior to chlorine exposure did not attenuate apoptotic load, which remained significantly greater than in air-exposed BALB/C controls.

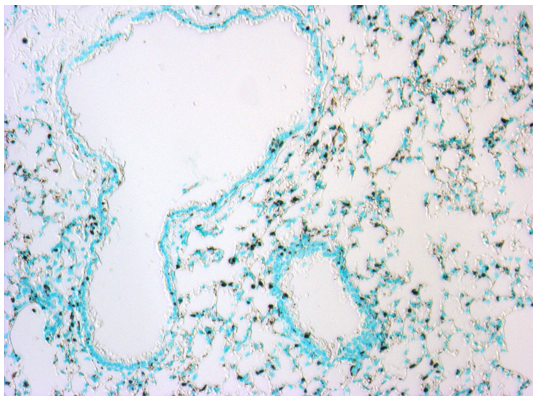
A. Air exposed 10x



B. 48 hours post chlorine exposure 10x



C. 48 hours post chlorine exposure + gefitinib (50mg/kg) 10x



D. Normalized counted PCNA positive nuclei counts

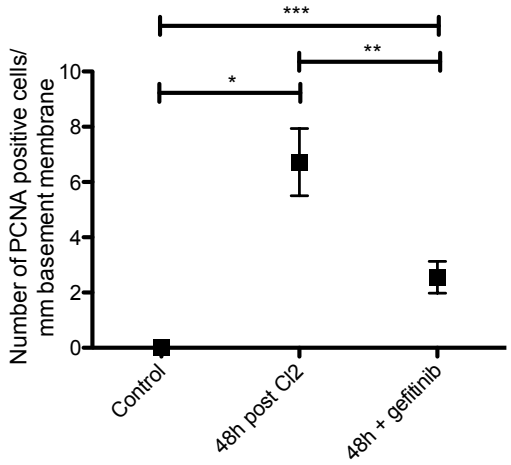
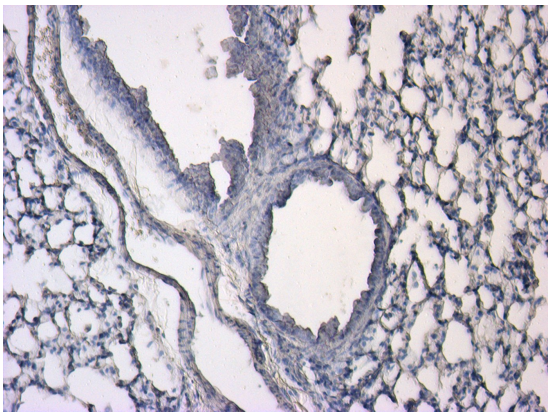


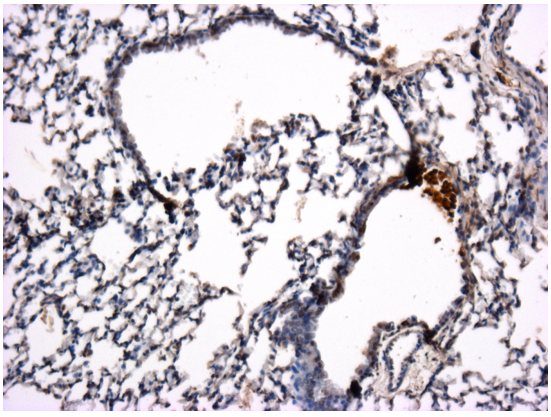
Figure 5: Epithelial proliferation occurs 48h after chlorine exposure and is inhibited by prior gefitinib administration.

Epithelial cell proliferation was assessed with immunostaining for proliferative cell nuclear antigen (PCNA) in 5μm thick sections of airways of the mouse. Nuclei that were immunoreactive for PCNA were counted in 6 bronchial airways per section and normalized to the length of the basement membrane in millimeters (mm) that was measured using commercial software from air exposed mice (A), mice 48h following chlorine (B), or mice 48h following chlorine but treated with gefitinib (50mg/kg) 1h prior to exposure (C). All statistical differences between groups were analyzed with a one-way ANOVA. *$p<0.05$, **$p<0.05$, ***$p<0.05$ $n = 6$ per group.

