Oxidative stress and chlorine
induced airway dysfunction

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Abstract

The aim of this work was to examine the effects of chlorine (Cl₂) gas inhalation on airway dysfunction in a mouse model of irritant-induced asthma. Cl₂ inhalation induces airway hyperresponsiveness (AHR), bronchial epithelial cell damage, increased oxidative stress and airway inflammation, characterized by neutrophilia. While the effects of Cl₂ inhalation had been previously characterized, mechanisms driving airway injury induced by Cl₂ remained elusive. We hypothesized that oxidative stress following Cl₂ was pivotally involved in mechanisms leading to airway injury. First, using antioxidants we aimed to further characterize the effects of Cl₂ inhalation by evaluating airway mechanics, pulmonary antioxidant activity, lung inflammatory profiles and bronchial epithelial cell proliferation. We established that administration of antioxidants post-Cl₂ inhalation was effective at ameliorating AHR, oxidative stress, bronchial epithelial cell proliferation, and airway inflammation, suggesting indirect mechanisms were responsible for Cl₂-induced airway damage. Next, we depleted several inflammatory cell types in an attempt to determine which cell type, if any, was contributing to the observed effects of Cl₂. We depleted macrophages, eosinophils and neutrophils and found that only depletion of neutrophils was effective at reducing airway hyperresponsivness and prevented endogenous antioxidant activity as measured by nuclear factor (erythroid-derived 2)-like 2 (NRF2) nuclear translocation. Concomitant experiments established Cl₂ increased the pro-inflammatory lipid mediators cysteiny1-leukotrienes and mRNA levels of cysLT-related biosynthetic genes.
Given the role of cysLTs and their receptor cysLTr1 as established pro-asthma mediators, we chose to explore the role of cysLTr1 in our model by utilizing cysLTr1 deleted mice (cysLTr1-/-). We found that deletion of cysLTr1 worsened measured outcomes following Cl₂. Compared to wild-type mice, cysLTr1-/- demonstrated increased AHR, airway neutrophilia and epithelial cell apoptosis. In contrast to wild-type mice, we observed that cysLTr1-/- did not show nuclear translocation of NRF2 or increased antioxidant mRNA levels following Cl₂ exposure. At baseline levels, we observed that cysLTr1-/- mice had increased e-cadherin protein expression on bronchial epithelial cells compared to wild-type mice. We propose that deletion of cysLTr1 in mice results in an increased susceptibility to Cl₂ induced airway damage and dysfunction and this damage is mediated via an aberrant endogenous antioxidant response. This vulnerability to oxidant damage, likely due to increased neutrophil numbers and NRF2 idleness, may be due to an innate increase in e-cadherin, which is partially mediated via cysLTr1. Finally, we use in vitro studies to examine how exogenous cysteinyl-leukotriene D₄ stimulates bronchial epithelial cells to release the neutrophil chemoattractant IL-8. We found this pathway to be dependent on the activation of the epidermal growth factor receptor. These in vitro studies establish the basis for further examination of specific pathways that may be involved in how epithelial cells may be involved in neutrophil recruitment.
Résumé

L’objectif du travail présenté dans cette thèse était d'examiner les effets de l'inhalation de chlore gazeux (Cl\textsubscript{2}) sur la dysfonction des voies respiratoires chez la souris, un modèle d’asthme induit par un irritant. L’inhalation de Cl\textsubscript{2} induit une hyperréactivité bronchique (HRB), des lésions des cellules épithéliales bronchiques, une augmentation du stress oxydatif et l'inflammation des voies aériennes qui se caractérise principalement par une neutrophilie. Même si les effets de l'inhalation de Cl\textsubscript{2} sont bien caractérisés, en revanche, les mécanismes entraînant les lésions des voies aériennes induites par le Cl\textsubscript{2} sont toujours méconnus. Nous émettons l'hypothèse que le stress oxydatif qui survient suite à une exposition au Cl\textsubscript{2} joue un rôle clef dans l’apparition des lésions des voies aériennes. Tout d'abord, les effets de traitements antioxydants sur l’inhalation de Cl\textsubscript{2} ont été caractérisés en évaluant les fonctions respiratoires, l’activité antioxydante et le profil inflammatoire pulmonaire ainsi que la prolifération des cellules épithéliales bronchiques. Nous avons établi que l'administration d'antioxydants à la suite d’une inhalation de Cl\textsubscript{2} améliore l’HBR, le stress oxydatif, la prolifération des cellules épithéliales bronchiques et l’inflammation des voies respiratoires. Ces résultats suggèrent que des mécanismes indirects sont responsables des lésions des voies respiratoires induites par Cl\textsubscript{2}. Ensuite, afin de déterminer quel(s) type(s) de cellules contribuent, le cas échéant, aux effets du Cl\textsubscript{2}, nous avons effectué une déplétion de plusieurs types de cellules inflammatoires : macrophages, éosinophiles et neutrophiles. Ainsi, nous avons constaté que seule la déplétion des neutrophiles
permet de réduire l’hyperréactivité bronchique et de prévenir l'activation des facteurs antioxydants endogènes, telle que mesurée par la translocation nucléaire du facteur nucléaire (erythroid-derived 2)-like 2 (NRF2). L’exposition au Cl₂ augmente le taux de médiateurs pro-inflammatoires lipidiques cystéinyll-leucotriènes (cysLTs) ainsi que la quantité d’ARNm liés à la biosynthèse de ces médiateurs, dans le poumon proximal. Étant donné que les cysLTs et leur récepteur cysLTr1 sont établis comme étant des médiateurs pro-asthmatiques, nous avons choisi d'explorer le rôle de cysLTr1 dans notre modèle en utilisant des souris mutées pour le gène cysLTr1 (cysLTr1-/-). Ainsi, nous avons constaté que la suppression de cysLTr1 aggrave les résultats obtenus suite à l’exposition au Cl₂. Par rapport aux souris de type sauvage, les souris cysLTr1-/- montrent une augmentation de l’HBR, une neutrophilie des voies respiratoires et une apoptose des cellules épithéliales. Aussi, contrairement aux souris de type sauvage, nous avons observé que les souris cysLTr1-/- ne montrent pas de translocation nucléaire de NRF2 ni d'augmentation des niveaux d'ARNm anti-oxydantes après une exposition au Cl₂. Enfin, nous avons observé que l'expression des protéines E-cadhérine dans les cellules épithéliales bronchiques des souris cysLTr1-/- est augmentée par rapport aux souris de type sauvage. Nous proposons que la suppression de cysLTr1 chez la souris cause une sensibilité accrue aux lésions et aux dysfonctionnements des voies respiratoires induites par le Cl₂. Nous pensons qu’une réponse aberrante des antioxydants endogènes est à l’origine de ces dommages. Cette vulnérabilité aux dommages oxydatifs, probablement causée
par l'augmentation du nombre de neutrophiles et l'absence de translocation NRF2, peut s'expliquer par une augmentation innée de l'e-cadhérine, partiellement contrôlée par cysLTr1. Contrairement à une carence chronique en cysLTr1, une exposition aiguë à LTD₄ des cellules épithéliales respiratoires humaines provoque une cascade d'événements conduisant à la libération d’HB-EGF (Heparin-binding epidermal growth factor), l’activation du récepteur d’EGF et la libération d’interleukine 8. À la fois protecteurs et pro-inflammatoires/régénérateurs, les leucotriènes s’avèrent jouer un rôle complexe dans la réponse de l'hôte à une exposition au Cl₂.
Chapter 1

Introduction and Literature Review
1.1. Introduction and Definition of Asthma

Asthma is an airway disease characterized by inflammation, mucus hypersecretion, airway narrowing, increased airway responsiveness and changes in the structure of the airway wall. For patients, these pathologic features manifest as wheezing, chest tightness, cough and airflow obstruction.[1] While several definitions of asthma have been used since the late 1950’s, the most widely accepted definition was proposed by the Global Strategy for Asthma Prevention and Management. This definition characterizes asthma from the perspective of functional consequences attributed to specific pathologic characteristics. It states: “Asthma is a chronic inflammatory disease of the airways in which many cell types play a role, in particular mast cells, eosinophils and T lymphocytes. In susceptible individuals the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough particularly at night and/or early morning. These symptoms are usually associated with widespread but variable airflow obstruction that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli.”[2] This definition encompasses a variety of asthma subtypes including those driven by immune-mediated responses and typically induced by allergens, as well as non-allergen induced forms including irritant-induced asthma (IIA). However, this definition regards asthma as a “chronic inflammatory disease”, an endotype that fails to
include an important subset of IIA that develops acutely from exposure to various irritants and includes a rapid neutrophilic response.

In the overall spectrum of stimuli that induce asthma pathogenesis, the disease can be divided into allergen-induced and non-allergen induced. While allergen and non-allergen induced asthma subtypes share phenotypic similarities including airway narrowing, hyperresponsiveness, airway inflammation and structural changes, there are critical differences in both pathogenesis and mechanisms that will be introduced.

1.2. Allergic Asthma

1.2.1 Th1-Th2 Cell Skewing

T helper lymphocytes are categorized based on their functional capabilities as well as the profile of regulatory factors they produce including cytokines, growth factors and peptides. Th1 and Th2 subsets develop from a common precursor cell, naïve CD4+ T lymphocytes. Their subsequent differentiation from naïve T cells into either Th1 or Th2 subsets is a function of the type of stimulus presented and of cytokines present during the immune response. Naïve T lymphocytes differentiate into Th2 cells in the presence of IL-4, potentially produced by mast cells, basophils or natural killer T lymphocytes.[3-5] Th2 cells produce IL-4, IL-13 and IL-5, which promote rapid IgE production by B cells, activate and recruit eosinophils and lead to increased mucus production.[6, 7] T helper 1 (Th1) cells secrete IL-2,
interferon gamma (IFN-γ), tumor necrosis factor (TNF) and mediate delayed-type hypersensitivity. The presence of a Th2-dominated immune response is a hallmark of patients suffering from allergen-induced asthma. These individuals usually test positive for serum total immunoglobulin E (IgE) antibodies that typically develop in response to exposure to low levels of allergen, have a positive skin prick test, and are often referred to as being “atopic”. The development of allergic asthma and differentiation of T cell subsets occurs following sensitization in which allergen is introduced presumably through the respiratory tract. Antigen is taken up by antigen-presenting cells (APCs) (dendritic cells, macrophages, eosinophils) in the airways. Antigen-loaded dendritic present antigen to naïve T-cells, and in the presence of IL-4, Th2 cells will develop and are then considered “primed” to that antigen. Subsequent exposure (s) to an allergen in an environment with primed Th2 cells induces a rapid and robust release of IgE, activation of inflammatory cells, and B cell production.

Beyond Th1 and Th2 there is another subset of T cell that influences the nature of the immune response to pathogens. This subset of cells are Th17 cells, named for the main cytokines produced which are IL-17A and F. Th17 cell development occurs in the presence of IL-1β and IL-23 and can be enhanced by IL-6. It seems the primary role of Th17 cells is the recruitment and activation of neutrophils. This recruitment may occur directly, through the production of IL-8. Th17 cells may also recruit neutrophils...
indirectly as IL-17 induces surrounding cells like fibroblasts and epithelial cells to upregulate production and release of neutrophil chemo-attractants including CXCL1, MIP-2, CXCL5, and IL-8.[14] Furthermore, IL-17 induces the upregulation of granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) which promotes neutrophil production.[15]

1.2.2 Inflammation in Allergic Asthma

Many of the cell types that are recruited and/or activated from exposure to allergens are related to mechanisms involving IgE and its two principal receptors, FcεRI, the high-affinity Fc receptor for IgE, and CD23. Because IgE and mast cells are found in high concentrations in mucosal tissues, IgE antibodies are among the first molecules an allergen encounters. This immediate encounter results in IgE forming crosslinking complexes with FcεRI,[16] resulting in mast cell degranulation and hypersensitivity reactions. Numerous mediators including pro-inflammatory cytokines, chemokines and growth factors (e.g., TNF-α, granulocyte-monocyte colony stimulating factor (GM-CSF), IL-1, IL-3, IL-4, IL-5) are released and/or release and synthesis of bronchoconstrictors (e.g histamine, cysteinyl leukotrienes) occur as a result of this degranulation.[17, 18] Additionally, IgE sensitized antigen presenting cells are activated by the presence of allergen which stimulates B cells to increase IgE production and replace any IgE used during the allergic response thus maintaining IgE levels and mast cell sensitization. These responses,
degranulation, hypersensitivity and increased IgE production are often referred to as the “early phase” allergic reaction and initiate the next phase or “late phase” through recruitment and activation of additional inflammatory cells that respond to the increased concentration of cytokines and chemokines.

Among the first cells recruited to the airways following allergen exposure are macrophages and eosinophils. Once recruited, FcεRI, is upregulated in these cells by the presence of IgE. CD23, or FcεRII, the low affinity Fc receptor, is also upregulated in the presence of cytokines released by Th2 cell and eosinophils including IL-4 and IL-13, and can enhance the allergic reaction.[19] Upregulation of these receptors results in activation and an increase in phagocytic activity.[20] Eosinophils are capable of releasing an impressive collection of cytokines including IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, and TGF (transforming growth factor)-α/β, chemokines (RANTES and eotaxin-1), and lipid mediators such as platelet-activating factor and leukotriene C4 (LTC₄).[21] These pro-inflammatory factors increase cell adhesion, vascular permeability, mucus production, and smooth muscle contractility.[22] Presence of activated eosinophils results in damage and dysfunction in tissue through secretion of granules that contain cytotoxic cationic proteins including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN).[23-25] These cationic proteins contribute to airway damage and to promoting airway hyperreactivity by stimulating of mast cell degranulation and airway mucus secretion, altering smooth muscle
contractility through dysregulation of vagal muscarinic receptors, and contributing to the formation of reactive oxygen species by catalyzing the oxidation of pseudohalides [thiocyanate (SCN⁻), halides [chloride (Cl⁻), bromide (Br⁻), iodide (I⁻)], and nitric oxide (nitrite).[24, 26-29] Given the broad range of effects, pharmacological interventions have been developed to target eosinophils using anti-IL5 (mepozilumab) and have been shown to effectively reduce eosinophil numbers. Promising clinical results have been reported, with anti-IL5 reducing the number of exacerbations in atopic, steroid-dependent asthmatics.[30, 31]

In addition to their phagocytic activity, macrophages release pro-inflammatory and pro-survival cytokines, chemokines and growth factors and play an important role in antigen presentation.[32] High levels of chemokines thought to be produced in large quantities by macrophages are found in the sera of asthmatic patients such as RANTES, monocyte chemoattractant protein-1 and -3, and IL-17.[33]

1.2.3 Airway Remodeling

The concept of airway remodeling is used as a collective term that encompasses changes in airway tissue composition in an asthmatic individual as compared to normal airways. First described by Huber and Koessler 90 years ago when examining the lung structure of patients with fatal asthma [34], the concept of airway remodeling has evolved to include airway wall thickening, subepithelial
fibrosis, goblet cell metaplasia, angiogenesis, epithelial cell hyperplasia and smooth muscle cell hypertrophy and hyperplasia.[35]

Airway wall thickening is seen in abundance when examining asthmatic lungs compared to non-asthmatic lungs with increases of 50–300% in cases of fatal asthma and 10–100% in cases of non-fatal asthma.[36] Clinical studies as well as mathematical modeling suggest that airway wall thickening correlates with disease severity and degree of AHR.[37] These studies have highlighted the consequences of increased thickness of the outer airway wall and subsequent uncoupling of the airway from the tethering forces in surrounding lung parenchyma. This may result in a greater degree of smooth muscle contraction and destabilization of the dynamic forces that control airway size, due to less opposing tension required to overcome intrinsic impedances and increased muscle shortening, which can result in airway closure.[38]

Increased airway smooth muscle mass has been shown in many asthma studies and is thought to be a hallmark of airway remodeling. There is evidence of hyperplasia as well as hypertrophy of smooth muscle cells, although causative mechanisms remain elusive. It has been shown that large airways of asthmatics appear to show smooth muscle hyperplasia, while hypertrophy can be detected throughout the bronchial tree and particularly in the smaller, peripheral airways of asthmatics.[39, 40] Regardless of mechanism, it is easy to infer the consequences of increased smooth muscle
mass in the lungs as being detrimental to airflow and a contributor to increased airway narrowing, thus exacerbating AHR.

Changes in epithelial cell phenotype are a well-documented phenomenon and potentially a strong contributor to the pathogenesis of asthma. Epithelial cell metaplasia results in the development of goblet cells leading to mucus hypersecretion. Mucus producing cells constitute a higher percentage of the lumen of the central airways in asthmatics promoting mucous plugging and decreased airflow.[41] Additionally, epithelial hyperplasia has been widely observed in asthmatics resulting in airway narrowing as the area of the airway epithelium is increased in asthmatic airways. [42]

Despite extensive research efforts, mechanisms that cause airway remodeling remain uncertain. This is due, most likely, to difficulties in obtaining sufficient long-term data sets as samples taken from patients are typically from a single time point and remodeling is a chronic, on-going process. Additionally, differentiating among cytokines, chemokines and inflammatory cells responsible for acute versus chronic responses is difficult. Nevertheless, it seems that several inflammatory mediators have been strongly correlated with remodeling processes. One such mediator is TGF-β1 as its expression consistently correlates with sub-epithelial basement membrane thickness and fibroblast hyperplasia.[43, 44] Also found in high quantities in asthmatic biopsies are granulocyte-macrophage colony-stimulating factor (GM-CSF) and epidermal growth factor (EGF), both thought to result in airway inflammation associated with increased eosinophils and fibrosis.[45] An
important cytokine that has emerged in regard to epithelial cell remodeling is IL-13 which may be secreted by T lymphocytes, mast cells, basophils, and certain B cells[46] or by airway epithelial cells and is thought to play a role in epithelial cell repair as well as goblet cell metaplasia.[47, 48]

1.3. Non-Allergic Asthma

Non-allergic asthma is characterized by the onset of asthma symptoms in the absence of allergic stimuli. This type of asthma may be intrinsic or triggered by exercise, cold air, smoke, or the inhalation of irritant substances. The induction of asthma by irritant substances is of particular interest to the work contained in this thesis. The notions of extrinsic and intrinsic asthma have fallen out of favor in recent years, perhaps because of the landmark paper of Burrows and colleagues that suggested that IgE was elevated above the normal age-adjusted values in most asthmatics and when IgE was completely absent from the serum, asthma was not present.[49]

1.3.1 Intrinsic Asthma

Patients with intrinsic asthma show no signs of atopy, have normal total and specific IgE in serum, and typically develop symptoms later in life.[50] However, IgE synthesis does occur locally in the airways, and the inflammatory cell profile is similar to that of atopic individuals with Th2 skewing, eosinophilia and mast cell activation. It is postulated that one potential mechanism may be the presence of “superantigens”, produced locally
in the airways by bacterial infection/colonization with organisms such as *Staphylococcus*.\[51\] Because intrinsic asthmatic patients are generally more difficult to treat and often steroid unresponsive, the idea that this form of asthma is triggered by the production of superantigens may be correct as this class of antigens typically leads to steroid resistance.\(\text{Reference}\) Lastly, cytotoxic autoantibodies have been detected in the lungs of intrinsic asthmatics, including against cytokeratin-18, which targets epithelial cells. These autoantibodies most likely exacerbate epithelial cell damage and may result in an environment more susceptible to bacterial colonization due to compromised epithelial cell structure.

1.3.2 Occupational Asthma

Occupational asthma is a form of asthma that develops as a direct result of exposure to substances encountered in the workplace. There are two subtypes of occupational asthma, sensitizer-induced and irritant-induced. Sensitizer-induced occupational asthma accounts for greater than 90% of the reported cases and includes the development of asthma symptoms from workplace exposure to high and low weight sensitizers.\[52\] A latent period is present in this form of asthma which requires an initial sensitization and asthma develops in response to subsequent repeated exposures to the offending agent.

Irritant induced asthma (IIA) is a form of occupational asthma that develops without latency. One important cause of IIA is the inhalation of chlorine, the lung toxicity of which was first described during World War 1 in
soldiers [53, 54] and in industrial workers in the early part of the 20th century.[55] IIA is characterized by an acute onset and is independent of adaptive immune mechanisms. Hallmark attributes of chlorine toxicity include dyspnea, chest tightness, decreased airflow, pulmonary edema and occasionally rapid death.[55] IIA encompasses both the symptoms following single (acute) and multiple (chronic) exposures to either high or low concentrations of an irritant substance leading to persistent bronchial hyperresponsiveness, airway inflammation and damage.[56, 57] Typical causative agents of IIA include diesel exhaust, dimethyl sulfate and cleaning products such as ammonium compounds and amine compounds.[57] Particularly strong irritating substances leading to IIA include bleach (sodium hypochlorite), hydrochloric acid, and alkaline agents (ammonia and sodium hydroxide).[58] The irritating substances become even more dangerous when mixed, and lead to the generation of chloramines from the combination of bleach and alkaline products or chlorine gas (Cl₂) following the combination of bleach and acid-based cleaning materials. Many IIA exposures are reported by workers in pulp and paper mills as frequent use of Cl₂ gas to bleach paper can lead to acute accidental exposure and/or repeated minor exposures.[59]

A subset of IIA termed reactive airway dysfunction syndrome (RADS) refers to the development of IIA from a single high exposure to an irritant substance. Diagnostic criteria for RADS include: 1. Absence of a pre-existing asthma condition, 2. Onset of asthma following a single exposure to an irritant substance in high concentrations, 3. Onset of asthma within 24 hours
of exposure and, 4, A positive methacholine challenge test (a fall in the FEV\textsubscript{1} in response to concentrations of inhaled aerosolized methacholine<8mg/mL).[60]

1.3.3 Clinical Presentation of IIA

Upon clinical presentation, patients who have been exposed to irritant substances undergo pulmonary function testing using spirometry, and methacholine challenge to evaluate the presence, severity, and reversibility of airflow limitation.[61] Lung radiographs may be used to determine the presence of lung edema and inflammation may be assessed by examination of sputum.[62] Arguably the most significant difference between IgE-mediated asthma and IgE-independent asthma relates to the profile of the inflammatory cell types found in the airways as bronchoalveolar lavage fluid (BALF) and sputum analysis often reveals elevated levels of IL-8 and neutrophilia in the latter.[63] As previously discussed, IgE mediated asthma is characterized by a largely eosinophilic inflammation. Table 1 shows some of the fundamental differences between these asthma subtypes, with notable differences in stimulus, inflammatory cell profiles and the nature of cytokine/chemokine/growth factors.

1.4 Cl\textsubscript{2} Induces IIA

Among the substances that induce either RADS or IIA, as mentioned above, inhalational exposure to Cl\textsubscript{2} gas has been of particular interest. Cl\textsubscript{2} is a highly
utilized chemical that people may be exposed to through inappropriate use of cleaning products, in accidental spills in pulp and paper mills and in swimming pools, through transportation accidents and through its use a chemical weapon.[63] Damage from Cl₂ is not limited to its gaseous form, but extends to its by-products such as chloramines, hypochlorous acid (HOCI), and chlorine dioxide. Due to its chemical properties and high water solubility, Cl₂ is quickly hydrated into hypochlorous acid and hydrochloric acid when in contact with water, making it especially damaging to the moist lining of the respiratory tract. Because Cl₂ is rapidly converted to its by-products, it is rapidly scrubbed from the airways. This quick conversion and scrubbing of Cl₂ has been utilized in research by using both high and low exposure concentrations to partition damage to predominately central airways at low concentrations or a mix of central and proximal airways as well as alveolar damage at higher levels of exposure.[64] At concentrations below 50ppm, bronchospasm, mucus production, increased airway responsiveness and decreased respiratory rate may be reversible as evidenced in some human and animal studies.[65, 66] At these concentrations, there is little evidence of Cl₂ reaching peripheral airways and the alveolar spaces.[67] At concentrations above 50ppm Cl₂ penetrates into the distal lung, affecting large and small airways, and parenchyma and has a greater chance of causing irreversible damage.[68]

Upon inhalation, Cl₂ encounters the epithelial cell lining of the nasal cavities and airway walls, resulting in epithelial cell damage. Recovery from
this exposure may not be complete, and some studies have found long term effects of Cl₂ damage in the intrapulmonary airways after a single inhalation including goblet cell hyperplasia, increased interstitial collagen, ASM proliferation and persistent AHR.[69] Following acute exposure, histological assessment of bronchial biopsies taken from individuals exposed to Cl₂ shows that epithelial shedding persists for several weeks, and regeneration of airway epithelial cells may persist for months with bronchial hyperresponsiveness lasting years.[70, 71] Furthermore, chronic exposure to low levels of Cl₂, such as those experienced by high-level swimmers, have been correlated with bronchial responsiveness and epithelial cell shedding among these athletes as well as increased disease severity in atopic patients that frequent pools.[72, 73] Models using rodents to evaluate the damage induced by Cl₂ inhalation have found similar patterns of response to those observed in human studies. A single, acute exposure to 800 ppm Cl₂ gas for 5 minutes in Balb/C mice induced bronchial hyperresponsiveness to methacholine, airway inflammation, increases in airway smooth muscle (ASM) mass, epithelial cell proliferation and collagen deposition which persisted for at least a 10 day period following exposure.[74] C57/Bl6 and FVB/N mice have epithelial cell shedding, increased protein leak, a high degree of neutrophilia and elevated levels of KC (the murine ortholog of IL-8) in the hours immediately following Cl₂ exposure, and lasting 48 hours.[75] Few studies have been conducted on chronic, long-term Cl₂ exposure. One study exposing B6C3F1 mice and F344 rats to various concentrations of Cl₂ (0.4-2.5 ppm, 6h/day) over a two year period found much
of the damage confined to the nasal cavities with olfactory epithelial degeneration, septal fenestration, mucosal inflammation, respiratory epithelial hyperplasia, squamous metaplasia, goblet cell hypertrophy and hyperplasia, and secretory metaplasia of the transitional epithelium of the lateral meatus.[76] The restriction of injury to the nose is perhaps not surprising given the large surface area that this organ possesses in the rat as well as the mouse. Further studies using animal models to understand the long-term, chronic effects of Cl$_2$ inhalation including biomarkers and physiological outcomes are needed.

1.4.1 Mechanisms of Cl$_2$ damage

Cl$_2$ exposure affects several cell types within the respiratory system including structural and inflammatory cells. Several studies have explored the effects of Cl$_2$ inhalation in both human and animals in an attempt to elucidate causative mechanisms that result in airway damage and dysfunction following Cl$_2$ inhalation. It is hypothesized that Cl$_2$ causes damage through oxidative stress, exerting its effects through the production of reactive oxygen species (ROS).

Inflammatory cell recruitment is a hallmark of the Cl$_2$-induced airway response. Exposure to Cl$_2$ triggers inflammatory cascades, and Cl$_2$ by-products including hypochlorous acid (HOCL) and chloramines activate the MAP kinase pathway and upregulate Nf-$\kappa$B activity through inhibition of I$k$Ba.[77, 78] Upregulation of proinflammatory pathways such as Nf-$\kappa$B have a variety of consequences and may induce the production and release of
cytokines (tumour necrosis factor (TNF)-α, IL-1β, IL-6), chemokines (CCL2, CCL5, CXCL8, CXCL10 and CXCL12), increase adhesion molecules and proteins (intercellular adhesion molecule 1, vascular cell adhesion protein 1) and promote oxidative stress through iNOS enzyme production. \[79\] Increased NF-κb activity results in greater vascular permeability, apoptosis, and may promote airway remodeling including ASM hyperplasia and goblet cell metaplasia. \[80, 81\] Chemokines including CXCL8 (IL-8) are chemotactic for neutrophils and subsequent airway damage may be induced following recruitment as they produce myeloperoxidase-catalyzed HOCl. This may lead to on-going oxidative stress in the hours following the initial Cl₂ exposure. \[82\]

Among the structural cells, alveolar and bronchial epithelial cells are predominantly affected and their altered characteristics reflect the pathogenic effects of Cl₂ on the airways and the parenchyma. Typically, airway damage induced by irritant substances results in the shedding of airway epithelial cells into BALF, causing a denudation of the lining epithelium, exposing underlying structural cells and allowing for increased permeability. Loss of epithelial cell numbers and increased permeability have been directly correlated with clinical outcomes and prognosis. \[83, 84\]

### 1.4.2 Effects of Cl₂ on Alveolar Epithelial Cells

Exposure of alveolar epithelial cells to HOCl acid results in a decreased transepithelial resistance and functioning of Na⁺ and Cl⁻ channels. HOCl disrupts the oncotic gradient necessary for fluid resorption, resulting in
pulmonary edema by damaging epithelial cells and reducing protective endogenous antioxidants such as glutathione concentrations.[85] Furthermore, formation of partially reduced intermediates such as OCl− damage the sulfhydryl functional groups on proteins and amino acids, free amine groups of plasma amino acids and aromatic amino acids resulting in DNA damage, protein carbonylation and further cellular damage.[86-88] Balb/C mice exposed to 500ppm Cl2 for 30 minutes demonstrate decreased alveolar fluid clearance due to Na+/Cl− ion channel dysfunction and reduced ENaC channel function. While it appears HOCl does not affect ENaC channels directly, as confirmed by in vitro experiments, these effects are hypothesized to be due to HOCl-induced post-translational modifications of amino acids required for proper ENaC formation.[89] Isolated alveolar Type II (ATII) cells show that Cl2-induced disruptions in ENaC function are dependent on ERK1/2 phosphorylation. Antioxidant administration before and after Cl2 exposure resulted in a decrease of oxidant formation, thus increasing ERK1/2 phosphorylation and mitigated the decrease in amiloride-sensitive short-circuit currents and ENaC concentrations in ATII cells. These effects were confirmed in mice through in situ analysis of ERK1/2 activity following Cl2 inhalation.[90]

In addition to disruptions in channel function, exposure to Cl2 can induce long-term epithelial cell dysfunction through DNA damage and subsequent mutagenesis. A549 cells exposed to HOCl resulted in DNA single-strand breakage (ssDNA), as evidenced by increased levels of 8-oxo-dG and 3-
(2-deoxy-b-D-erythro-pentofuranosyl)pyrimido[1,2-a]purin-10(3H)-one (M1dG), as well as HPRT1 mutations.\[91\] Damage to DNA has also been found in bronchial epithelial cells which, following exposure to HOCl, exhibit deamination of purine bases in cellular DNA.\[92\]

1.4.3 Effects of Cl\(_2\) on Bronchial Epithelial Cells

Bronchial epithelial cells are critical structural cells, significant contributors to the regulation and release of mediators, and the first line of defense in the lungs against inhalational injury from noxious irritants. Upon inhalation, Cl\(_2\) quickly dissolves into the airway surface fluid (ASF) maintained above the bronchial epithelial cells and initiates the formation of by-products including HOCl. This event immediately disrupts the delicate balance of antioxidants maintained in the ASF including levels of reduced glutathione (GSH), the main antioxidant present in the extracellular compartment of bronchial epithelial cells.\[93\] Exposure to HOCl effectively depletes GSH by neutralization of HOCl via formation of GSH disulfide (GSSG), GSH thiosulfonate, and GSH sulfonamide, resulting in a constitutive decrease in GSH ASF content. Although GSH is a very efficient scavenger of HOCl, GSSG is not, and therefore GSH depletion increases epithelial cell vulnerability to the subsequent oxidative burden through either exogenous (continued Cl\(_2\) exposure) or endogenous (neutrophil-induced HOCl formation) oxidative injury.\[94\] Once the endogenous antioxidant defense has been depleted, epithelial cells become susceptible to the damaging effects of ROS, including
breakdown of tight junctions. Exposure of a human bronchial epithelial cell line (16HBE14o-) to 1 mM HOCl administered to the apical surface resulted in reduction in transepithelial resistance, short-circuit current and GSH efflux. Exogenous administration of GSH in the presence of HOCl prevented these alterations.[93] Exposure of primary human bronchial epithelial (NHBE) cells to HOCl induces formation of ROS, detected by 2′,7′-dichlorofluorescein diacetate a fluorochrome that measures hydroxyl, peroxyl activity within the cell and decreased GSH while increasing levels of GSSG.[95] Mechanisms postulated to mediate these effects on GSH are thought to be partially cAMP dependent, as HOCl reduces cAMP levels and GSH formation is enhanced by cAMP activity.[96]

In vivo, histological evidence of epithelial cell damage using 3-nitrotyrosine (3-NT) as an oxidative stress marker shows strong signals localized to the apical surface of the airway epithelium following exposure to increasing concentrations of Cl₂ in mice.[97] Following inhalational exposure to Cl₂, bronchial epithelial cells undergo acute apoptosis, detected by TUNEL staining, and there is evidence of epithelial cell shedding from BALF analysis followed by extensive epithelial cell proliferation, reflected in Ki-67 expression.[97]

A critical aspect of the endogenous response to oxidative insults is the upregulation of genes and the synthesis of a variety of antioxidants. Nuclear factor (erythroid-derived 2)-like 2 (NRF2) is considered the “master regulator of antioxidant gene expression”, and is activated during periods of high ROS
activity.[98] NRF2 has been shown to play an important role in ROS-mediated cell damage and repair and may contribute to mechanisms driving the ROS-induced response. Under basal conditions, NRF2 is maintained in the cytosol, forming a complex with the protein Kelch-like ECH-associated protein 1 (KEAP1) which acts as an adaptor protein for Cullin 3-based ubiquitination and NRF2 proteosomal degradation. If a cell encounters excessive oxidative or electrophilic stress the disulfide bond binding NRF2 to KEAP1 is oxidized to two thiol groups, dissociating NRF2 from KEAP1 and halting ubiquitination of NRF2 protein units. Liberated NRF2 is phosphorylated at serine and threonine residues by kinases including PI3K, PKC, JNK and ERK in the cytosol which results in its translocation to the nucleus.[99] There, it combines with a small Maf protein and binds to the Antioxidant Response Element (ARE) at the upstream promoter region controlling dozens of antioxidant genes and initiating transcription (Figure 1.1).[100] Transcriptional profiling of primary bronchial epithelial cells treated with HOCl (0.4, 1 and 4mM) showed dose-dependent increases in several NRF2-dependent genes including thioredoxin 1, hemeoxygenase, aldehyde dehydrogenase 1, NAD(P)H dehydrogenase, quinone 1 and glutamate-cysteine ligase. Time- and concentration-dependent nuclear translocation of NRF2 was observed in HOCl exposed cells as compared with untreated cells and specific knockdown of NRF2 using siRNA significantly increased HOCl toxicity in cells as evidenced by decreased cell viability.[95] Furthermore, siRNA directed against KEAP1,
effectively enhancing NRF2 activity, protected cells against the effects of HOCl by bolstering antioxidant enzyme activity.[95]

1.3.4 Role of Neutrophils in Cl₂ Induced Airway Injury

We, and others, have noted a striking neutrophilia in the hours following Cl₂ exposure suggesting this cell type played an important role,[101-103] including the fact that in the presence of ROS bronchial epithelial cells recruit large numbers of neutrophils to the airways.[104] Conceptually, the presence of neutrophils is beneficial, as they are able to phagocytose damaged cells, and produce reactive oxygen species, nitrogen species and proteolytic enzymes that help clear invading pathogens. These benefits, however, may have limited pertinence in a pathogen-free model of disease and this normally tightly-regulated system of neutrophil recruitment and activation appears to create a greater oxidative burden and worsening of lung damage. Through production of reactive oxygen species, neutrophils may inadvertently cause damage to healthy lung tissue, prolonging recovery and repair.

Neutrophils are typically recruited and activated by pro-inflammatory mediators including TNFα, IL-1β, IL-6, IL-8 and MCP-1 and epithelial cells are capable of synthesizing and releasing all of these factors.[105, 106] Neutrophil activation as well as neutrophil numbers found in the BALF have been directly correlated with a poorer prognosis for patients with acute lung injury.[107] Neutrophil populations in the lung of many of these patients reveal elevated nuclear accumulation of the transcription factor Nf-κB, an
important finding as major pro-inflammatory mediators are under the control of Nf-κB including TNF-α and IL-8.[108] Depletion of neutrophils has shown promising results in preventing much of the damage associated with acute lung injury, including lung inflammation, protein leak, and sepsis. [109-112]

Neutrophils typically survive 6-12 hours under normal conditions in the blood at which point they undergo apoptosis. In the presence of activating factors such as TNF-α, IL-8, IL-6, IL-1β and granulocyte–macrophage colony-stimulating factor (GM-CSF), which may be released in the presence of oxidative stress by epithelial cells and macrophages, the lifespan of neutrophils increases and they become resistant to apoptosis.[113] This prolonged lifespan and activity may contribute to the ongoing damage observed in models of Cl₂-induced lung injury.