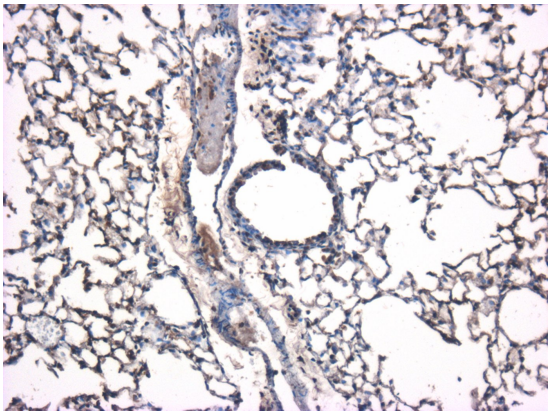
A. Air exposed 10 x



B. 6 hours post chlorine exposure 10 x



C. 6 hours post chlorine exposure + gefitinib (50mg/kg) 10x



D. Normalized TUNEL positive nuclei counts

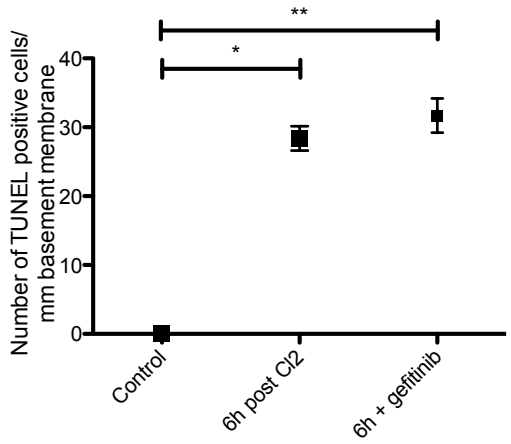


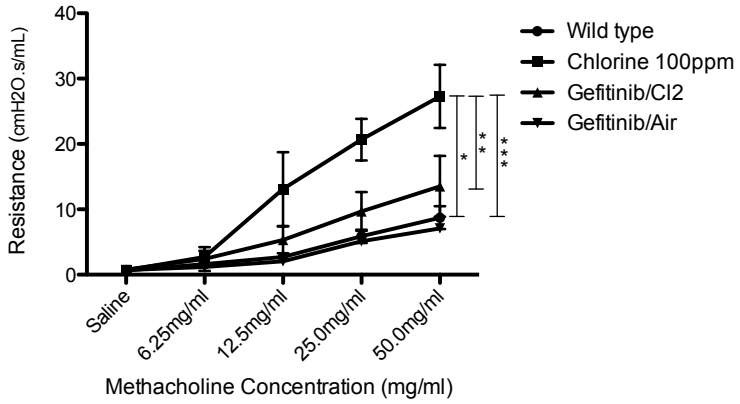
Figure 6: Gefitinib administration prior to chlorine does not affect bronchial epithelial apoptosis.

Bronchial epithelial apoptosis was assessed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in 5μm thick sections of airways of the mouse. Nuclei that were immunoreactive for TUNEL were counted for 6 bronchial airways per section and normalized to the length of the basement membrane in millimeters (mm) that was measured using commercial software from air exposed mice (A), mice 6h following chlorine (B) or mice 6h following chlorine but treated with gefitinib (50mg/kg) 1h prior to exposure (C). All statistical differences between groups were analyzed with a one-way ANOVA. *p <0.05, **p<0.05, n = 4 per group.

Gefitinib and DMTU inhibit airway hyperresponsiveness in BALB/C mice following chlorine exposure

Airway mechanics were measured 24h following chlorine exposure using a small animal ventilator. Respiratory system resistance (Rrs) and elastance (Ers) were measured using the single compartment model of the lung. Methacholine was nebulized for 10 seconds and delivered to the animal during inspiration in increasing concentrations of 6.25, 12.5, 25 and 50 mg/ml. Chlorine-exposed animals demonstrate clear airway hyperresponsiveness (AHR) relative to BALB/C air-exposed controls (Figure 7), demonstrating increases in respiratory resistance and elastance in response to a methacholine challenge. Gefitinib administration 1h prior to chlorine exposure attenuated the increases in resistance evoked by methacholine challenge in BALB/C mice following chlorine gas exposure (Figure 7). However, gefinitib did not significantly attenuate the increases in elastance subsequent to chlorine exposure. Similarly, the role of oxidative stress in the development of AHR was also studied. DMTU administration 1h prior to chlorine exposure also attenuated increases in resistance following a methacholine challenge in BALB/C mice following chlorine gas exposure (Figure 8), but the increases in elastance were not diminished.