Cellular damage to epithelial cells and apoptosis consistently appears following exposure to irritants. High levels of Fas and FasL have been detected in the BALF of patients with ARDS and BALF isolated from these patients induces apoptosis in otherwise healthy epithelial cells in vitro.[114] Infiltrating neutrophils are a source of Fas ligand (FasL) and thereby may contribute to epithelial cell apoptosis by triggering pathways downstream of Fas. Fas and FasL mutant animals exhibit less pulmonary epithelial cell apoptosis in response to high levels of oxidative stress when compared with wild-type animals.[115]

Upon activation, neutrophils undergo an “oxidative burst”, resulting in the production of superoxide by membrane-associated NADPH oxidase.
Upon activation, NADPH oxidase is assembled in the plasma membrane leading to the generation of superoxide through the transfer of electrons from NADPH inside the cell across the membrane where the electron is coupled to oxygen to produce the superoxide anion. Superoxide is rapidly converted to H$_2$O$_2$ and O$_2$ through a non-enzymatic dismutation. Myeloperoxidase (MPO), a peroxidase enzyme, catalyzes the formation of HOCl, simultaneously released at the time of H$_2$O$_2$ formation utilizing the H$_2$O$_2$ and a chloride ion to form HOCl.[116] MPO is capable of oxidizing a variety of substrates using H$_2$O$_2$ by removing a single electron during the formation of radical products. These substrates include: tyrosine, tryptophan, sulfhydryls, phenol, and indole derivatives, nitrite, hydrogen peroxide and xenobiotics.[117-119] However, the majority (70%) of H$_2$O$_2$ produced is converted by MPO to HOCl.[120] HOCl is cell permeable, readily interacting with thiols, thioethers and amino groups affecting intracellular function and causing DNA damage.[121] Specifically, this DNA damage is thought to involve deoxyguanosine, which becomes hydroxylated in cells exposed to HOCl. MPO-derived HOCl interacts with iron related protein 1, leading to transferrin receptor mRNA degradation and disruptions in proteins that regulate cellular energy and iron metabolism.[122, 123]

Neutrophils have sensors on their cell surface capable of detecting soluble molecules generated in response to tissue injury.[124] Activation of neutrophil receptors CD11b and CD35 promotes adherence to endothelial cells and aids in targeting of damaged cells for destruction and phagocytosis.[125]
In addition to stimulating adhesion to damaged cells, activation of these receptors induces neutrophil degranulation, release of MPO and tumor necrosis factor (TNF), and increased MAPK activity.[126, 127]

1.5 Cysteinyl-leukotrienes as asthma mediators

CysLTs belong to a larger family termed “eicosanoids” that includes leukotrienes, prostaglandins, thromboxanes, lipoxins and other related compounds. The nomenclature for this family of compounds is taken from the fact that they are derived from 20 carbon (eicosa) unsaturated fatty acids, thus the term eicosanoids. The term “leukotriene” is derived from the original cell type in which they were first identified, namely polymorphonuclear leukocytes, and the four double bonds each of the leukotrienes has in common, and of which three are conjugated in the triene structure. CysLTs differ from leukotrienes (LTB₄) in that they contain a cysteine residue, thus the “cys” prefix.

Originally discovered in the 1940’s, cysteiny1-leukotrienes (cysLTs) were originally termed “slow-reacting substance of anaphylaxis” (SRS-A).[128] Their identification as peptide-conjugated lipid mediators came later, in 1970, following experiments demonstrating their potent contractile properties [129, 130] and designed to measure the contractile responses of guinea pig ileal smooth muscle suspended in organ baths.[131] These experiments, performed by Brocklehurst, were aimed at gaining better understanding of the newly isolated SRS-A and its role in muscle contraction.
By rendering the muscle unresponsive to histamine, through the use of H1 antagonists, Brocklehurst found that in the presence of SRS-A, the muscle responded with slow contractions in a dose-dependent fashion. In light of Brocklehurst’s findings, and on a self-described “gut-feeling”, Frank Austen began to perform experiments on bronchial smooth muscle using SRS-A with the hope of finding a key mediator involved in histamine-independent bronchoconstriction observed in the lungs of asthmatics. Austen and his collaborators first sought to identify the cellular source of SRS-A in the lungs finding that hapten-specific IgG1, but not IgG2, antibodies elicited the response of antigen induced SRS-A synthesis in guinea pig lungs, implicating mast cells as a source of SRS-A.[132] Mast cell production of SRS-A was subsequently confirmed through experiments in both monkey and human lung fragments.[133] Next, in a key experiment which resulted in a more precise identification of SRS-A in the UV spectrum, spark-source mass spectroscopy and electron probe analysis showed high-levels of sulfur in purified SRS-A.[134] These findings led to experiments that allowed purified SRS-A to be isolated from mouse mastocytoma cells that were exposed to [3H] arachidonic acid and [35S]cysteine and stimulated with a calcium ionophore, ultimately allowing for the identification the structure of leukotriene C4 (LTC4).[135, 136] Once LTC4 was discovered, the other cysLTs, LTD4 and LTE4 were identified and importantly, each cysLT induced contraction of guinea pig tracheal and lung parenchymal strips with varied potency, suggesting their affinity for the same receptor.[137, 138] Drazen et al., performed critical experiments which
further consolidated the importance of cysLTs as asthma mediators, establishing two key aspects of cysLT activity through experiments using inhaled LTC₄ and LTD₄. First, inhaled cysLTs were 1000-fold more potent at inducing bronchoconstriction than histamine and second, the onset of clinical symptoms was slow and prolonged compared to histamine, resulting in an audible wheeze, rather than a histamine-stimulated cough.[139]

1.5.1 Biosynthesis of CysLTs and Leukotriene B₄

The biosynthesis of the cysLTs and LTB₄ is summarized in Figure 1.2. CysLTs and LTB₄ are formed de novo and originate from arachidonic acid (AA). Biosynthesis begins when AA is released from the outer nuclear membrane by cytosolic phospholipase A₂ (cPLA₂), a step initiated by calcium mobilization in the cell.[140] AA is then converted by the enzyme 5-lipoxygenase (5-LO) and 5-lipoxygenase–activating protein (FLAP) to 5-hydroperoxyeicosatetraenoic acid (HPETE). 5-LO and FLAP then convert HPETE in a second reaction to an epoxide, LTA₄. LTA₄ can be converted to LTB₄ by LTA₄ hydrolase or to LTC₄ by LTC₄ synthase (LTC₄S).[141] This differential conversion depends on the cell type in which LTA₄ is synthesized. Cells containing LTA₄ hydrolase, such as neutrophils, preferentially produce LTB₄,[142] whereas cells containing LTC₄S including mast cells, basophils, macrophages and eosinophils produce LTC₄.[143-145] The conversion of LTA₄ to LTC₄ occurs through the conjugation of glutathione (GSH) to LTA₄. LTC₄ is exported to the cell surface through an energy-dependent step[146]
involving multi-drug resistance associated protein.[147] LTD₄ is formed following extracellular conversion involving sequential amino acid removal from the glutathione tripeptide moiety by γ-glutamyl transpeptidase.[148] Formation of LTE₄ requires removal of glycine by a dipeptidase or by the functionally specific enzyme, γ-glutamyl leukotrienease.[149] Once LTE₄ is formed, no further modifications take place, making LTE₄ the most stable of the cysLTs. It is acetylated in the liver, effectively stabilizing it before being excreted in the urine.[150]

1.5.2 Transcellular metabolism of cysteinyl-leukotrienes

Transcellular metabolism allows for the production of eicosanoids including cysLTs in cells that lack the complete set of enzymes necessary to complete the biosynthetic cascade. Cells that are able to produce and export intermediates, such as LTA₄, are taken up by surrounding cells and metabolized.[151] Folco et al., describes five criteria to describe cell-cell interactions leading to transcellular metabolism. These include: “1. Two or more cells must occur in a tissue in vivo, in an appropriate ratio to each other, so that together they have the complete enzymatic cascade to generate a particular eicosanoid. 2. The donor cell must have the enzymatic activity to convert a cellular stimulus into free arachidonate or an arachidonate intermediate (precursor). 3. The acceptor cell must have the enzymatic capacity to convert the arachidonate intermediate into the biologically active eicosanoid but need not have the capacity to generate the initial precursor
(intermediate). 4. The donor cell must be activated to begin this process, but the acceptor cell need not be. 5. Both donor and acceptor cells need specific mechanisms to export and import the highly lipophilic precursor and the acceptor cell must be able to release the active eicosanoid.”[151]

Cellular production of 5-lipoxygenase, a key enzyme involved early in the biosynthesis of eicosanoids is found in circulating neutrophils, monocytes, eosinophils, macrophages, and mast cells. While the expression of 5-LO is limited in regards to the variety of cells which express it, all of the aforementioned cells are capable of releasing LTA₄ and participate in transcellular biosynthesis of either LTC₄ or LTB₄.[151] Of the cell types involved in transcellular metabolism, neutrophils have been found to be particularly active in this process by being avid LTA₄ donors. The first evidence of LTC₄ generation by transcellular biosynthesis was reported in cocultures of endothelial cells and human neutrophils.[152] While endothelial cells do not contain LTC₄ synthase, following incubation with neutrophil-derived LTA₄, LTC₄ was detected by reverse phase HPLC and ultraviolet spectroscopy.[153] Platelets have also been found to produce LTC₄ upon incubation with exogenous LTA₄ and when in co-culture with neutrophils. The quantity of LTC₄ produced was shown to be dependent on the number of platelets present.[154] Alveolar macrophages readily take up LTA₄, either administered exogenously or in co-culture with neutrophils and preferentially generate LTB₄, suggesting a higher concentration of LTA₄ hydrolase in comparison to LTC₄ synthase in this cell type.[155]
Cell-cell interactions resulting in production of arachidonate metabolites can become complex and in addition to the production of pro-inflammatory mediators such as LTB$_4$ or LTC$_4$, transcellular metabolism-driven anti-inflammatory activity has also been found. Interactions between airway epithelial cells and neutrophils typify these complexities. Klockmann et al. co-cultured bronchial epithelial cells with neutrophils in an effort to identify mechanisms of interaction between these cell types in the context of arachidonic acid product formation.[156] Under monoculture conditions, epithelial cells predominately released PGE$_2$, PGF$_{2\alpha}$, and 15-hydroxyeicosatetraenoic acid (15-HETE), whereas neutrophils synthesized LTB$_4$. However, when the cells were co-cultured, and stimulated with calcium ionophore, LTB$_4$ levels decreased by 45%. This effect was attributed to the production of epithelial-cell derived PGE$_2$, which has an inhibitory effect on LTB$_4$ production. Interestingly, the observed reduction of LTB$_4$ did not require cell-cell contact, suggesting transcellular metabolism can act in a paracrine fashion and that products transferred maintain stability despite being metabolic intermediates. A reduction in ROS, including superoxide anion, was observed, suggesting that epithelial cells are pivotal in direct reduction of the oxidative burden imposed by activated neutrophils. Ultimately, the airway epithelium contributes to anti-inflammatory mechanisms driven by processes involving transcellular metabolism of AA metabolites.[156]

Much of the knowledge gained in understanding mechanisms and consequences of transcellular metabolism has been achieved through *in vitro*
studies. While the results of these studies have been compelling, these methods may lack relevance in the context of in vivo systems. Recently, studies utilizing chimeric mice to explore transcellular metabolism in vivo have shed light on how these mechanisms operate in the whole animal. First LTA<sub>4</sub> hydrolase knockout (LTA<sub>4</sub>-/-) and LTC<sub>4</sub> synthase knockout (LTC<sub>4</sub>S-/-) mice were generated as they cannot produce LTB<sub>4</sub> or LTC<sub>4</sub>, respectively.[156] Next, 5-lipoxygenase knockout mice (5-LO/-/-), unable to produce either LTB<sub>4</sub> or cysLTs, were lethally irradiated. Bone marrow from LTA<sub>4</sub>-/- or LTC<sub>4</sub>S-/- was transplanted into two groups of 5-LO/-/- mice. Zymosan was injected into the peritoneal cavity to induce an inflammatory response. Assays for cysLTs, LTs and prostaglandins were performed on isolated peritoneal fluid. Both LTB<sub>4</sub> and cysLTs were detected in mice transplanted with either LTA<sub>4</sub>-/- or LTC<sub>4</sub>S-/- bone marrow, respectively.[157] This study proved unequivocally that transcellular metabolism occurred in vivo as no mouse used in the study possessed all of the machinery required to produce either LTB<sub>4</sub> or cysLTs.

1.5.3 Cysteinyl-Leukotriene Receptor Structure and Tissue Distribution

CysLTs exert their effects on cell surface receptors. Two cysLT receptors, cysLTr1 and cysLTr2, were identified through the use of pharmacologic profiling studies using mammalian tissues.[138, 158] Following their discovery, these receptors were cloned in both the mouse[159, 160] and human[161][162] and found to be members of the G protein-coupled receptor family (GPCR) and include both pertussis toxin-sensitive and pertussis toxin-insensitive G protein signaling cascades.[163] This categorization was based
on experiments performed showing ligand binding of these receptors were enhanced by divalent cations, and inhibited by sodium ions and non-hydrolyzable GTP analogs.[164] The structure of cysLT receptors follows a typical GPCR pattern including 7 hydrophobic transmembrane spanning domains bound to 6 hydrophilic loops. Based on the ligand binding structure, cysLT receptors belong to a subcategory of GPCRs classified as rhodopsin/β2-adrenergic receptor-like receptors. Structural homology between cysLTr1 and cysLTr2 is relatively low, at 38% in the human, with major differences being found in transmembrane domains 4-6 while transmembrane domains 3 and 7 are relatively similar. It is hypothesized that these transmembrane structural differences account for the varied ligand binding affinity between LTC₄ and LTD₄, with cysLTr2 binding LTC₄ and LTD₄ with equal affinity while cysLTr1 binds LTD₄ with highest affinity and LTC₄ and LTE₄ to a lesser extent. These differences can be localized to the 2nd and 3rd transmembrane loops.[165]

Receptor structural homology between species, especially among human and rodent species, has revealed a relatively high level of similarity. Human cysLTr1 and mouse cysLTr1 share 87% homology. Human and mouse cysLTr2 share 65% homology and human and rat share 73% homology at the protein level.[166] Receptor distribution among the cysLTr1 and cysLTr2 among tissues and between species indicates overlapping distributions, however, these distributions correlate well with pharmacological studies predicted from tissue profiling.[167]
There are two distinct transcripts for cysLTr1 in the mouse giving rise to two isoforms, a long and short form. The mouse cysLTr1 gene consists of four exons and three introns, with the long isoform including all four exons whereas the short form includes only exon I and IV. Notably, mouse cysLTr1 includes an in-frame start codon (ATG) found 39-bp upstream from the ATG start site for translation which corresponds with the start site of the human cysLTr1. This sequence results in a 13 amino acid extension in the N-terminus end of the mouse cysLTr1, a sequence missing in the human cysLTr1 due to the presence of a stop codon 6 nucleotides upstream of the ATG start site and therefore only one isoform of human cysLTr1.[161] As with cysLTr1, two splice variants of mouse cysLTr2 exist, with a single isoform existing in humans. The human cysLTr2 gene is located on chromosome 13q14 and arises from a single exon. In contrast, the mouse cysLTr2 contains 6 exons and is located on chromosome 14, with its entire coding sequence localized to exon 6.[168]

Recently a third cysLT receptor has been identified. This receptor was discovered in porcine pulmonary arterial ring that showed responsiveness to LTC4 and LTD4, but not LTE4.[169] This receptor, an orphan GPCR named GPR17 was cloned by human genomic DNA screening with the IL-8[170] receptor with subsequent cloning in chicken P2Y1 and murine P2Y2 receptors.[171] A phylogenetic analysis screening P2Y receptors showed that GPR17 was homologous to the cysLT receptors 1 and 2 and had 31% and 36% homology to these receptors, respectively. Additionally, the amino acid sequence of human GPR17 was found to be 90.3% identical to that of both mouse and rat GPR17
orthologs.[172] Lecca et al., conducted experiments utilizing transfectants based on GTPyS binding assays confirming human, rat, and mouse GPR17 as a dual receptor for uracil nucleotides as well as cys-LTs. Initial experiments confirming GPR-17 as an additional cysLT receptor utilized GRP17-expressing 1321N1 and COS-7 cells to show through single-cell calcium imaging that these cells respond to 100nM of LTD₄ and 100uM of UDP-glucose.[173] However, Maekawa et al. were not able to recapitulate these results in any of 1321N1, CHO and HEK-293T cell types using exogenous LTD₄, nor were they able to elicit a response using UDP-glucose. It was then reasoned by Maekawa et al., that heterodimerization of GPCRs had the potential for a duality that included an either a positive or negative regulatory role in transfectants.[174, 175] It was then hypothesized that GPR17 may act as a negative regulator of cysLTr1, controlling its calcium signaling function. Maekawa et al. conducted a series of in vitro experiments showing: GPR17 inhibits membrane function of cysLTr1, but not expression; GPR17 inhibits LTD₄-induced, cysLTr1-mediated ERK phosphorylation; GPR17 silencing induces upregulation of gene expression and function of cysLTr1 in bone-marrow derived mast cells; GPR17 and cysLTr1 co-localize on human derived monocytes and; cysLTr1 and GPR17 co-immunoprecipitate in CHO cells co-transfected with both receptors.[176] Subsequent experiments were performed in vivo following the development of a GRP17/- mouse model to assess the role of GPR17 with respect to its ability to regulate pulmonary inflammation in a house dust mite model of asthma. In this model, the role of GPR17 was further confirmed as results indicated that GPR17/- mice developed exaggerated cysLTr1-mediated airway
inflammation including neutrophil, eosinophil and lymphocyte migration following house dust mite sensitization and challenge. Additionally Th2/Th17 cytokine mRNA levels (IL-4, IL-5, IL-13, IL-17A) were elevated in GPR17 deficient mice. These effects were noted in the presence of increased cysLTr1 expression in the lungs, thus implicating the lack of GPR17 in unchecked expression and function of cysLTr1, resulting in the deleterious effects observed.[177]

1.5.4 Regulation of cysLT receptors

Regulation of cysLT receptors is controlled through immunological mechanisms which are mediated by cytokines, chemokines and growth factors. The eosinophil-derived cytokine IL-5, an important cytokine in the pathogenesis of allergic asthma, has been shown to be important in the upregulation of cysLTr1 in HL-60 cells.[178] The proposed mechanism being that IL-5 induces the production and release of cysLTs, which then stimulate cysLTr1 upregulation through autocrine pathways. This cytokine-driven cysLTr1 upregulation is observed in other cell types, including human monocytes and monocyte-derived macrophages which secrete IL-4 and IL-13, which also induce increases in cysLTr1 expression.[179] The regulation cysLTr1 in human fibroblasts and ASMC by IL-13 has been reported[180, 181] and these observations have led to more sophisticated in vitro experiments utilizing the possibility of paracrine mechanisms of cysLTr1 upregulation. Studies on human mast cells which produce cysLTs in abundance when activated and express both cysLTr1 and cysLTr2 have shown increases in Ca^{2+}
levels inducing the production of cysLTs through store-operated calcium-release-activated calcium (CRAC) channels. [182] Studies reveal that activated mast cells are capable of paracrine signaling that releases Ca$^{2+}$ in spatially separate resting mast cells. CysLTs were identified as the molecules inducing this paracrine signaling resulting in further production of cysLTs and thus a positive feedback cascade. In humans with allergic rhinitis, acutely isolated mast cells responded to cysLTs and exhibited store-operated Ca$^{2+}$ influx through CRAC channels. [182] These observations suggest a mechanism for continuous mast cell activation through a cysLT-dependent system.

Of importance, it has been observed that both IFN-$\gamma$ and IFN-$\beta$ are both capable of inducing cysLTr1 and cysLTr2 up-regulation in airway smooth muscle cells. [183] This observation is critical as up-regulation of these cytokines is generally associated with inhibition of synthesis and activities of pro-allergic Th2 associated cytokines. This paradoxical action suggests a more complex role of the cysLT receptors than previously thought and requires further investigation.

CysLTr2 seems to have similar mechanisms to cysLTr1 with regard to cytokine stimulation and the resulting increase in transcript levels. IL-4 stimulation induces an up-regulation of cysLTr2 in human umbilical vein endothelial cells, and it is down-regulated and partially reversible by IFN-$\beta$. [184, 185] CysLTr2 was found to be up-regulated on lung eosinophils during an asthma exacerbation, an effect that was reproduced in vitro through the stimulation of eosinophils using IFN-$\gamma$, suggesting that cysLTr2 may also play
a positive regulatory role in the pathogenesis of asthma.[186] The regulation of GPR17 appears to be dependent on cysLTr1, with its up-regulation being triggered by increases in cysLTr1 transcript, as observed through transgenic mouse studies using GPR17-/- mice.

1.5.5 CysLTs in the pathogenesis of asthma

The pathogenesis of asthma is complex and involves the interactions of numerous cell types, mediators, and exacerbating stimuli which commonly vary from person to person. However, despite these complexities, cysLTs are consistently recognized as potent and critical mediators in acute and chronic asthma. They are the most potent bronchoconstrictors discovered to date, affecting the tone and contractile properties of the airways and an important factor when considering asthma pathogenesis. The tone and contractile properties of airways is maintained through a delicate balance, orchestrated by contractile mediators which include cysLTs and histamine which are off-set by relaxant mediators such as PGE2.[187, 188]

CysLTs also affect vascular permeability, increasing protein leak into the airways which results in tissue edema, which further lends credence to the concept that their presence is deleterious. Through the study of cysLTs their roles have evolved from proinflammatory to remodeling regulatory roles. The presence of cysLTs increases mucus secretion through an IL-13-dependent mechanism, impairs the ciliary action of bronchial epithelial cells and induces ASMC smooth hyperplasia and airway remodeling.[189-191]
CysLTs are produced by eosinophils and mast cells in high concentrations, and this concentration increases during asthma exacerbations as the number of eosinophils recruited to the lung increases as does the activity of mast cells. This increase is evidenced by the correlation of increased eosinophils and the concentration of cysLTs in BAL fluid and excreted in urine during an asthma exacerbation.[192] And, while cysLTs are produced by these inflammatory cell types, they are also implicated in chronic inflammation due to their role as pro-survival mediators. This includes increasing eosinophil survival by inducing the release of cytokines such as GM-CSF[193], as well as bolstering cellular adhesion through stimulation of P-selectin production.[194] CysLTs may also contribute to promoting transendothelial migration of inflammatory cells across the vessel wall into the airways in response to paracrine signals from mast cells and lymphocytes.[166] In vitro, the use of cysLT receptor antagonists like montelukast has shown a diminution of eosinophil survival through indirect mechanisms including the inhibition of epithelial-derived GM-CSF, IL-6 and IL-8 and directly, by reducing extracellular matrix-induced eosinophil survival.[195] In contrast, cysLTs are also capable of activating eosinophils to secrete eosinophil cationic protein (ECP), which can worsen asthma exacerbations through the induction of apoptosis and damage to bronchial epithelial cells and intrapulmonary neurons.[196]

In addition to eosinophils, cysLTs have a potent effect on macrophages. Stimulation of human macrophages with LTD₄ results in the release of
chemokines including MIP1α and MIP1β through an ERK-dependent signaling cascade.[197] These critical chemokines activate granulocytes such as eosinophils, neutrophils and basophils and have been shown to induce acute neutrophilic inflammation.[198] Dendritic cells are also affected by cysLTs, both expressing cysLTr1 as well as having the cellular machinery required for cysLT production.[199] Allergen-stimulated dendritic cells are potently affected by cysLTs and stimulation of bone marrow derived dendritic cells with stimulated with cysLTs induced the release of IL-10, IL-12, IL-5, and IFN-γ.[199, 200] In addition to cytokine and chemokine release, cysLTs are capable of enhancing dendritic cell-stimulated antigen presentation, T cell proliferation, and T cell cytokine production.[201, 202] CysLTs may also promote dendritic cell migration indirectly, through stimulating the release of dendritic cell chemoattractants from macrophages such as RANTES, MIP-1 and MIP-3. [203] It should be noted that the relationship between cysLTs and dendritic cells resulting in the development of allergic asthma is an area of great interest at the moment, however; in maintaining focus on the subject of this thesis and the role of cysLTs in non-allergic asthma further discussion on this topic will be minimal.

CysLTs have been shown to increase sensitivity of activated human neutrophils in vitro through increased calcium mobilization. Activated neutrophils stimulated with LTC₄ and LTD₄ produce more superoxide anion, neutrophil elastase and MMP-8 in comparison with activated neutrophils not exposed to exogenous LTC₄/LTD₄, a reaction that is attenuated by
montelukast.[204] This important study lends significance to the ability of cysLTs to play a role in both atopic and non-atopic related inflammation. Mechanisms by which cysLTs affect neutrophil activity may be cyclic AMP (cAMP) dependent as addition of montelukast to activated neutrophils led to inhibition of cAMP synthesis. [205] Anderson et al. demonstrated that neutrophils activated with the chemoattractant N-formyl-L-methionyl-L-leucyl-L-phenylalanine and incubated with montelukast showed a reduction in the generation of reactive oxygen species including superoxide and hypochlorous acid. Interestingly, montelukast was also able to prevent LTB₄ release from neutrophils stimulated with platelet-activating factor, suggesting several possibilities regarding the specificity of montelukast as well as the feed-forward mechanisms by which neutrophils respond to cysLTs.[205]

With regard to the contribution of cysLTs to structural cells in the lung, it has been established that cysLTs affect smooth muscle cells by increasing motor tone as well as an asthmatic phenotype through promotion of hypertrophy and hyperplasia in chronic severe asthma.[206] CysLTs induce the production of vascular endothelial growth factor (VEGF) in fibroblasts through mechanisms involving JNK and ERK. These findings appear to be cysLTr1 specific as HEK293 cells stably transfected with cysLTr1 and exposed to cysLTs had enhanced production of VEGF while exposure to JNK and ERK inhibitors attenuated this production.[207] Human fetal lung fibroblasts exposed to cysLTs and TGF-β increased collagen production and extra-cellular matrix as well as cysLTr1 and α-smooth muscle actin mRNA in exposed
In fibroblasts treated with IL-13, LTC₄ stimulation resulted in the production of eotaxin and striking upregulation of cysLTR1 mRNA, an effect attenuated by pre-treatment with montelukast. In this study, the stimulation of fibroblasts with LTC₄ in the absence of exogenous IL-13 was unable to increase eotaxin release, and cysLTR1 upregulation was minimal. The combination of IL-13, TGF-β and cysLTs resulted in bronchial smooth muscle cell proliferation, which was not achieved when any one compound was given individually. The findings of the aforementioned studies support the concept of synergistic roles that cysLTs play as mediators of airway remodeling and angiogenesis. Through stimulation with these various cytokines, chemokines and growth factors, the consequences of cysLTR1 upregulation, including increased binding capacity for cysLTs and therefore increased calcium mobilization results in increased contractility and proliferation. Though there is ongoing debate surrounding the exact contribution to the etiology of asthma, it seems reasonable that these cysLT-related changes in ASM affect the pathophysiology of asthmatics through creating hypersensitivity in the airways as well as loss of airway luminal space due to increased smooth muscle mass.

Studies showing the effects of cysLTs on epithelial cells have demonstrated a variety of roles and responses including increased production of eosinophil adhesion molecules such as ICAM-1, induction of mucin-related genes, and activation of pro-survival and proliferative pathways. Several biochemical pathways have also been explored including signal
transduction mechanisms. ERK1/2 and STAT-1 pathways are involved in pathways relating to cysLT actions in epithelial cells, with downstream effects such as cysLT-induced adhesion molecule production and MUC2 gene expression being inhibited by various inhibitors of these pathways including the protein kinase C (PKC) inhibitor (bisindolylmaleimide), a mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor (PD98059), an ERK-2 inhibitor (AG126). We also showed in the Brown Norway rat, exogenously administered LTD₄ resulted in increased goblet cell hyperplasia and bronchial epithelial cell proliferation and that an EGFR inhibitor (AG1478) attenuated this effect. Other groups have shown that alveolar epithelial cells exposed to LTD₄ have increased Na, K-ATPase activity and protein release in vitro, and reduced alveolar fluid clearance in the perfused rat lung.[211]

1.6 CysLTs and ROS

1.6.1 Correlation of cysLTs and Reactive Oxygen Species in Lung Disease

While many of the asthma-related studies examining the role of cysLTs is geared towards understanding a Th-2 driven model, there are effects of cysLTs that may be translated to other forms of asthma including IIA in the context of ROS as they relate to cysLTr1 signaling. Several studies have found correlative results between cysLT levels and oxidative stress in both human and animal models. In exhaled breath condensate of asthmatics 8-isoprostane, a marker of lipid peroxidation synthesized through the arachidonic acid pathway,[212] was found to strongly correlate with the concentration of
cysLTs and to increase with clinical severity of asthma.[213] Similar correlations between 8-isoprostane and cysLTs as well as increased levels of LTB₄, and PGE₂ were identified in the exhaled breath condensate of aspirin-sensitive asthmatics. Both cysLT and 8-isoprostane levels were reduced by steroid treatment while no reduction was seen in either PGE₂ or LTB₄ levels suggesting involvement of mechanisms beyond the 5-LO pathway.[214] A study of COPD patients during infectious exacerbation showed that exhaled hydrogen peroxide levels also correlated with elevated cysLT levels, and these levels were reduced by antibiotic therapy. Again, LTB₄ and PGE₂ levels remained stable and unaffected by treatment, despite decreases in cysLT and ROS and improved FEV₁.[215] In cystic fibrosis patients experiencing acute exacerbation, increased levels of 8-isoprostane, cysLT and PGE₂ have been observed, with strong correlations between degree of neutrophilic inflammation and cysLT concentrations in sputum. In this case, antibiotic treatment did not reduce isoprostane, cysLT, or PGE₂ levels while a reduction was seen in inflammatory cell numbers.[216] In a model of acute lung injury, hydrochloric acid was instilled into the lungs of TLR4 deficient mice with subsequent evaluation of respiratory mechanics, inflammatory cell influx, cysLT and oxidative stress levels. In TLR4 deficient mice, levels of cysLTs and 8-isoprostane were lower as well as neutrophil influx than in wild-type mice. These changes also correlated with improved respiratory mechanics including reduced levels of respiratory system resistance and elastance.[217]
1.6.2 CysLTs induce endogenous production of ROS

Direct evidence implicating cysLTs and ROS was demonstrated by Ravasi et al. who showed that cysLTr1-mediated ASMC proliferation required the activation of EGFR, ERK1/2 phosphorylation and importantly, the generation of ROS.[218] Stimulation of ASMC with LTD₄ triggered a four-fold increase in the production of ROS within 5 minutes of stimulation. Pre-treatment with montelukast prevented LTD₄-induced ROS generation and pre-treatment with NAC, an antioxidant, attenuated the LTD₄-induced increase in EGFR phosphorylation, ERK1/2 phosphorylation and BrdU incorporation (a measurement of DNA synthesis) and prevented proliferation. While excess ROS is deleterious, the activation of EGFR has also been shown to be important in preventing oxidative-stress induced apoptosis,[219] which may include cysLTr1-driven ROS-dependent activation. In epithelial cells, LTD₄ is capable of inducing increases in NADPH dehydrogenase activity and ADP/ATP ratio.[220] These effects result in the activation of pro-survival pathways. Specifically, LTD₄ induced the translocation of β-catenin to the mitochondria, where it associated with Bcl-2, activating T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription machinery and activating Nf-κb pathways.[220] In a murine model of oxidative-stress induced acute lung injury, β-catenin expression was found to be critical in regulating epithelial cell regeneration and matrix repair, again suggesting a role for endogenously produced cysLT-dependent ROS production as a protective mechanism against acute lung injury and facilitation of tissue repair.[221]
1.6.3 CysLTr1 Role in Oxidant-Driven Lung Injury

While there have been no studies that seek to directly support cysLTs and cysLTr1 as protective against oxidative injury, some studies have reported surprising results when attempting to demonstrate the deleterious role of cysLTr1. One such study published in 2004 utilized bleomycin to induce pulmonary fibrosis.[222] While treatment with bleomycin does induce fibrosis, it also causes epithelial cell apoptosis, neutrophil influx, lipid peroxidation and lactate dehydrogenase accumulation through oxidative stress-dependent mechanisms.[223, 224] In this study, cysLTr1-/- and cysLTr2-/- and LTC₄s -/- mice were generated to evaluate if chronic pulmonary injury was mediated via cysLTr2. Unexpectedly, the study determined that although LTC₄s-/- mice showed attenuated responses to bleomycin, cysLTr1-/- mice exhibited an exacerbated response. This response included increased septal thickening, inflammatory cells and extracellular matrix. They also found no difference in the concentrations of cysLTs, LTB₄ or PGE₂ in the BALF of cysLTr1-/- and wild-type controls. This finding contrasts with previous work showing that 5-LO-/- mice were protected against bleomycin-induced injury and did show elevated concentrations of PGE₂.[225] This finding further suggests that in an oxidative-stress driven model, functional cysLTr1 may be necessary to mount an appropriate response. Given the protection afforded to LTC₄s-/- animals, it can also be speculated that cysLTs themselves may not directly exert these protective effects, rather, the downstream signaling that is cysLTr1 and/or
cysLTr1 receptor interactions may be critical. Indeed, it has been shown that cysLTr1 interacts with P2Y receptors and are co-expressed on a variety of inflammatory cells which, when activated, result in the trafficking of cysLTr1 into the cell. It is postulated that, in the absence of this internalization process, the cell is unable to regulate and fine-tune its response to the increase of inflammatory mediators found in several pathological processes.[226]

1.7 Summary

Irritant induced asthma has emerged as an important and widespread asthma subtype. Although there are distinct mechanisms driving its pathogenesis, these mechanisms are not completely understood. It is likely that ROS may play dual roles as they may be a causative agent to induce airway injury but may also be important in reparative mechanisms. Neutrophils appear to play a pivotal role in the development of lung damage associated with IIA although their exact contribution to airway damage and AHR remains understudied. Neutrophils are potent producers of ROS, through the production of HOCl, and are recruited to sites of injury following exposure to inhaled irritants, making them a reasonable cell type to consider when exploring IIA mediated mechanisms.

Lipid mediators, and cysLTs in particular, as well as their receptors, have long been associated with allergic asthma, but remain understudied in the context of IIA despite strong correlative evidence associating them with ROS. As potent bronchoconstrictive agents, and given AHR as a hallmark of IIA, the release and mediation of cysLT synthesis in the context of IIA proves a
pertinent topic. Epithelial cells appear to be involved in damage and repair mechanisms during irritant-induced lung injury. These cells express high levels of cysLTr1 and may recruit neutrophils following cysLTr1 activation.

The purpose of this thesis was four fold. First, we sought detailed characterization of the mechanism of Cl₂ induced IIA in the mouse lung from the perspective of airway inflammation, AHR and induction of ROS. This approach included administration of a variety of anti-oxidants given as either preventive or rescue treatments following Cl₂ exposure. Second, we focused our attention on the role of inflammatory cells recruited to the airways following Cl₂ induced lung injury. We used various strategies to explore the role of macrophages, eosinophils and neutrophils to determine which cell type had the greatest influence on the development of AHR following Cl₂ inhalation in the mouse. Third, using *in vitro* methods, we established cysLT-stimulated epithelial cells readily release IL-8, a potent neutrophil chemoattractant, through an EGFR dependent mechanism. Lastly, we evaluated the role of cysLTr1 in a Cl₂ induced model of airway injury through utilization of cysLTr1 deleted mice. The work presented in this thesis sheds light on the mechanisms responsible for IIA induced-airway injury, through the use of a model using Cl₂ gas in mice. We have focused on the role of neutrophils during Cl₂ induced injury and the role cysLTr1 plays in their recruitment and activity in the lung.
# Table. 1.1

## Allergic vs. Non-allergic Asthma

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Figure 1.1

NRF-2 mechanism of action

Under basal conditions, NRF2 is restricted to the cytoplasm of the cell by remaining bound to Kelch-like ECH-associated protein 1 (KEAP1). NRF2 is targeted for ubiquitination and proteasomal degradation by the Cullin3-Rbx2 E2 ubiquitin ligase. Under oxidative stress conditions, KEAP1 dissociates from Nrf2 by the formation a disulfide bridge thus freeing NRF2 to translocate to the nucleus. NRF2 then forms a heterodimer with the small protein Maf and then they bind to the antioxidant response element (ARE), resulting in transcription of target genes.
Figure 1.2

Biosynthesis of leukotrienes from arachidonic acid pathway

Cytosolic phospholipase A2 (cPLA2) catalyzes the release of the lipid mediator arachidonic acid (AA) from the membrane phospholipids in which AA can in turn generate prostaglandins, lipoxins, or leukotrienes. Prostaglandins (e.g. PGE2) are lipid mediators generated from AA by the action of cyclooxygenase 1 or 2 (Cox 1/2) and prostaglandin synthases such as mPGES-1 (Ptges), which gives rise to PGE2. Lipoxins (e.g. LXA4) are AA metabolites formed by a combination of 15-lipoxygenase (15-LO) and 5-lipoxygenase (5-LO or Alox5). AA can also be metabolized by Alox5 and downstream enzymes such as leukotriene A hydrolase, which forms LTB4, or Cys-LTs (LTC4, LTD4, and LTE4).
Figure 3. Cytosolic phospholipase A2 (cPLA2) catalyzes the release of the lipid mediator arachidonic acid (AA) from membrane phospholipids in which AA can in turn generate prostaglandins, lipoxins, or leukotrienes. Prostaglandins (e.g. PGE2) are lipids mediators generated from AA by the action of cyclooxygenase 1 or 2 (Cox 1/2) and prostaglandin synthases such as mPGES-1 (Ptges), which give rise to PGE2. Lipoxins (e.g. LXA4) are AA metabolites formed by a combination of 15-lipoxygenase (15-LO) and 5-lipoxygenase (5-LO or Alox5). AA can also be metabolized by Alox5 and downstream enzymes such as leukotriene A hydrolase, which forms LTB4 or Cys-LTs (LTC4, LTD4, LTE4).
1.6 References


138. Lee, T.H., et al., Leukotriene E4-induced airway hyperresponsiveness of guinea pig tracheal smooth muscle to histamine and evidence for


Chapter 2

Dimethylthiourea Protects Against Chlorine Induced Changes in Airway Function in a Murine Model of Irritant Induced Asthma
2.1 Prologue