A. Total Respiratory System Resistance Following A Methacholine Challenge



B. Total Elastance System Resistance Following A Methacholine Challenge

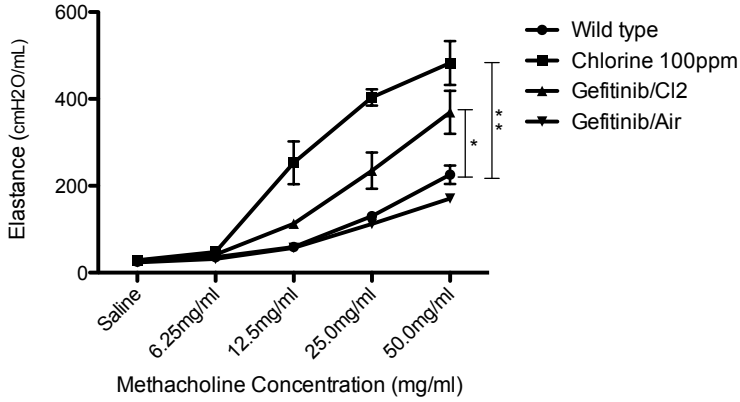
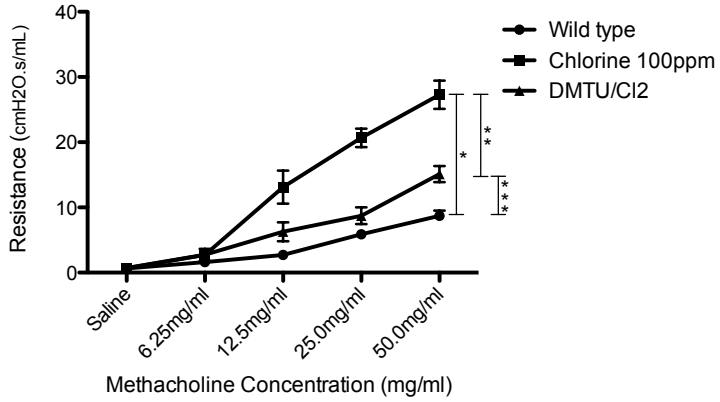


Figure 7: Gefitinib inhibits airway hyperresponsiveness in BALB/C mice following chlorine exposure.

Airway mechanics were measured in BALB/C mice 24h following 5 minutes of 100ppm chlorine exposure using a small animal ventilator. Respiratory system resistance (Rrs) and elastance (Ers) were estimated using the single compartment model. Methacholine was nebulized for 10 seconds and delivered to the animal during inspiration in increasing concentrations of 6.25, 12.5, 25 and 50 mg/ml. BALB/C mice were either exposed to air, chlorine gas or chlorine gas preceded by geftinib administration 1h prior to exposure. All statistical differences between groups were analyzed with a one-way ANOVA. *p <0.05, **p <0.05, ***p <0.05 n = 6 per group.