As discussed in chapter 1, exposure to Cl₂ induces IIA, characterized by airway hyperresponsiveness (AHR), airway inflammation and increased oxidative stress. Here, experiments are presented that characterize the airway effects of Cl₂ exposure in mice and establish the model. In addition we evaluate the effects of treatment with an antioxidant, dimethylthiourea (DMTU). Mice were exposed to Cl₂ gas using a nose-only exposure chamber for 5 minutes at varying concentrations of Cl₂. We sought to achieve AHR in response to inhaled methacholine (MCh) and airway inflammation, both hallmarks of IIA. We found that at higher concentrations of Cl₂, epithelial cells were denuded from the airways. Because we sought in later experiments to examine the role of bronchial epithelial cells, we selected 100ppm as our optimal concentration, giving increased AHR, inflammation, and without gross morphological damage to the bronchial epithelial cells. Twenty-four hours following Cl₂ exposure, mice were evaluated for AHR in response to increasing doses of MCh using a FlexiVent. We found that Cl₂ exposure increased respiratory system resistance and elastance. Additionally, inflammatory cell numbers were increased, with striking increases in neutrophils. It should be noted that during the experiments the mice recovered well and despite the Cl₂ exposure, maintained normal body condition and behavior both immediately following the Cl₂ exposure and at the 24 hour time point. Once we had established that Cl₂ induced AHR and inflammation, we selected the hydroxyl radical scavenger DMTU to administer either one hour pre- or one hour post-Cl₂
exposure. Because Cl₂ is scrubbed from the airways within about one hour, administration at these time points would allow us to evaluate if Cl₂ was directly or indirectly induced the effects observed at 24 hours. Results showed that pre-treatment with DMTU reduced total inflammatory cells 1 hour following Cl₂, but at 24 hours this effect was lost. Post-Cl₂ treatment with DMTU was able to prevent influx of total inflammatory cells, however. Regardless of treatment timing, DMTU was able to prevent neutrophil and lymphocyte influx at 24 hours, suggesting that neutrophil migration signals are triggered by events indirectly related to Cl₂ exposure. With regard to oxidative stress, 100ppm of Cl₂ may not have been a sufficient insult to induce the release of molecules resulting from oxidative processes, as demonstrated by our lack of signal in an Oxyblot and nitric oxide analysis on BAL samples. We did find strong signals in lung tissue for 4-HNE, and this effect was completely prevented by DMTU in both pre- and post-treatment scenarios. We last examined glutathione levels in both BAL supernatant and in BAL cellular fractions at 10 minutes, 1 hour and 24 hours following Cl₂. Changes in GSH and GSSG were observed at 10 minutes post exposure only and were prevented by DMTU treatment but the transient nature of the changes rendered this marker less useful than the more robust 4-HNE signal. Taken together, these results suggest that Cl₂ causes indirect airway damage resulting in increased AHR, neutrophil influx and increased oxidant stress in lung tissue. We show for the first time that these events can be prevented by post-treatment with the antioxidant DMTU.
2.2 Abstract

Exposure to chlorine (Cl\textsubscript{2}) causes airway injury, characterized by oxidative damage, an influx of inflammatory cells and airway hyperresponsiveness. We hypothesized that Cl\textsubscript{2}-induced airway injury may be attenuated by antioxidant treatment, even after the initial injury. Balb/C mice were exposed to Cl\textsubscript{2} gas (100 ppm) for 5 minutes, an exposure that was established to alter airway function with minimal histological disruption of the epithelium. Twenty-four hours after exposure to Cl\textsubscript{2}, airway responsiveness to aerosolized methacholine (MCh) was measured. Bronchoalveolar lavage (BAL) was performed to determine inflammatory cell profiles, total protein, and glutathione levels. Dimethylthiourea (DMTU; 100 mg/kg) was administered one hour before or one hour following Cl\textsubscript{2} exposure. Mice exposed to Cl\textsubscript{2} had airway hyperresponsiveness to MCh compared to control animals pre-treated and post-treated with DMTU. Total cell counts in BAL fluid were elevated by Cl\textsubscript{2} exposure and were not affected by DMTU treatment. However, DMTU-treated mice had lower protein levels in the BAL than the Cl\textsubscript{2}-only treated animals. 4-Hydroxynonenal analysis showed that DMTU given pre- or post-Cl\textsubscript{2} prevented lipid peroxidation in the lung. Following Cl\textsubscript{2} exposure glutathione (GSH) was elevated immediately following exposure both in BAL cells and in fluid and this change was prevented by DMTU. GSSG was depleted in Cl\textsubscript{2} exposed mice at later time points. However, the GSH/GSSG ratio remained high in chlorine exposed mice, an effect attenuated by DMTU. Our data show that the anti-oxidant DMTU is effective in
attenuating Cl\textsubscript{2} induced increase in airway responsiveness, inflammation and biomarkers of oxidative stress.
2.3 Introduction

Respiratory health is adversely affected by exposure to strong irritant substances such as chlorine (Cl\(_2\)) or ozone [1]. A single, acute exposure of persons to Cl\(_2\) in an industrial or domestic context may trigger asthma in a proportion of those exposed and is termed irritant-induced asthma [2,3]. High dose exposures may lead to acute lung injury and death [4]. Although the mechanism of the induction of asthma by irritants is uncertain, this form of asthma may be a significant contributor to the current rising prevalence of this disease. Some of the irritants that induce symptoms of asthma such as ozone and Cl\(_2\) cause oxidant injury, in particular to the airway epithelium. Desquamation of the airway epithelium and prolonged sub-epithelial inflammation accompanied by airway hyperresponsiveness has been documented following a single acute Cl\(_2\) inhalational exposure [5]. Epithelial shedding may adversely affect barrier function of the epithelium and may diminish the influence of epithelial-derived bronchodilator substances such as nitric oxide [6]. Cl\(_2\) is a highly reactive substance and has been documented to cause airway injury in mice that is associated with oxidant stress, as evidenced by the finding of peroxynitrite in the airway tissues and carbonylation of proteins [7]. There may be additional contributions to oxidant injury through activation of inflammatory cells [8]. The causative role of oxidative stress in the changes in airway function and airway inflammation caused by a potent oxidant like Cl\(_2\) is relatively under-investigated. Recently a combination of anti-oxidants (ascorbic acid, desferroxamine and N-acetylcysteine) was found
to attenuate signs of respiratory dysfunction, in particular gas exchange and microvascular leak, in the rat [9].

The current study was designed to examine the relationship between oxidant damage, airway hyperresponsiveness and inflammation caused by Cl₂ by testing the efficacy of an anti-oxidant in protecting against these effects. For this purpose we used dimethylthiourea (DMTU), an oxygen metabolite scavenger [10], that is highly cell-permeable [11-13]. We also wished to examine the effects of Cl₂ on markers of oxidative stress and whether DMTU attenuated these effects. We hypothesized that treatment with DMTU would ameliorate the inflammatory and pathophysiological effects induced by Cl₂ gas exposure whether administered before or after exposure.
2.4 Materials and Methods

Animals and protocol

Male Balb/C mice (18-22 g) were purchased from Charles River (Wilmington, Massachusetts) and housed in a conventional animal facility at McGill University. Animals were treated according to guidelines of the Canadian Council for Animal Care and protocols were approved by the Animal Care Committee of McGill University.

Mice were exposed to either room air (control) or Cl₂ gas diluted in room air for 5 minutes using a nose-only exposure chamber. An initial experiment was performed to assess an exposure level required to effect changes in airway responsiveness to methacholine (MCh) that was well tolerated by the animals. For this purpose we exposed mice to 100, 200 or 400 ppm Cl₂, and 24 hours later we performed MCh challenge and removed the lungs for histological analysis. Based on the results of this experiment we tested the effects of DMTU on animals exposed to 100 ppm Cl₂. The control mice were exposed to room air (Control; n = 6) and test mice were exposed to Cl₂ (Cl₂; 100 ppm; n = 6) with DMTU (100 mg/kg) treatment intraperitoneally either one hour before (DMTU/Cl₂; n = 6) or one hour after Cl₂ exposure (Cl₂/DMTU; n = 6). DMTU was prepared fresh prior to each exposure and a dose of 100 mg/kg in 500 μL of sterile phosphate buffered saline (PBS) was administered i.p. either one hour before or one hour following exposure to Cl₂. Control (air exposed) mice received 500 μL PBS i.p and Cl₂ exposed mice received 500 μL PBS i.p. either one hour before or one hour following
exposure. We chose the dose of DMTU based on previous observations of efficacy against an oxidant pollutant in mice [11]. At 24 hours after Cl$_2$ exposure, lung function measurements including responsiveness to aerosolized MCh were performed and bronchoalveolar lavage fluid was obtained for assessment of inflammatory cell counts, total protein, nitrate/nitrite (nitric oxide) and glutathione levels. The lungs were removed for analysis of carbonylated proteins and 4-hydroxynonenal (4-HNE). Measurements of inflammatory cell counts and glutathione levels in BAL fluid were made also at 10 min and at 1 hr after Cl$_2$. Following exposure animals were returned to the animal facility and allowed food and water ad libitum.

*Exposure to Cl$_2$*

Mice were restrained and exposed to Cl$_2$ gas for 5 minutes using a nose-only exposure device. Cl$_2$ gas was mixed with room air using a standardized calibrator (VICI Metronics, Dynacalibrator®, model 230-28A). The Cl$_2$ delivery system has two main components, a gas generator, which includes a heated permeation chamber and air flow generator. Dynacal permeation tubes designed specifically for operation with the Dynacalibrator were used and contain the Cl$_2$. The permeation chamber and air flow generator control accuracy of the Cl$_2$ generated to within 1-3% of the desired concentration (manufacturer's specifications). Within the gas chamber, permeation tubes containing Cl$_2$ are housed for gas delivery. The Teflon permeation tubes contain Cl$_2$ in both gas and liquid phases. When the tube is heated the
Cl₂ reaches a constant and increased vapor pressure and it permeates the tube at a constant rate. The desired concentration is delivered at an appropriate flow rate, as specified by the manufacturer. The device is attached to the exposure chamber and allowed to calibrate for 30 minutes until the optimum temperature of 30°C is reached and the Cl₂ flow is constant. Following removal of the animals from the exposure chamber, the chamber was continually flushed with the gas mix to ensure that the desired concentration of Cl₂ was maintained.

_Evaluation of Respiratory Responsiveness_

Mice were sedated with an intraperitoneal (i.p) injection of xylazine hydrochloride (8 mg/kg) and anaesthetized with i.p. injection of pentobarbital (30 mg/kg). Subsequently, the animal was tracheostomized using at 18 gauge cannula and connected to a small animal ventilator (FlexiVent, Scireq, Montreal, Canada). Muscle paralysis was induced with pancuronium bromide (0.2 mg/kg i.p.). The mice were ventilated in a quasi-sinusoidal fashion with the following settings: a tidal volume of 10 mL/kg, maximum inflation pressure of 30 cmH₂O, a positive end expiratory pressure (PEEP) of 3 cmH₂O and a frequency of 150/min. Following an equilibration period of 3 minutes of tidal ventilation two lung inflations to a transrespiratory pressure of 25 cm H₂O were performed and baseline measurements were taken. The respiratory mechanics were estimated using a single compartment model and commercial software (Scireq). Baseline was established as the average of three perturbations. Following establishment of baseline, MCh was administered using an in-line
nebulizer (Aeroneb Lab, standard mist model, Aerogen Ltd, Ireland) and progressively doubling concentrations ranging from 6.25 to 50 mg/ml were administered over 10 seconds synchronously with inspiration. Six perturbations each taken over 1.5s, at a frequency of 2.5Hz were used and data were calculated at each dose of MCh to establish the peak response. The highest value was kept for analysis subject to a coefficient of determination above 0.85. Respiratory system resistance (Rrs) and respiratory system elastance (Edyn) were determined before challenge and after each dose of MCh.

Bronchoalveolar Lavage Fluid Analysis

Following euthanasia (60 mg/kg pentobarbital, i.p.), the lungs were lavaged with 600 μl of sterile saline, followed by four separate aliquots of 1 ml each as previously described [7]. The first 600 μl/mL aliquot of BAL fluid was centrifuged at 1500 rpm for 5 minutes at 4°C and the supernatant was retained for measurements of nitric oxide, glutathione levels and protein levels using a Bradford Assay. The separate 1 mL aliquots were spun at 1500 rpm for 5 min at 4°C and the supernatant removed. The cell pellets were pooled for differential cell counts using 100 μl of the re-suspended cells. Cytospins were prepared, air-dried and stained (Diff-Quik® method, Medical Diagnostics, Düdingen, Germany). A differential cell count was determined on a minimum of 300 cells.
*Histology*

Following harvesting, the lungs were perfused with saline until the effluent was clear. The right lung was inflated with 1 mL 10% buffered formalin, fixed overnight with formalin. Tissues were embedded in paraffin blocks, cut into 5 μm sections and stained with hematoxylin and eosin. Sections were evaluated for epithelial morphological changes. The absolute number of epithelial cells in the airways was determined by counting cells on hematoxylin and eosin stained slides at 200× magnification and data were expressed as the number of epithelial cells per mm of basement membrane perimeter (P_{BM}). Epithelial cell height was determined by measuring the distance between the basement membrane and the top of the epithelial cell in the four quadrants for each airway and averaged.

*Measurement of Nitrite/Nitrate in BAL*

For the evaluation of nitric oxide, 0.6 N trichloroacetic acid was added to the supernatant of the BAL fluid to give a final concentration of 0.12 N to precipitate any protein. Samples were centrifuged for 10 minutes at 10,000 RPM followed by removal of the supernatant for analysis using previously described methods [7]. Total NOx was measured in BAL as an index of NO production using the Griess reaction. Briefly, 80 μl of sample were pre-incubated with 20 μl of NO3-reductase and 10 μl of its enzyme cofactor for 3 h at room temperature and then incubated with 100 μl of Griess reagent for 10 min. NOx concentrations were determined using standard curves obtained from
different concentrations of NaNO$_2$ or NaNO$_3$. Absorbance was measured at 540 nm with a plate reader (SLT 400 ATC; SLT Lab Instruments, Salzburg, Austria). No NO$_x$ was detected in saline solutions using this assay.

_Carbonylated protein residues (Oxyblot)_

An Oxyblot was performed on left lung tissue extracts taken 24 hours following Cl$_2$ challenge. Extracted proteins were denatured with 12% sodium dodecylsulfate (SDS) before derivatization with the addition of DNPH (2,4-dinitrophenylhydrazone-hydrazone). DNPH-derivatised proteins were separated on a 10% SDS-PAGE gel at 140 V for 2 h. Proteins were then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane with 11.6 mM Tris (Fisher), 95.9 mM glycine (Fisher) and 20% methanol (Fisher) at 25 V for 2 h. Membranes were then blocked with 1% bovine serum albumin-TTBS solution (0.02 M Tris base, 0.5 M NaCl, and 0.1% of Tween 20; Sigma) and were probed for 90 min with rabbit anti-DNP antibody (Intergen Company, Purchase, NY). The membranes were then rinsed in TTBS and incubated with HRP-conjugated goat anti-rabbit IgG (Intergen Company, Purchase, NY) for 1 h. A chemiluminescence detection system (ECL Plus; Amersham), Hyperfilm (Amersham), and Fluorochem 8000 software (Alpha Innotech Corporation, San Leandro, CA) were used for antibody detection and quantification by densitometry.

_Lung 4-hydroxynonenal (4-HNE) assay_
All reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Frozen tissue, or a known amount of 4-HNE standard (Cayman Chemical, Ann Arbor, MI, USA), was placed in 2 ml of cold methanol (Thermo Fisher) containing 50 μg/ml butylated hydroxytoluene, with 10 ng d3-4-HNE (Cayman Chemical) internal standard added just before homogenization with the Ultra-Turrax T25 (Thermo Fisher). An EDTA solution (1 ml of 0.2 M, pH 7) was added. Derivatization was accomplished by the addition of 0.2 ml of 0.1 M HEPES containing 50 mM O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride, pH 6.5. The mixture was then vortexed and held at room temperature. After 5 min, 1 ml of hexanes (Thermo Fisher) was added, and the samples were shaken vigorously. Brief centrifugation was performed to achieve phase separation and the O-pentafluorobenzyl-oxime derivatives were extracted from the upper hexane layer. The sample was dried under a stream of N2 gas and further derivatized into trimethylsilyl ethers by the addition of 15 μl each of pyridine and N, O bis(trimethylsilyl)trifluoroacetamide. The samples were vortexed and heated to 80°C for 5 min and then analyzed for 4-HNE content by GC/MS. GC/MS analysis was performed using a Focus GC coupled to a DSQ II mass spectrometer and an AS 3000 autosampler (Thermo Fisher). A 15-m TR-5MS column (0.25-mm i.d., 0.25-μm film thickness; Thermo Fisher) was used with ultrahigh-purity helium as the carrier gas at a constant flow rate of 1.0 ml/min. Two microliters of sample was injected into the 270°C inlet using split mode with an injection ratio of 10 and a split flow of 10 ml/min. The initial oven temperature was 100°C and then ramped to
200°C at 15°C/min, followed by an increase in temperature to 300°C at 30°C/min, and held for 1 min. The MS transfer line temperature was held constant at 250°C and the quadrupole at 180°C. Analysis was done by negative-ion chemical ionization using 2.5 ml/min methane reagent gas. Ions were detected using SIM mode with a dwell time of 15.0 ms for each fragment of 4-HNE at m/z 152, 283, and 303, and d3-4-HNE at m/z 153, 286, and 306. Under these conditions, the larger, second peak of the two 4-HNE isomers was used for quantification and exhibited a retention time of 7.18 min, which was just preceded by the elution of d3-4-HNE at 7.17 min. Quantification was performed using a standard curve generated by graphing the area ratio of 4-HNE to d3-4-HNE versus concentration.

Measurement of glutathione (GSH and GSSG) in BAL fluid and cells

BAL fluid from control, chlorine exposed and DMTU pre-treated chlorine exposed mice was collected for glutathione evaluation by HPLC. Both glutathione (GSH) and glutathione disulfide (GSSG) were measured to determine if GSH had converted to GSSG. As GSH is found almost exclusively in its reduced form, a conversion to GSSG, which is inducible following oxidative stress, would indicate an increase in oxidative stress in the lung. BAL samples were collected at 10 minutes, one hour and 24 hours after Cl₂ challenge. Phosphoric acid (60 μL; 1 M) was added to BALF samples to prevent GSH degradation. BAL was centrifuged at 1500 RPM for 5 minutes, and the supernatant was removed for evaluation of extracellular GSH/GSSG
and 150 μL of PBS and 15 μL 1 M phosphoric acid added was used to reconstitute the pellet for analysis of intracellular GSH and GSSG. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (150 μL; 6 mM) was added to lyse the cells. GSH and GSSG were measured by RP-HPLC using a post-column derivatization procedure modified from the literature [14]. GSH and GSSG levels were determined in 50 μl aliquots by RP-HPLC using a gradient prepared from 0.05% trifluoroacetic acid (TFA) in water (solvent C) and 0.05% TFA in acetonitrile (solvent D) as follows: 0 min, 0% D; 10 min, 15% D. The flow rate was 1 ml/min and the stationery phase was a column (150 × 4.6 mm) of Ultracarb ODS (31% carbon loading; 5 μm particle size; 150 × 4.6 mm; Phenomenex, Torrance, CA). The eluate from the column was mixed with o-phthalaldehyde (370 μM) in 0.2 M tribasic sodium phosphate, pH 12, which was pumped into a T-fitting using an auxiliary pump (Waters Reagent Manager). The mixture then passed through a loop of PEEK tubing (6 m × 0.5 mm, i.d.; volume, 1.2 ml) that was placed in a water bath at 70°C. Under these conditions both GSH and GSSG are converted to a fluorescent isoindole adduct, which is measured using excitation and emission wavelengths of 336 and 420 nm, respectively. Prior to introduction into the fluorescence detector (Waters model 2475 Multi wavelength Fluorescence Detector), the mixture was cooled in a small ice-water bath and passed through a filter containing an OptiSolv 0.2 μm frit (Optimize Technologies). The amounts of GSH and GSSG were determined from a standard curve using the authentic compounds as external standards.
Statistical analysis

Data were analyzed using an analysis of variance and for post hoc comparisons of means a Newman-Keuls test was used. A p < 0.05 was accepted as significant. All values are expressed as the mean + one standard error of the mean.
2.5 Results

Concentration-dependent changes in airway responsiveness following Cl₂

To establish a suitable submaximal concentration of Cl₂ for subsequent experiments animals were exposed to 100 ppm, 200 ppm or 400 ppm of Cl₂ for 5 minutes. The next day, the animals were challenged with doubling doses of MCh ranging from 6.25 to 50 mg/ml. Respiratory system resistance (Figure 2.1A) and elastance (Figure 2.1B) were evaluated. There was a dose-dependent increase in responsiveness to MCh reflected in both of the above parameters of lung function.

Histological changes in the airways after Cl₂ exposure

The effects of Cl₂ on airway architecture were assessed on hematoxylin and eosin stained lung sections obtained 24 hours after exposure (Figure 2.2). Lower concentrations of Cl₂ (100 ppm and 200 ppm) did not result in any detectable change under light microscopy to the airway epithelium (Figure 2.2A and 2.2B). There was an obvious thinning of the airway epithelium at a concentration of 400 ppm (Figure 2.2C). There were statistically significant differences observed in epithelial cell height caused by exposure to Cl₂ (Figure 2.2E). We also quantified the number of epithelial cells in the airway walls. While there was no significant difference in cell following exposure to Cl₂ at 100 ppm compared to control (Figure 2.2D), at 400 ppm, there were fewer epithelial cells compared to both control and 100 ppm (Figure
2.2D). Given the lack of gross histological change induced by 100 ppm of Cl₂ we chose to perform further studies using this concentration.

Effect of DMTU on MCh responsiveness following Cl₂ challenge

Airway responses to increasing doses of MCh (6.25-50 mg/ml) were elevated 24 h following Cl₂ challenge (Figure 2.3A). This effect was attenuated by administration of DMTU given both prior to and post Cl₂-exposure. Changes in respiratory system elastance in response to MCh paralleled those observed for resistance (Figure 2.3B). DMTU alone had no significant effect on MCh responsiveness.

Changes in bronchoalveolar lavage cells after Cl₂ gas exposure

To assess the effects of Cl₂ on airway inflammation and epithelial cell shedding bronchoalveolar lavage was performed at 10 minutes, one hour and at 24 hours after Cl₂ exposure. The fluid recovered by BAL averaged 75% of the volume instilled and did not differ significantly among the groups. Total cell counts were not significantly different at 10 minutes after exposure to Cl₂ (Figure 2.4A) but were significantly increased in Cl₂ treated groups by one and 24 hours compared to control (Figure 2.4B and 2.4C). At one hour, pre-treatment with DMTU reduced the total number of inflammatory cells present in the airways compared to Cl₂ only mice. At 24 hours, total cell counts were persistently elevated after Cl₂ and were attenuated only in mice post-treated with DMTU after Cl₂ exposure (Figure 2.4C). Cl₂ caused a significant increase
in neutrophils and lymphocytes 24 hours following challenge, an effect attenuated by both pre- and post-treatment with DMTU (Figure 2.5C and 2.5D). There were no significant changes in any of the cell subsets at 10 mins (Figure 2.5A, 2.5B and 2.5E).

Changes in protein level following Cl\(_2\) exposure

We measured the total protein level in BAL fluid harvested at 1 and 24 hours after Cl\(_2\) exposure to assess the effects of Cl\(_2\) on cell damage and protein levels. At both time points following Cl\(_2\) exposure there was a significant increase in total protein in the BAL fluid as assessed by the Bradford assay. Treatment with DMTU, both before and after Cl\(_2\) exposure reduced protein levels in BAL (Figure 2.6).

Effects of Cl\(_2\) on markers of oxidative stress

Nitric oxide concentrations were determined using the Griess reaction and no significant change was seen between any of groups 24 hours following Cl\(_2\) challenge (Figure 2.7A). An OxyBlot was performed on lung extracts to detect proteins modified by oxygen metabolites 24 hours following Cl\(_2\) exposure. Levels of carbonylation were quantified by densitometry and no substantial difference was seen among control, Cl\(_2\) treated or DMTU treated animals (Figure 2.7B). Lungs were removed 24 hours following Cl\(_2\) treatment for analysis of 4-HNE by GC-MS. Cl\(_2\) induced a significant increase in 4-HNE
levels (Figure 2.7C). DMTU given either pre- or post- Cl₂ treatment prevented any significant changes in 4-HNE levels (Figure 2.7C).

**Effects of Cl₂ and DMTU treatments on GSH and GSSG intracellularly and extracellularly in the bronchoalveolar compartment**

Cl₂ increased both intracellular (Figure 2.8A) and extracellular (Figure 2.8B) GSH levels in BAL after 10 min, but had no effect on GSH levels after 1 and 24 hours (Figure 2.8C and 2.8D). Treatment with DMTU prior to administration of Cl₂ blocked the increase in GSH in both compartments at 10 min (Figure 2.8A and 2.8B) but had no effect on GSH levels at the later time points (Figure 2.8C and 2.8D). Cl₂ induced a significant increase in GSSG levels in the intracellular and extracellular compartments at 10 min (Figure 2.9A and 2.9B). At 1 and 24 hours there was a decrease in GSSG levels in Cl₂ treated groups compared to control and DMTU treated groups that were restored by DMTU treatment (Figure 2.9C and 2.9D). The ratio of GSH/GSSG was significantly higher in the cell fraction of BAL in Cl₂ exposed mice than control and DMTU treated mice at 10 minutes (Figure 2.10A). There was a trend towards a decrease in GSH/GSSG ratio in the extracellular compartment of the BAL at the same time point, but this was not statistically significant. Additionally, at 24 hours, the GSH/GSSG ratio remained high in the Cl₂ treated mice but was attributable to a decline in GSSG at this time (Figure 2.10D). This effect was prevented by treatment with DMTU (Figure 2.10A and 2.10D).
2.6 Discussion

In the current study we have shown that Balb/C mice exposed to Cl$_2$ gas for 5 min develop concentration-dependent airway hyperresponsiveness to inhaled, aerosolized MCh. At concentrations of Cl$_2$ greater than 100 ppm there is evidence of epithelial damage with flattening of the cells and the shedding of ciliated cells into the BAL. However, at a concentration of Cl$_2$ (100 ppm), despite the lack of gross morphological changes in epithelial cells there was still a substantial degree of airway hyperresponsiveness, an effect potentially attributable to increased oxidative stress. The effect of Cl$_2$ on airway function was attenuated by pre-treating the mice one hour before Cl$_2$ exposure with an intraperitoneal injection of DMTU. Treatment with DMTU 1 hour after exposure to Cl$_2$ also ameliorated the adverse effects on airway function. Oxidative injury to lung tissue was detected 24 hours post-Cl$_2$ exposure and indicated by an increase lipid peroxidation in Cl$_2$ exposed mice, an effect attenuated by pre- or post-Cl$_2$ treatment with DMTU. Additionally, DMTU treatment maintained GSH/GSSG levels at those of control mice, whereas Cl$_2$ only treated mice showed significant changes in both GSH and GSSG at various time points.

Airway hyperresponsiveness has been previously demonstrated to follow Cl$_2$ exposure in both rat and mouse models of irritant induced asthma [15,16]. Pathological changes including airway remodeling occur following a single exposure to a high concentration of Cl$_2$ in rats [17]. It seems likely that epithelial damage is a major contributor to the altered responsiveness to inhaled
MCh. The epithelium could serve as a barrier that could reduce access of MCh to the smooth muscle or might attenuate the responsiveness to MCh through the release of relaxant substances such as NO or prostaglandins [18-20]. The mechanism of AHR following Cl₂ may be similar to that of ozone in that both forms of injury are associated with oxidant damage to the tissues. Natural killer cells and interleukin-17 have been shown recently to be essential in the protection against airway damage and hyperresponsiveness following repeated ozone exposures [21]. Cl₂ potentially causes toxicity through its highly reactive nature. However, it is also known to cause damage through the generation of hydrochloric acid (HCl). Indeed HCl has been shown to cause airway hyperresponsiveness in mice when administered into the airways, by mechanisms that have been suggested to relate to epithelial barrier function. However, it has been shown that HCl is much less toxic than Cl₂ so it is likely that the effects of Cl₂ induced oxidants are more likely to account for its adverse effects [22,7].

Irrespective of the mechanism of Cl₂ induced airway hyperresponsiveness, DMTU was highly effective in preventing its development when given either as a pre-treatment or as a rescue treatment. Assuming that the therapeutic effects of DMTU are indeed mediated by antioxidant properties, the data suggest that the initial direct oxidative stress caused by Cl₂ is only part of the oxidative burden and that another source of reactive oxygen is important in the time period between 1 and 24 h following Cl₂ exposure. For example, secondary activation of neutrophils, macrophages
or epithelium and various chemokines, cytokines and growth factors they secrete could conceivably contribute to airway damage in a mechanism similar those shown for respiratory viral infection [23].

Measures of oxidant injury such as nitric oxide production, as reflected in BAL nitrates/nitrites, and protein carbonylation were not detectably different from control animals at 24 hours after Cl₂ exposure, consistent with a relatively mild injury compared to previous results [7]. However, presence of oxidative stress was apparent following assessment of lung tissue levels of 4-HNE, an indication of lipid peroxidation. 4-HNE levels were reduced to baseline by pre- and post-Cl₂ treatment with DMTU, suggesting that lipid peroxidation is a prolonged effect of exposure to Cl₂ further supporting the conclusion that the amelioration of markers of airway injury is likely mediated by anti-oxidant properties of DMTU.

Glutathione is an important endogenous antioxidant and changes in its intracellular and extracellular concentrations are expected following an oxidant challenge such as Cl₂. Generally oxidant stress is noted to diminish GSH both intracellularly and extracellularly in the lung (reviewed in [24]) although glutathione increases as an adaptive response to oxidative stress associated for example with cigarette smoking or pulmonary infection [25,26]. We found that Cl₂ exposure induced rapid and transient changes in glutathione concentrations. Ten minutes following exposure there was a surge in both intra- and extracellular GSH levels in BAL, presumably attributable to GSH synthesis and export into the extracellular milieu. Additionally, Cl₂ may induce lysis of
pulmonary cells, especially epithelial cells which might also contribute to the large amount of extracellular GSH. Epithelial cells are known to contain high levels of GSH [25] and high doses of Cl₂ have been shown to cause epithelial cell shedding and/or lysis. However the changes in GSH observed in the current experiment occurred in the absence of significant changes in epithelial cell counts in BAL fluid or in epithelial cell numbers enumerated in the airway walls themselves. The changes in GSH were transient and had resolved by 1 hour. The rapid rise in GSH was prevented by pre-treatment with DMTU prior to Cl₂ exposure, suggesting a measure of relief against the effects of oxidative stress.

In addition to the early spike in GSH concentration in BAL cells and fluid, we also noted a significant increase in GSH in its oxidized form, glutathione disulfide (GSSG), both intra-and extracellularly at 10 minutes, presumably indicative of oxidative stress in the lung. These changes were abrogated by DMTU supporting the idea that the mechanism of protection was through neutralization of oxygen metabolites. Furthermore, the protection provided by delayed treatment with DMTU further suggests that delayed oxidative stress is also a significant contributor to the response to injury. By 1 and 24 hours, GSH levels were restored but GSSG levels showed a significant decrease in chlorine exposed groups. It is not clear what the significance of this finding is for airway function. Despite the GSSG levels being depleted at this time point, the ratio of GSH/GSSG was higher in chlorine exposed mice compared with controls and DMTU treated animals. The anti-oxidants ascorbic
acid, desferroxamine and N-acetyl-L-cysteine have been show to ameliorate the injury caused by Cl₂ in the rat [9]. In these experiments there was evidence of depletion of GSH by Cl₂, an observation that we have not confirmed. However the exposure in the rat was substantially greater (400 ppm for 30 minutes).

Consideration of oxidative stress as a target in irritant-induced asthma caused by potent oxidants is reasonable. However, oxidative stress-induced damage may also contribute to other forms of asthma. Asthmatic subjects manifest evidence of oxidative stress, as evidenced by a variety of changes including increased superoxide generation from leukocytes, increased total nitrites and nitrates, increased protein carboxyys, increased nitric oxide in exhaled breath condensate, increased lipid peroxidation products and decreased protein sulphhydryls in plasma [26]. They also show increased superoxide dismutase activity in red blood cells, increased total blood glutathione, and decreased glutathione peroxidase activity in red blood cells and leukocytes. A recent epidemiological study of childhood asthma demonstrated significant decreases in glutathione and amino acid precursors of glutathione as well as various other components of both enzymatic and non-enzymatic endogenous antioxidant defense mechanisms [27]. Thioredoxin, a reducing protein, may also inhibit experimental allergic asthma and airway remodeling [28].

In conclusion, exposure to modest levels of Cl₂ (100 ppm) leads to an increase in airway responsiveness in mice. Mice exposed to Cl₂ showed
increases in total inflammatory cells, in particular neutrophils and lymphocytes. Despite lack of increases in nitrate/nitrite or carbonylated proteins, lipid peroxidation levels (4-HNE) were significantly higher in Cl₂ exposed animals. Importantly, there was also evidence of a salutary treatment effect when DMTU was administered as late as 1 hour after the exposure to Cl₂ suggesting that oxidative damage is an ongoing process following the initial injury. Treatment with anti-oxidants shortly after acute exposure to highly irritant oxidant substances such as Cl₂ may have therapeutic utility.
Figure 2.1

Dose-response effect of Cl₂ on respiratory responsiveness to methacholine

Mice were either unchallenged (Control; n = 6) or challenged with 100 (n = 6), 200 (n = 6) or 400 (n = 6) ppm Cl₂ gas. After 24 h, total respiratory system resistance (A) and respiratory system elastance (B) in response to saline (Sal) and doubling doses of MCh were assessed using a small animal ventilator (FlexiVent). Baseline (Base) values obtained from untreated mice are shown for comparison. Mice treated with all three concentrations of Cl₂ showed significantly higher respiratory system resistance and at 12.5, 25, and 50 mg/ml of MCh as compared with control. * p < 0.05, n = 6 per group.
Figure 2.2

Effects of Cl₂ on airway histology

Twenty-four hours following Cl₂ exposure lungs were collected, paraffin embedded and lung sections cut (5 μM). Sections were then stained with hematoxylin and eosin. Representative pictures of airway sections from control mice (A) mice treated with 100 (B), or 400 ppm (C) Cl₂. Total epithelial cells were quantified in each airway and corrected for PBM and showed no difference between control and 100 ppm, but significantly fewer epithelial cells at 400 ppm (D). Epithelial cell height was also calculated and showed that mice given 100 ppm and 400 ppm had shorter epithelial cells than control (E).
Figure 2.3

Effects of Cl₂ on methacholine respiratory system resistance and elastance

Panel A shows the effects of 100ppm Cl₂ exposure on total respiratory system resistance in mice that were treated with before and 1 hour after exposure with DMTU. A two-way ANOVA showed that there is a significant difference between mice pre- or post-treated with DMTU when compared to animals receiving Cl₂ only. Panel B shows the effects of Cl₂ exposure and DMTU treatment on total respiratory system elastance. DMTU/Cl₂ treated animals had elastance levels similar to control whereas Cl₂ only treated mice had significantly higher values compared to control: n = 6 per group; * p < 0.05.
Figure 2.4

Effects of Cl₂ exposure on the numbers of cells in BAL fluid

Data for control and Cl₂ (100 ppm) exposed animals that were sacrificed 10 minutes (A), 1 hour (B) and 24 hours (C) after Cl₂ exposure. Cl₂ exposure caused a significant increase in total leukocytes compared to controls at 1 hour and 24 hours, the effect of which was attenuated by pre-treatment with DMTU at one hour and post treatment with DMTU at 24 hours. (n = 6 per group; * p < 0.05, **p < 0.01, ***p < 0.001).
Figure 2.5

Cellular composition of BAL fluid following Cl₂ exposure at 100 ppm

Differential cell counts were done at 10 minutes and 24 hours. No cell subset was significantly different at 10 min (data not shown). At 24 hours neutrophils and lymphocytes were significantly elevated in Cl₂ groups. Treatment with DMTU was limited increases in these cell types. There was no difference between control and DMTU treated groups. Control (n = 9), Cl₂ 100 ppm (n = 7), DMTU/Cl₂ (n = 7), Cl₂/DMTU (n = 6); * <0.05.
A. Macrophages 24 h

B. Eosinophils 24 h

C. Neutrophils 24 h

D. Lymphocytes 24 h

E. Epithelial cells 24 h
Figure 2.6

Effects of Cl₂ exposure and DMTU treatment on BAL fluid protein

Protein levels in BAL fluid were assessed by Bradford assay. There was a significant increase in total protein at 1 and 24 hours after 100ppm Cl₂ exposure. Pre-treatment with DMTU attenuated the increase in protein at both time points and at 24 hours when given one hour post- Cl₂ exposure. (n = 6-9/group; * p < 0.05, **p < 0.01, ***p < 0.001).
Figure 2.7

Effects of 100ppm Cl₂ exposure and DMTU treatment on markers of oxidative stress

Nitric oxide was also measured 24 hours following Cl₂ exposure using a Griess reaction and no significant change was seen between any of groups. (B) Twenty-four hours following Cl₂ exposure BAL was collected and an OxyBlot was performed on lung tissue homogenates to detect carbonylated proteins. No significant differences were detected among the groups. (C) Twenty-four hours following chlorine exposure, lungs were collected for 4-HNE analysis. Chlorine caused a significant increase in 4-HNE levels over control and DMTU treated groups. There were no differences between DMTU groups and baseline. (n = 6-10, * p < 0.05).
Figure 2.8

Effects of 100ppm Cl₂ exposure and DMTU treatment on glutathione levels in BAL fluid and cells.