A. Total Respiratory System Resistance Following A Methacholine Challenge



B. Total Elastance System Resistance Following A Methacholine Challenge

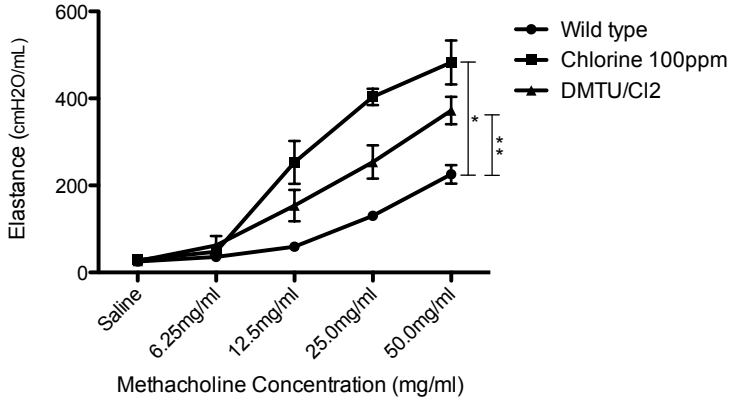


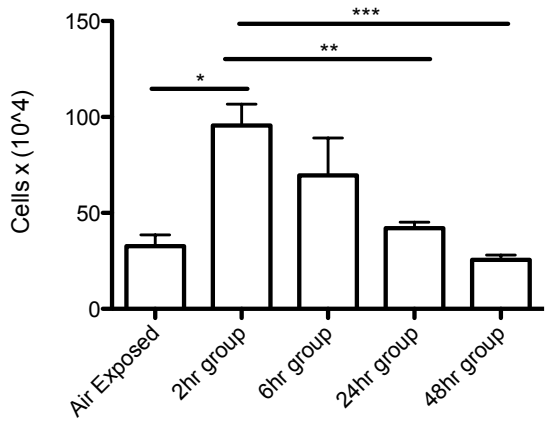
Figure 8: DMTU inhibits airway hyperresponsiveness in BALB/C mice following chlorine exposure.

Airway mechanics were measured in BALB/C mice 24h following 5 minutes of 100ppm chlorine exposure using a small animal ventilator. Respiratory system resistance (Rrs) and elastance (Ers) were estimated using the single compartment model. Methacholine was nebulized for 10 seconds and delivered to the animal during inspiration in increasing concentrations of 6.25, 12.5, 25 and 50 mg/ml. BALB/C mice were either exposed to air, chlorine gas or chlorine gas preceded by DMTU administration 1h prior to exposure. All statistical differences between groups were analyzed with a one-way ANOVA. *p <0.05, **p<0.05, ***p<0.05 n = 6 per group.

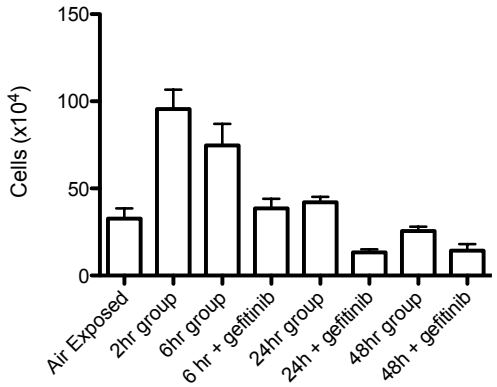
Chlorine gas induces the influx of inflammatory cells into the airways of mice with neutrophil influx being partially dependent on the EGFR.

Inflammatory cell counts were performed at the various time points following chlorine exposure. Following a BAL and creation of cytospin slides, the total number of inflammatory cells in the airways of mice at 2h following chlorine exposure was increased relative to air-exposed BALB/C controls (Figure 9). There was a progressive decline in total inflammatory cells at 6h, 24h and 48h, respectively (Figure 9). Gefitinib administration (50mg/kg) 1h prior to chlorine exposure did not significantly alter total inflammatory cell counts at the previous time points. Neutrophil BAL numbers were increased at 2h following chlorine exposure, with a peak measured at 6h along with a subsequent reduction at 24h following chlorine exposure (Figure 9). Gefitinib administration (50mg/kg) prior to chlorine challenge attenuated neutrophilia at 6h and 24h post exposure relative to air-exposed BALB/C controls (Figure 9).

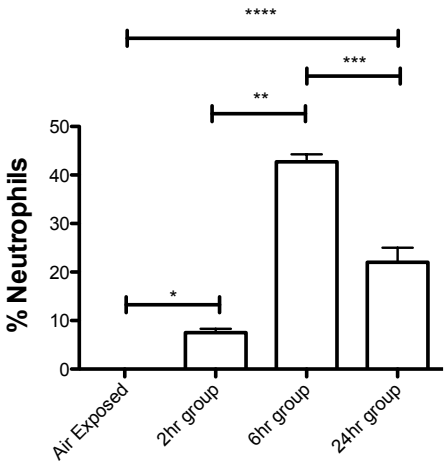
A. Total Cell Counts Following Cl_2 exposure