(A) 10 minutes following Cl₂ exposure, GSH levels in the BAL cell fraction show a significant increase that was attenuated by pre-treating the mice with DMTU one hour prior to Cl₂ challenge. (B) 10 minutes following Cl₂ challenge, the same significant increase of GSH is seen in the BAL supernatant. (C) GSH levels 1 hour following Cl₂ exposure and (D) 24 hours after Cl₂ exposure were not different among groups. (n = 6-9; * p < 0.05, **p < 0.01, ***p < 0.001).
Figure 2.9

Effects of Cl₂ exposure and DMTU treatment on oxidized glutathione in BAL fluid cells and supernatant

Ten minutes after 100ppm Cl₂ exposure (A-B), oxidized GSSG levels were determined. Animals exposed to Cl₂ had increased GSSG in the BAL fluid and intracellularly 10 min following Cl₂ exposure (A & B). Extracellular GSSG was reduced at one hour and 24 hours following Cl₂ challenge, but no differences were found between control and DMTU treated groups.(n = 6-9, *p < 0.05, **p < 0.01, ***p < 0.001).
Figure 2.10
Effect of Cl₂ exposure and DMTU on ratio of GSH/GSSG.

(A) Ten minutes following 100ppm Cl₂ exposure the ratio of GSH/GSSG in the intracellular fraction of the BAL was significantly increased in Cl₂ exposed mice compared to control and DMTU/Cl₂ treated animals. (B-C) The extracellular fractions of the BAL at ten minutes and 1 hour showed no differences between groups. (D) Cl₂ exposure induced a significant increase in the ratio of GSH/GSSG, an effect attenuated by DMTU. (n = 6-9; * p < 0.05, ** p < 0.01, *** p < 0.001).
2.7 References


Chapter 3

AEOL10150: a novel therapeutic for rescue
treatment after toxic gas lung injury
Prologue 3.1

In Chapter 3, we elaborate on the model established in Chapter 2 to induce airway damage following Cl₂ exposure in mice. For these experiments, we utilized a novel antioxidant, AEOL10150 (AEOL). This compound is unique in its action as it is a catalytic metalloporphorin that has peroxynitrite SOD and catalase-like activities as well as inhibiting peroxynitrate. AEOL was synthesized by our collaborator, Dr. Brian Day, at the National Jewish Health in Denver, Colorado as part of the CounterACT program (NIH funded counterterrorism program) as a potential rescue treatment for soldiers exposed to Cl₂ during combat. Results presented show that we again we successful at inducing AHR and airway inflammation, characterized by severe neutrophilia by exposing mice to Cl₂ at 100ppm for 5 minutes. AEOL was administered 1 and 9 hours following exposure to Cl₂. Twenty-four hours later, we found that AEOL was successful at preventing AHR in response to inhaled methacholine, inflammatory cell influx and 4-HNE increases in lung tissue. We evaluated airways for proliferating bronchial epithelial cells 72 hours following Cl₂ exposure using an immunohistochemical stain for Ki-67, which labels nuclei of cells that are actively proliferating. We observed that Cl₂ exposure results in bronchial epithelial cell proliferation and this effect was prevented by post-Cl₂ treatment with AEOL. This result is significant in that it again highlights the indirect effects of Cl₂ exposure. We conclude that AEOL is successful as a rescue treatment following Cl₂ exposure and has potential to be a novel and pertinent therapeutic.
3.2 Abstract

New therapeutics designed as rescue treatments after toxic gas injury such as from chlorine (Cl2) are an emerging area of interest. We tested the effects of the metalloporphyrin catalytic antioxidant AEOL10150, a compound that scavenges peroxynitrite, inhibits lipid peroxidation, and has SOD and catalase-like activities, on Cl2-induced airway injury. Balb/C mice received 100 ppm Cl2 gas for 5 minutes. Four groups were studied: Cl2 only, Cl2 followed by AEOL10150 1 and 9 hours after exposure, AEOL10150 only, and control. Twenty-four hours after Cl2 gas exposure airway responsiveness to aerosolized methacholine (6.25–50 mg/ml) was measured using a small-animal ventilator. Bronchoalveolar lavage (BAL) was performed to assess airway inflammation and protein. Whole lung tissue was assayed for 4-hydroxynonenal. In separate groups, lungs were collected at 72 hours after Cl2 injury to evaluate epithelial cell proliferation. Mice exposed to Cl2 showed a significantly higher airway resistance compared to control, Cl2/AEOL10150, or AEOL10150-only treated animals in response to methacholine challenge. Eosinophils, neutrophils, and macrophages were elevated in BAL of Cl2-exposed mice. AEOL10150 attenuated the increases in neutrophils and macrophages. AEOL10150 prevented Cl2-induced increase in BAL fluid protein. Chlorine induced an increase in the number of proliferating airway epithelial cells, an effect AEOL10150 attenuated. 4-Hydroxynonenal levels in the lung were increased after Cl2 and this effect was prevented with AEOL10150. AEOL10150 is an effective rescue treatment for Cl2-induced airway
hyperresponsiveness, airway inflammation, injury-induced airway epithelial cell regeneration, and oxidative stress.
3.3 Introduction

Chlorine (Cl\textsubscript{2}) is a highly reactive oxidant gas that is used in the bleaching of paper, in the production of hydrocarbon solvents, in the disinfection of swimming pools, and as a chemical weapon (1-3). Five-year cumulative data between 1988 and 1992 from the American Association of Poison Control Centers' National Data Collection System reported 27,788 exposures to Cl\textsubscript{2} in the United States (1). Acute human exposures have occurred as a result of industrial accidents or during wartime that have led to long-term respiratory dysfunction and even death (2). Residual effects after acute Cl\textsubscript{2} damage can persist for years and include decreased vital capacity, reduced diffusing capacity, and lowered total lung capacity with a trend toward higher airway resistance (4) and (5). There are no effective pharmacological rescue treatments currently available.

There have been several experimental and case studies performed in both animal and human models characterizing the effects of Cl\textsubscript{2} gas exposure on the respiratory system (3-7). After initial exposure, injury is generally characterized by an influx of inflammatory cells into the airways, specifically neutrophils, lymphocytes, eosinophils, and macrophages. In addition, epithelial apoptosis and necrosis and airway hyperresponsiveness can occur (7) and (8). Epithelial cell damage has been observed in rodents exposed to Cl\textsubscript{2} gas, including denudation of the epithelium, followed by repopulation of the epithelial cell layers (8).
The molecular properties of Cl\(_2\) are such that it has an extremely high propensity to oxidize. It has been shown to have greater toxicity than nitrogen dioxide (NO\(_2\)), oxygen (O\(_2\)) or ozone (O\(_3\)), a property that may be related, in part, to its high water solubility (9). The hydration of Cl\(_2\) leads to the production of hydrochloric acid (HCl) and hypochlorous acid (HOCl). It is therefore likely that oxidative injury is also involved in the damage and repair processes (10) and (11). Consistent with this idea, Cl\(_2\) gas is about 30-fold more potent than hydrochloric acid, further emphasizing its oxidant, rather than acidic, properties as being the predominant mechanism responsible for its actions (3) and (12). When administered into the airways hydrochloric acid causes airway hyperresponsiveness in mice by mechanisms that have been suggested to relate to epithelial barrier function (13). Epithelial cells are particularly susceptible to Cl\(_2\) damage and have been implicated as key targets in the damage and repair process. They are among the first cells to encounter Cl\(_2\) in the airway and may be affected by the direct toxicity of Cl\(_2\) or indirectly through its by-products HOCl and HCl. Additionally, epithelial cells are capable of storing, producing, and releasing large quantities of the antioxidant glutathione in response to oxidative stress (13).

The aim of this study was to assess the efficacy of a novel catalytic antioxidant in ameliorating airway damage when administered after an acute exposure to inhaled Cl\(_2\) gas. For this purpose, we utilized a catalytic metalloporphyrin that is a member of a novel class of low-molecular-weight antioxidants. The compound, Mn(III) tetrakis-(N,N'-diethylimidizolium-2-yl)
porphyrin, AEOL10150 (AEOL), efficiently scavenges peroxynitrite, inhibits lipid peroxidation, and has SOD and catalase-like activities(14). Recent studies using the sulfur mustard analog 2-chloroethylethyl sulfide (CEES), a compound that also can induce oxidative stress in the lung, have demonstrated that AEOL is effective in reducing cytotoxicity and mitochondrial dysfunction when given 1 h after CEES exposure. In vivo, AEOL has shown promising therapeutic properties in protecting the lungs of rats exposed to CEES against inflammatory cell infiltration, reactive oxygen species, and DNA damage (14). In this study, we used a mouse model to further characterize the effects of Cl₂ gas as well as the ability of AEOL to rescue the lungs from damage caused by Cl₂ inhalation by examining airway function, airway inflammation, bronchial epithelial cell proliferation, and markers of oxidative stress.
3.4 Materials and Methods

Animals and protocol

Male Balb/C mice (18–22 g) were purchased from Charles River (Wilmington, MA, USA) and housed in a conventional animal facility at McGill University. Animals were treated according to the guidelines of the Canadian Council for Animal Care and protocols were approved by the Animal Care Committee of McGill University. Animals were provided with water and food ad libitum throughout the experiment. Four groups were studied: Cl₂ only (n = 10), Cl₂ followed by AEOL (n = 10), AEOL only (n = 10), and control (n = 10). Mice in groups treated with AEOL were given 5 mg/kg intraperitoneally (ip) 1 and 9 hours after Cl₂ exposure. Mice in control or Cl₂-only groups were given 1 ml of phosphate-buffered saline (PBS; pH 7.4) ip 1 and 9 hours after air or Cl₂ exposure. Mice were studied at 24 hours after initial Cl₂ exposure. In separate groups (n = 6/group) mice were studied at 72 hours after initial Cl₂ exposure to evaluate epithelial cell proliferation in the lungs. There was no significant weight loss or behavioral signs of distress at any time point after Cl₂.

AEOL10150 pharmacokinetics and safety profile

Male C57Bl/6 mice were given a single bolus dose of AEOL intravenously by tail-vein injection and blood samples were drawn upon sacrifice by cardiac puncture (two mice/time point). Blood samples were obtained at 5, 10, 15, and 30 minutes and 1, 2, 4, 8, 12, and 24 hours after dosing. Plasma AEOL concentrations were measured using a previously described HPLC analytical
method (14). Pharmacokinetic data fitting was done using a WinNonlin noncompartmental model (Pharsight, Cary, NC, USA) (Table 3.1).

AEOL10150 safety assessments were performed in mice, rats, nonhuman primates, and humans as described in FDA IND No. 67,741. In mice, AEOL’s no observable adverse effect level was 40 mg/kg/day and the maximum tolerated dose was 160 mg/kg/day (data not shown). The structure of AEOL is shown in (Figure 3.1)

*Exposure to Cl*₂

Mice were restrained and exposed to Cl₂ for 5 minutes using a nose-only exposure device. Cl₂ gas was mixed with room air using a standardized calibrator (VICI Metronics, Dynacalibrator, Model 230-28A). The Cl₂ delivery system has two main components, a gas generator, which includes a heated permeation chamber, and an air-flow generator. Dynacal permeation tubes designed specifically for operation with the Dynacalibrator were used and contained the Cl₂. The permeation chamber and air-flow generator control the accuracy of the Cl₂ generated to within 1–3% of the desired concentration according to the manufacturer’s specifications. Teflon permeation tubes containing Cl₂ in both gas and liquid phases are contained within the permeation chamber. When the tube is heated the Cl₂ reaches a constant vapor pressure such that it permeates the tube at a constant rate. The desired concentration is delivered at an appropriate flow rate, as specified by the manufacturer. The device is attached to the exposure chamber and allowed to
calibrate for 30 min until the optimum temperature of 30 °C is reached and the Cl₂ flow is constant. After removal of the animal from the exposure chamber, the chamber was continually flushed with the gas mix to ensure that the desired concentration of Cl₂ was maintained between mouse exposures.

*Methacholine responsiveness*

Mice were sedated with xylazine hydrochloride (8 mg/kg, ip) and anesthetized with pentobarbital (30 mg/kg, ip). Subsequently, the animals were given tracheotomies using an 18-gauge cannula and connected to a small-animal ventilator (FlexiVent; Scireq, Montreal, QC, Canada). Muscle paralysis was induced with pancuronium bromide (0.2 mg/kg ip). The mice were ventilated in a quasi-sinusoidal fashion with the following settings: a tidal volume of 10 ml/kg, maximum inflation pressure of 30 cm H₂O, a positive end expiratory pressure of 3 cm H₂O, and a frequency of 150/min. After an equilibration period of 3 min of tidal ventilation, two lung inflations to a transrespiratory pressure of 25 cm H₂O were performed and baseline measurements were taken. The template used was Snapshot-150 version 5.2 and mechanics were calculated using the single-compartment model. Baseline was established as the average of three perturbations. After establishment of baseline, methacholine (MCh) was administered using an inline nebulizer (Aeroneb Lab, standard mist model; Aerogen Ltd., Galway, Ireland), and progressively doubling concentrations ranging from 6.25 to 50 mg/ml were administered over 10 s synchronous with inspiration. Six measurements were made at each
dose of MCh to establish the peak response. The highest value was kept for analysis subject to a coefficient of determination above 0.85. Respiratory system resistance and respiratory system elastance were determined before challenge and after each dose of MCh.

**Bronchoalveolar lavage**

Twenty-four hours after Cl₂ exposure the mice were euthanized with an overdose of sodium pentobarbital (30 mg/kg, ip). The mice were given tracheotomies and 0.5 ml of sterile saline was instilled into the lungs and the fluid recovered was placed in 1.5-ml Eppendorf tubes and kept on ice. Fluid recovered from the first wash was centrifuged at 1500 rpm for 5 minutes at 4 °C and the supernatant was retained for extracellular glutathione analysis. Three subsequent washes were done with 1-ml aliquots of sterile saline to recover lung inflammatory cells and placed in a 15-ml tube for centrifugation. The bronchoalveolar lavage fluid (BAL) was centrifuged at 1500 rpm for 5 min at 4 °C and total live and dead cells were counted using trypan blue exclusion. Cytospin slides were prepared using a cytocentrifuge (Shandon, Pittsburgh, PA, USA) and stained with Diff Quick (Jorgensen Labs, Loveland, CO, USA). Differential cell counts were determined based on a count of 300 cells/slide. Total protein in the BAL supernatant was quantified using a Bradford colorimetric assay (Bio-Rad, Hercules, CA, USA) and determined by spectrophotometry at 620 nm against a standard curve of bovine serum albumin.
**Tissue preparation for assessment of epithelial regeneration**

Mice were allowed to recover for 72 hours in separate groups after Cl₂ exposure before they were euthanized using an overdose of sodium pentobarbital (30 mg/kg, ip). The pulmonary circulation was flushed with sterile saline via the right ventricle until the effluent was clear. After removal the lungs were fixed by intratracheal perfusion with 10% buffered formalin at a constant pressure of 25 cm H₂O for a period of 24 hours. Immunohistochemistry was done after cutting of 5-μm paraffin-embedded sections, which were stained with Ki-67 nuclear staining to determine epithelial cell proliferation.

**Immunohistochemistry**

Cells undergoing proliferation were detected in tissue sections by immunostaining for Ki-67. Immunohistochemical staining for Ki-67 was performed with the Vectastatin avidin–biotin peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA). After deparaffination of sections, the slides were immersed in antigen unmasking solution (Vector Laboratories) for 8 minutes. Sections were washed for 5 minutes twice with Tris-buffered saline (TBS; 0.5 M Tris–HCl, 1.5 M NaCl, pH 7.6). Lung sections were permeabilized using 0.2% Triton X-100 for 20 minutes. After being washed, nonspecific binding sites were saturated with Universal Blocking Solution (Vector Laboratories) for 20 minutes. Primary antibodies against goat Ki-67 (Cayman Chemical, Ann Arbor, MI, USA) and control normal goat serum
(Vector Laboratories) used at a dilution of 1:250 were applied to tissue sections for incubation in a humidified chamber at room temperature for 1 hour. The sections were then washed with TBS twice for 5 min. Biotinylated rabbit anti-goat IgG (Vector Laboratories) was applied to the tissue sections at a concentration of 1:50 and incubated at room temperature for 45 minutes. Sections were washed twice with TBS for 5 minutes. Then the slides were incubated with avidin–biotin complex alkaline phosphatase (Vector Laboratories) for 45 minutes. Last, Vector red alkaline phosphatase (Vector Laboratories) was used to develop the sections for 15 minutes. Sections were dehydrated by moving slides through three baths of xylene and mounted with Vectamount mounting medium (Vector Laboratories). Lung sections were visualized for positive Ki-67 staining by light microscopy.

**Quantitative morphology on airway sections**

Numbers of Ki-67-positive cells were quantified after staining. Positive cells were defined as cells that showed strongly positive nuclear staining for Ki-67. Only airways with a major/minor (long axis/short axis) diameter ratio of < 2.5 in cross section were selected for analysis. Airways analyzed ranged in size from 0.5 to 3 mm. The number of Ki-67-positive cells in the epithelium was quantified under a light microscope using a 40× objective. The airway basement membrane length was measured by superimposing the image of the airway onto a calibrated digitizing tablet (Jandel Scientific, Chicago, IL, USA), with a microscope equipped with a camera lucida projection system (Leica)
Microsystems, Richmond Hill, ON, Canada). The numbers of proliferating cells corrected for airway size were expressed as Ki-67+ cells/mm of basement membrane perimeter (PBM).

**Glutathione measurements**

The BAL fluid from control, Cl2-exposed, and AEOL-treated mice was collected for glutathione evaluation by HPLC. BAL samples were collected 24 hours after Cl2 challenge. Phosphoric acid (60 μl; 1 M) was added to BAL fluid samples to prevent glutathione (GSH) degradation. BAL was centrifuged at 1500 rpm for 5 minutes, and the supernatant was removed for evaluation of extracellular GSH/GSSG. The pellet was reconstituted with 150 μl of PBS, 150 μl of 12 mM Chaps, and 15 μl of 1 M phosphoric acid for analysis of intracellular GSH and GSSG. GSH and GSSG were measured in 50-μl aliquots by RP-HPLC using postcolumn derivatization as described previously (14). The mobile phase was a gradient between 0 and 15% acetonitrile containing 0.05% trifluoroacetic acid over 10 min at a flow rate of 1 ml/min. The stationary phase was a column (150 × 4.6 mm) of Ultracarb ODS (5-μm particle size, 150 × 4.6 mm; Phenomenex, Torrance, CA, USA). GSH and GSSG in the column eluate were converted to a fluorescent isoindole derivative by continuously mixing the column eluate with 'o-phthalaldehyde (370 μM) in 0.2 M tribasic sodium phosphate, pH 12, at 70 °C. Fluorescence was monitored using excitation and emission wavelengths of 336 and 420 nm, respectively.
The amounts of GSH and GSSG were determined from a standard curve using the authentic compounds as external standards.