B. Total Cell Counts Following Cl_2 exposure with gefitinib



C. Neutrophil Cell Counts Following Cl_2 exposure



D. Neutrophil Cell Counts Following Cl_2 exposure with gefitinib

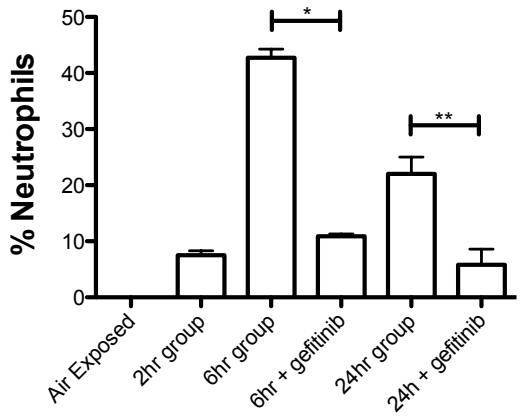


Figure 9: Chlorine gas induces the influx of inflammatory cells into the airways of mice with neutrophil influx being partially dependent on the EGFR.

Assessment of inflammatory cells in the lumen of BALB/C mice was performed using total and differential cell counts at 2, 6, 24 and 48h following chlorine exposure. BALB/C mice were either exposed to air, chlorine gas or chlorine gas preceded by geftinib administration 1h prior to exposure. All statistical differences between groups were analyzed with a one-way ANOVA. *p <0.05, **p<0.05, ***p<0.05, ****p<0.05 *n* = 6 per group.

DISCUSSION

This study describes the time-dependent effect of chlorine gas exposure on the EGFR axis in the mouse. Chlorine gas inhalation amplified EGFR mRNA transcription at 6 and 24h following exposure compared to controls. Protein levels of the EGFR were not significantly altered following analysis of Western blots, although a small decrease in EGFR protein may have occurred at 2h post exposure with recovery to control levels at subsequent time points. Recycling of the EGFR at the apical membrane requires mechanisms that are needed for homeostatic control of the cell and will be discussed later. In particular, the mitogenic activity of several EGFR ligands such as HB-EGF requires internalization of the receptor and attenuation of the proliferative signals to the cell. Measurements of HB-EGF in the bronchoalveolar lavage fluid (BALF) revealed increased levels of HB-EGF following chlorine with a peak at 6h following exposure. Interestingly, HB-EGF levels in the airway lumen were reduced if an antioxidant was given to mice prior to challenge with chlorine gas, suggesting an oxidative stress mediated release mechanism. A tyrosine kinase inhibitor specific for the EGFR, gefitinib, attenuated epithelial proliferation seen at 48h post chlorine exposure, implicating the EGFR axis in regenerative mechanisms of the airway epithelium. However, bronchial epithelial cell apoptosis at 6h post-chlorine was not reduced with gefitinib, which we hypothesized would occur due to the pro-survival pathways downstream of the EGFR. Systemically, we showed the gefitinib was capable of attenuating AHR, measured 24h post exposure, and neutrophilia at 6h and 24h post exposure induced by chlorine gas inhalation. Attenuation of AHR and reduction of neutrophilia was also seen with

administration of DMTU, an antioxidant, 1h prior to exposure, consistent with previously published work [31].

Normal rodent and human airway epithelial cells have a low basal expression of EGFR primarily localized to the basolateral membrane [79]. Stimulation of airway epithelial cells with pro-inflammatory molecules such as cigarette smoke extract and TNF-α as well as oxidative stress can increase EGFR expression on the apical membrane [79]. Following chlorine exposure, we detected a slight decrease in EGFR expression from proximal lung protein extraction although we did not study translocalization of the receptor. We reported an increase in EGFR mRNA expression at 6h and 24h post chlorine exposure, although we did not demonstrate increases in HB-EGF and AREG mRNA in response to chlorine. EGFR ligands are known to be stored in large quantities in an inactive pro-ligand form on the apical membrane, making them available for rapid release through shedding mechanisms. Cigarette smoke and various stimuli inducing oxidative damage have also been reported to increase expression of EGFR and EGFR ligands *in vitro* and *in vivo* [79-82]. Oxidative stress has been implicated in upregulating mRNA transcription of HB-EGF and AREG in a rat gastric epithelial cell line and requires functioning EGFR protein [83]. The importance of the receptor in increasing the growth factor response following stress highlights the need to synthesize new, functioning receptor in instances of cellular injury. Upregulation of the EGFR immediately following injury may mitigate further damage by increasing the potential proliferative signals to the cell. Studies have demonstrated aberrant and irreversible activation of the EGFR following oxidative stress, highlighting the need to eliminate the constitutively activated receptors and synthesize new, normal functioning proteins.

Although mechanisms mediating apical levels of the EGFR have not been well elucidated, it is plausible to consider that they are linked to mechanisms monitoring the functional status of the cell such as the wingless (Wnt)/β-catenin pathway. Epithelial cell injury will cause disassembly of E-cadherin from the cell-cell adhesion complexes, which will allow for stabilization of β-catenin [84]. β-catenin can then translocate to the nucleus under the influence of the Wnt pathway [84]. During dedifferentiation of the epithelial cells, a robust increase in β-catenin has been shown [85] and *in vitro* studies of prostate cancer cells have demonstrated that activated β-catenin is capable of upregulating transcription of the EGFR gene [86]. Evidence suggests that β-catenin can enhance proliferation and survival of cells through upregulation of the EGFR pathway. Oxidative stress may activate the EGFR axis via receptor activation or increasing receptor number on the apical membrane through pathways such as that involving β-catenin following the breakdown of cell-cell junctions. Limitations of our study include not measuring the proportion of activated EGFR by calculating the p-EGFR/EGFR ratio on the apical bronchial airway epithelial membrane in the mouse. This makes it difficult to assess whether oxidative stress dependent EGFR activation was the trigger for EGFR mRNA transcription.

Chlorine gas triggers a substantial release of HB-EGF into the airway lumen from the apical surface of the airway epithelium with highest levels occurring 6h post exposure. We abrogated the release of HB-EGF with administration of an antioxidant 1h prior to chlorine gas, suggesting an oxidative-stress dependent release mechanism in the context of chlorine-induced airway injury. Mechanisms responsible for shedding activity that have been proposed include activation of metalloproteinases and mechanical forces.

Activation of metalloproteinases (ADAMs 9, 10, 12, 17), MMP-3 and MMP-7 can cause release of HB-EGF. Compressive forces on epithelial cells *in vitro* induce EGFR phosphorylation in an HB-EGF dependent manner [87]. Soluble HB-EGF and membrane-bound HB-EGF have differing functions with the former responsible for cell migration and decreased cell-cell adhesions and cell-matrix interactions while the latter does the opposite by promoting cell-matrix, cell-cell interactions and decreasing cell migration [88]. Oxidative stress contributes both to the synthesis of new HB-EGF as mentioned above as well as its release into the airway lumen. Limitations of this study include not studying the temporal release of other EGFR ligands such as AREG and TGF-α and their contributions to EGFR activation in chlorine-induced airway injury.

Deregulation of the EGFR axis has been implicated in oncogenesis and several cancers. Appropriate control of signaling of cellular pathways downstream of the receptor is required to maintain cellular homeostasis. Endocytosis and recycling of the EGFR is a mechanism that is geared towards attenuation of the signal once the receptor is activated or damaged. Activated EGFR is internalized into a multivesicular body through the early endosome. At this stage, the receptor can be recycled to the membrane surface or processed into the lysosome where it will be hydrolyzed into its constituent amino acids [89]. In the context of chlorine-induced airway injury, the EGFR may be constitutively and aberrantly activated independently of ligand due to oxidative stress, which would require internalization of the receptor. In addition, we measured increased HB-EGF levels in the airway lumen at 2h following Cl_2, which can also activate the EGFR and contribute to its internalization subsequent to activation. It is plausible to consider that the slight decrease in EGFR seen at 2h would coincide with increased internalization and

degradation of the aberrantly activated receptor. Intuitively, the mRNA expression seen at 6h post Cl_2 could be a response to increased EGFR degradation. Restoration of EGFR protein levels measured at 6h and 24h to air control levels suggests that activation of EGFR axis may be involved in resolution of airway injury.

At 48h post exposure, we measured epithelial regeneration by quantifying PCNA positive airway epithelial cells. The reduction in epithelial proliferation using gefitinib suggests that the EGFR is contributing to regeneration of the epithelium following resolution of chlorine-induced airway injury. Increased levels of EGFR have been reported in airways of asthmatics and were thought to contribute to airway remodeling [90]. Previous *in vitro* work established a role of the EGFR in epithelial repair where inhibition of the receptor with AG1478 lead to increased levels of fibrogenic mediators such as TGF-β2 following mechanical injury [90]. Evidence suggests that sub-basement membrane collagen thickness correlates with asthma disease severity and hyperresponsiveness. We hypothesized that the inhibition of the EGFR would exacerbate damage and prevent resolution of injury because of anti-apoptotic downstream pathways, which may serve to preserve airway function. This may also lead to excessive collagen deposition in the airways if injury is not resolved, which would compromise airway mechanics. Quantification of apoptosis revealed no change in bronchial epithelial apoptosis with or without an EGFR inhibitor following chlorine gas exposure. This may be attributable to the extreme damage to the epithelium that overcomes the EGFR axis. In addition, other growth factors such as hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) have been proposed as contributors to epithelial repair[59, 62, 91]. Although likely involved, their contributions

and interactions with the EGFR axis require further study. We cannot exclude the possibility that exploring these time points might have revealed differences.

We found attenuation of AHR and neutrophilia with administration of an antioxidant, consistent with published work [31], or an EGFR inhibitor prior to chlorine exposure. Using the single compartment model, both total respiratory system resistance and elastance was significantly decreased compared to chlorine-exposed animals. The exact mechanisms underlying airway hyperresponsiveness are unclear. However, it is plausible to consider that the epithelium, which shows damage following chlorine, is likely involved. Unlike humans, the airways of the mouse do not have a great deal of smooth muscle so perhaps tissues other than ASM may be the driving force for the development of AHR. Damage to the epithelial layer may increase the ability of methacholine to access the ASM layer, increasing responsiveness of the tissue. Decreased epithelial functioning will reduce the capabilities of the airways to produce relaxant substances such as nitric oxide and certain prostaglandins. The contribution of both impaired epithelial barrier and synthetic functions may contribute to the development of AHR. Another potential possibility is AHR is driven by neutrophil influx into the airways following chlorine exposure. The development of neutrophilia may be caused by the release of CXCL1 downstream of the EGFR [92], which has been showed previously. *In vitro* studies have shown that BEAS-2B cells are capable of releasing IL-8 through Nf-κB and AP-1 transcription factors, independently of EGFR [93]. We established that gefitinib was capable of attenuating neutrophilia induced by chlorine gas inhalation, similar to DMTU. It is unclear if gefitinib is inhibiting neutrophilia by reducing CXCL1 release downstream of the EGFR and whether or not it is solely the EGFR that is driving

neutrophil influx. We hypothesized that the influx of neutrophils 6h post chlorine would contribute to airway injury. Release of powerful oxidants such as HOCl and proteases from neutrophils may contribute to epithelial injury and damage. Paradoxically, the activated EGFR may exacerbate the injury by recruitment of neutrophils while providing downstream anti-apoptotic signals to the cells. It would have been important to study the roles of neutrophils in the airways and chemoattractant release to establish a stronger connection. However, other work from our lab group examined the effect of neutrophil depletion on chlorine-induced airway injury using antibodies directed against Gr-1, a marker expressed on neutrophils. Following chlorine exposure, the anti-Gr-1 treated mice did not show increases in total respiratory system resistance but did have similar levels of total respiratory system elastance and tissue damping [94]. Further work needs to be done in establishing the role of neutrophils in the development of AHR, which may provide greater insight into the mechanisms underlying irritant-induced asthma. The chronicity of irritant-induced asthma and its associated symptoms following an acute exposure or several low dose exposures may involve the innate immune response featuring primarily neutrophilic inflammation. The establishment of a causative link between the neutrophil response to inhaled irritants and AHR development would provide a therapeutic target for this specific form of asthma.

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