**Lung 4-hydroxynonenal (4-HNE) assay**

4-HNE assay was performed as described previously (14). Frozen tissue, or a known amount of 4-HNE standard (Cayman Chemical, Ann Arbor, MI, USA), was placed in 2 ml of cold methanol (Thermo Fisher, Waltham, MA, USA) containing 50 µg/ml butylated hydroxytoluene, with 10 ng d3-4-HNE (Cayman Chemical) internal standard added just before homogenization with the Ultra-Turrax T25 (Thermo Fisher). An EDTA solution (1 ml of 0.2 M, pH 7) was added. Derivatization was accomplished by incubation with 0.2 ml of 0.1 M Hapes containing 50 mM O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride, pH 6.5, for 5 min at room temperature. The O-pentafluorobenzyl–oxime derivatives were then extracted with hexane, converted to trimethylsilyl ethers by the addition of 15 µl each of pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide, and analyzed by gas chromatography/mass spectrometry using a Focus gas chromatograph coupled to aDSQ II mass spectrometer (Thermo Fisher). The stationary phase was an A15-m TR-5MS column (0.25-mm i.d., 0.25-µm film thickness; Thermo Fisher) and the carrier gas was helium (1.0 ml/min). Two microliters of sample was injected into the 270 °C inlet using split mode with an injection ratio of 10 and a split flow of 10 ml/min. The initial oven temperature was 100 °C and then ramped to 200 °C at 15 °C/min, followed by an increase in
temperature to 300 °C at 30 °C/min, and held for 1 min. The MS transfer line temperature was held constant at 250 °C and the quadrupole at 180 °C. Analysis was done by negative-ion chemical ionization using 2.5 ml/min methane reagent gas. Ions were detected using SIM mode with a dwell time of 15.0 ms for each fragment of 4-HNE at m/z 152, 283, and 303 and d₃-4-HNE at m/z 153, 286, and 306. Under these conditions, the larger, second peak of the two 4-HNE isomers was used for quantification and exhibited retention time of 7.18 min, which was just preceded by the elution of d₃-4-HNE at 7.17 min. Quantification was performed using a standard curve generated by graphing the area ratio of 4-HNE to d₃-4-HNE versus concentration.

**Statistical analysis**

Differences in responsiveness to methacholine were analyzed by repeated-measures ANOVA and a post hoc Bonferroni test. One-way analysis of variance was used to determine the drug treatment effect on other variables and the post hoc comparisons were performed using a Dunnett multiple comparison test for differential cell counts. P < 0.05 was considered significant.
3.5 Results

*Effect of AEOL on MCh responsiveness after Cl₂ challenge*

Airway responsiveness to inhaled aerosolized MCh in increasing concentrations (6.25–50 mg/ml) was elevated 24 hours after Cl₂ exposure. Cl₂ significantly increased respiratory system resistance over control and AEOL-treated groups at the two highest doses of MCh (Figure 3.2A). Respiratory system elastance was increased in the Cl₂-only exposed group compared with control at the two highest doses of MCh (Figure 3.2B). AEOL did not significantly attenuate the increase in elastance compared to Cl₂-only treated mice (Figure 3.2B). However, there was also no significant difference between control groups and Cl₂/AEOL-treated mice (Figure 3.2B).

*Changes in bronchoalveolar lavage cells after Cl₂ gas exposure*

To assess the airway inflammation induced by Cl₂, bronchoalveolar lavage was performed at 24 hours after Cl₂ exposure. The BAL fluid recovered averaged 75% of the initial volume instilled and did not vary significantly between groups. Total cell counts were significantly increased in the Cl₂-exposed group compared to control groups as well as Cl₂/AEOL (Figure 3.3). Although AEOL was able to reduce total inflammatory cell influx after Cl₂ exposure compared with Cl₂-only exposed mice, there was still a significant difference between Cl₂/AEOL-treated groups and control groups. AEOL alone had no effect on total inflammatory cell numbers.
Differential cell counts were performed to examine the pattern of inflammatory cell recruitment to the airways after Cl₂ exposure. Cl₂ exposure significantly increased total numbers of macrophages, neutrophils, and eosinophils (Figure 3.4A-C) above control groups but did not significantly increase lymphocytes or epithelial cells (Figure 3.4D & E). Treatment with AEOL attenuated the increase in macrophage and neutrophil numbers recruited to the airway after Cl₂ exposure but did not significantly affect eosinophil, lymphocyte, or epithelial cell numbers. Changes in protein level after Cl₂ exposure

Total protein levels were measured in BAL fluid using a Bradford assay 24 hours after Cl₂ exposure to assess cell damage and microvascular leak. Cl₂ caused a twofold increase in the total protein present in the BAL compared to control groups. Treatment with AEOL after Cl₂ exposure significantly reduced the amount of protein in the BAL fluid (Figure 3.5).

**Epithelial cell proliferation**

We have previously reported that epithelial proliferation occurs as early as 48 hours after high-level Cl₂ exposure (8). We measured the level of cellular hyperplasia as an indirect method of assessment of epithelial damage. We chose to quantify the level of proliferation of epithelial cells at 72 hours after exposure. Ki-67 nuclear staining was performed to determine numbers of proliferating epithelial cells. The total numbers of positive cells were quantified in each airway and corrected for airway size by dividing by the
perimeter of the basement membrane of the airway. A minimum of six animals totaling at least 24 airways were studied in each group. Cl₂ induced an increase in the total number of proliferating epithelial cells in the airways (Figure 3.6A). Treatment with AEOL significantly reduced the number of proliferating epithelial cells compared to Cl₂-only treatment. Figure 3.6 B-D show representative pictures of control (Figure 3.6B), Cl₂-only (Figure 3.6C), and Cl₂/AEOL (Figure 3.6D) cells. Darker nuclei indicate Ki-67-positive cells.

**BAL GSH and GSSG levels at 24 h are unaffected by Cl₂**

At 24 hours after Cl₂ and AEOL treatments, extracellular GSH levels were measured in BAL fluid (Figure 3.7A) There were no significant differences in the levels of either GSH (Figure 3.7A) or GSSG (Figure 3.7B) among the four groups.

**AEOL protects against Cl₂-induced increases in 4-NHE**

Twenty-four hours after Cl₂ exposure, lungs were harvested and lung homogenates analyzed for 4-HNE. Cl₂ induced a significant increase in 4-HNE levels compared to control and AEOL-treated groups (Figure 3.8). AEOL alone had no effect on 4-HNE levels, but completely blocked the response to Cl₂.
3.7 Discussion

In this study we wished to explore whether rescue treatment with the synthetic metalloporphyrin AEOL could prevent the deleterious effects of Cl2 on the lungs, including airway hyperresponsiveness to MCh, inflammatory cell influx into the airways, epithelial cell proliferation, and oxidative damage. Our results showed that although AEOL itself had no effect on the lungs, it was effective in attenuating the effects of Cl2 on all of the above parameters. BAL glutathione levels were not perturbed at 24 hours after Cl2. However, at the same time, there was clear evidence of oxidative lung damage measured by 4-HNE that was prevented by AEOL. The data show that post hoc treatment is effective and support the concept that oxidative damage continues beyond the immediate effects of acid and oxidant damage caused by Cl2.

Previous studies have shown that exposure to Cl2 causes increased airway responsiveness (AHR) in human, rat, and mouse models (3), (8), (15) and (16). The mechanism of AHR after Cl2 may be similar to that of ozone in that both forms of injury are associated with oxidant damage to the tissues (17). In our study, mice received a single dose of Cl2 and although baseline mechanical parameters were unchanged there was an increase in respiratory system resistance and elastance in response to MCh. Airway epithelial damage most probably contributed to the AHR to inhaled MCh. We noted a marked increase in numbers of proliferating epithelial cells in Cl2-exposed mice compared to control and AEOL-treated groups, an indication of the intensity of the epithelial regeneration necessitated by prior damage by Cl2 exposure.
AEOL attenuation of this effect indicates a protective effect on the airways, making epithelial cell repair less necessary. An alternative interpretation of AEOL’s action on the airways is that it inhibits epithelial cell repair. However, this is improbable considering AEOL’s favorable effects on other study outcomes. We did not, however, see high numbers of shed epithelial cells in the BAL fluid after Cl₂, which suggests that shed cells may already have been removed by phagocytosis or that we may not have sampled BAL fluid early enough to detect the signal. Whether airway hyperresponsiveness is caused by loss of epithelial-derived bronchorelaxant factors or through disruption of barrier function remains to be determined.

Consistent with previous studies, we noted an increase in macrophage, neutrophil, and eosinophil populations after Cl₂ exposure (8). In addressing potential mechanisms associated with chlorine damage, these cells, especially neutrophils, produce reactive oxygen species from activation of endogenous enzymes such as myeloperoxidase and NADPH oxidase. Activated neutrophils exacerbate airway damage, causing an increase in microvascular permeability with the escape of proteinaceous fluid into the airways. AEOL was effective at reducing BAL fluid protein, probably reflecting favorable effects on cellular necrosis and microvascular permeability after Cl₂ exposure (18) and (19). Production of reactive oxygen species may contribute to Cl₂-induced AHR. Previous studies report that exposure to ozone induces several genes such as thyroid hormone-β receptor, nitric oxide synthase, and glutathione reductase (20). Nitric oxide synthase has been implicated as a potential mechanism
leading to airway hyperresponsiveness in response to Cl₂, because a nitric oxide synthase inhibitor (1400 W) abrogated the Cl₂-induced changes in responsiveness (11). The increase in eosinophils is a result consistent with previous reports showing oxidative stress induces eosinophilia (21). Eosinophilia in an oxidative injury model may be of consequence, as oxidative stress induces production of 5-oxo-6,8,11,14-eicosatetraenoic acid, a potent chemoattractant for granulocytes that function through G-protein-coupled receptors, suggesting a possible mechanism by which oxidative stress may increase inflammatory cell influx (22).

Because the effects of Cl₂ exposure could be at least partially mediated by oxidative stress we measured two markers: glutathione and 4-HNE. The glutathione redox system responds rapidly to oxidative stress, resulting in the oxidation of GSH to GSSG. This can lead to an initial reduction in the ratio of GSH to GSSG, but this is often temporary, as there are compensatory changes in the enzymes involved in glutathione biosynthesis and metabolism. Although there are studies reporting an increase in glutathione 24 hours after Cl₂ inhalation, these studies used high concentrations of Cl₂ for longer periods of time (7), (8), (16) AND (23). In accordance with our findings, a recent study using similar doses of Cl₂ but for longer periods of exposure found that GSH/GSSG levels were reduced at 1 hour but had recovered by 24 hours after Cl₂ exposure (24). Additionally, our previous work has shown an increase in BAL fluid GSH levels 10 minutes after Cl₂, but not at 1 hour, indicating a rapid response to oxidative stress (25). The present study was designed to investigate
the longer term (≥24 h) protective effects of AEOL and it is likely that these times were too late to detect changes in GSH and GSSG.

4-HNE is a biologically active aldehyde and a stable end product of lipid peroxidation. The evaluation of lipid peroxidation through the assessment of 4-HNE is a highly sensitive way of measuring oxidative stress. Increases in 4-HNE levels have previously been reported to be associated with endothelial barrier dysfunction and increases in microvascular leak (26). Importantly, in this study mouse lung levels of 4-HNE were reduced to baseline by post-Cl\textsubscript{2} treatment with AEOL, suggesting that lipid peroxidation is an important mechanism for Cl\textsubscript{2}-induced lung damage.

AEOL10150 is a member of a class of catalytic metalloporphyrins characterized as small-molecular-weight antioxidants (14). AEOL effectively scavenges peroxynitrite and lipid peroxides and has high SOD and catalase-like activities (27). Additionally, AEOL is also effective as a scavenger of lipid hydroperoxides. It is unique as a potential therapeutic as it reacts with and detoxifies multiple reactive oxygen and nitrogen species in a catalytic rather than a stoichiometric fashion. As a highly effective superoxide scavenger AEOL may have prevented the neutrophil-induced damage to airway epithelial cells by reducing the toxicity of superoxide by-products released from activated neutrophils.

In summary, we have shown that AEOL is an effective rescue treatment against Cl\textsubscript{2}-induced markers of airway injury including airway hyperresponsiveness, airway inflammation, oxidative stress, and epithelial
proliferation. O'Neill et al. have shown that AEOL is also effective in preventing the effects of other chemical toxins such as half mustard (2-chloroethyl ethyl sulfide) (14), suggesting that AEOL may be effective as a therapeutic agent against a variety of reactive toxic chemical agents.
Table 1.1 Mouse Pharmacokinetics of AEOL 10150

<table>
<thead>
<tr>
<th>Mouse pharmacokinetics of AEOL10150</th>
<th>Plasma half-life (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (min)</th>
<th>Clearance (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEOL (2 mg/kg, iv)</td>
<td>6.6</td>
<td>4046</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

Values were calculated from curve fit data from two mice per time point.
Figure 3.1

Structure of AEOL10150

The skeletal formula for compound Mn(III) tetrakis-$(N,N'$-diethylimidizolium-2-yl) porphyrin, AEOL10150, is shown.
Figure 3.2

AEOL reduces airway responsiveness to methacholine after Cl₂ challenge

Airway responsiveness was determined by FlexiVent 24 h after Cl₂ exposure. (A) Total respiratory system resistance after MCh in control, Cl₂-only, Cl₂/AEOL, and AEOL-only treated mice. A two-way ANOVA showed a significant decrease between mice post-treated with AEOL compared to animals receiving Cl₂ only at the two highest doses of MCh. There was no difference between AEOL-treated mice or control groups. (B) The effects of Cl₂ exposure and AEOL treatment on total respiratory system elastance. There were differences only in control vs Cl₂-only mice at the two highest doses of MCh. AEOL-treated groups were not significantly different from control or Cl₂-only treated animals (n = 10 per group; **P < 0.01, ***P < 0.001).
Mice were sacrificed 24 h after Cl₂ exposure. Cl₂ led to a significant increase in total leukocytes compared to controls and AEOL-treated groups. AEOL only was not different from control; however, Cl₂/AEOL-treated mice had more total cells in their BAL compared to AEOL-only mice \((n = 10\) per group; \(*P < 0.05, **P < 0.01, ***P < 0.001\).
Figure 3.4

Cellular composition of BAL fluid after Cl₂ exposure

Differential cell counts were done 24 h after Cl₂ exposure. (A–C) Macrophages, neutrophils, and eosinophils were significantly elevated in Cl₂ groups compared to control. (A and B) AEOL attenuated this increase in macrophages and neutrophils. (A–E) There was no difference between control and AEOL-treated groups. (D and E) There were no differences in any of the other cell types assessed (n = 10 per group; *P < 0.05, **P < 0.01, ***P < 0.001).
Figure 3.5

Effects of Cl₂ exposure and AEOL treatment on BAL fluid protein

Protein levels in BAL fluid were assessed by Bradford assay. There was a twofold increase in total protein at 24 h after Cl₂ exposure compared to control groups. Treatment with AEOL Cl₂ significantly limited the increase in protein content in the BAL fluid (n = 10 per group; *P < 0.05).
Figure 3.6

Epithelial cell proliferation after Cl₂ exposure

Actively proliferating cells were labeled using Ki-67 staining 72 hours after Cl₂ exposure. (A) Cl₂ caused a significant increase in numbers of proliferating epithelial cells in the airways compared to control. AEOL treatment attenuated this effect; however, Cl₂/AEOL groups were significantly increased compared to controls. (B–D) Representative examples of airway sections stained with Ki-67 at 250× original magnification. Dark nuclei indicate a positive cell against the methyl green counterstain (n = 6 per group; *P < 0.05, ***P < 0.001).
Figure 3.7

Effects of Cl₂ exposure and AEOL treatment on glutathione levels in BAL fluid

24 h after Cl₂ exposure, GSH levels in the BAL cell fraction show no increase in any of the treatment groups. (B) GSSG levels show no change in any group 24 hours after Cl₂ exposure ($n = 10$ per group).
Figure 3.8

4-HNE levels in total lung homogenates

Lungs were collected 24 hours after Cl₂ exposure and snap-frozen. Total lung homogenates were assessed for 4-HNE levels. Cl₂ induced a significant increase in 4-HNE production compared to controls and AEOL-treatment groups. There were no differences between any of the control and the AEOL-treatment groups (n = 6 per group; *P < 0.05).
3.7 References


Chapter 4

Neutrophils Mediate Airway Hyperresponsiveness Following Chlorine-Induced Airway Injury in the Mouse
4.1 Prologue

In this chapter, we began to explore contribution of specific populations of inflammatory cells to airway dysfunction induced by Cl₂ exposure. In Chapters 2 and 3, we observe treatment with antioxidants is effective in preventing oxidative stress, airway inflammation and AHR one hour following Cl₂ exposure. We felt Cl₂ may not be directly responsible for these observations, and these effects were due, at least in part, to an endogenous response. Due to the high numbers of neutrophils observed in BAL and their capacity to release oxidants including HOCl, we chose to examine the effects of neutrophil depletion on airway inflammation, oxidative stress and AHR. Because eosinophils and macrophages have historically played a role in inflammatory processes we also depleted these cell types and assessed AHR and airway inflammation. We found that neutrophil depletion, but not depletion of eosinophils or macrophages effectively prevents Cl₂-induced AHR. Furthermore, we found that neutrophil depletion prevents NRF-2 nuclear translocation. These results suggest that neutrophils play a central role in increasing oxidative stress and AHR in the lungs following Cl₂, and that in our model, Cl₂ alone is not sufficient to induce NRF-2 translocation or AHR.
4.2 Abstract

**Rationale:** Inhalation of chlorine gas (Cl\textsubscript{2}) is known to cause oxidant stress and airway dysfunction leading to irritant-induced asthma (IIA). Airway dysfunction following Cl\textsubscript{2} exposure is characterized by damage to airway epithelial cells, increase in airway responsiveness and inflammation characterized by intense neutrophilia. We wished to explore the role of eosinophils, macrophages and neutrophils on airway responsiveness following Cl\textsubscript{2}. We hypothesized that Cl\textsubscript{2}-induced influx of neutrophils was primarily responsible for increased airway responsiveness following Cl\textsubscript{2}, and that their presence resulted in increased oxidative stress. **Methods:** A neutrophil depleting antibody (anti-Gr1; 100µg) was injected 6 hours prior to Cl\textsubscript{2} exposure to assess the contribution of neutrophils to airway responsiveness and NRF2 levels following acute lung injury induced by Cl\textsubscript{2}. Anti-IL-5 was injected i.p. 1 hour prior to Cl\textsubscript{2} exposure to deplete eosinophils. Clodronate loaded liposomes were administered intratracheally 48 hours prior to Cl\textsubscript{2} exposure to deplete macrophages. Balb/C mice were exposed to 100ppm Cl\textsubscript{2} gas for 5 minutes using a nose-only exposure system. Bronchoalveolar lavage (BAL) and lung tissue were collected for assessment of inflammation and for mRNA analysis, respectively, at 6 and 24 hours following Cl\textsubscript{2}. At 24 hours, respiratory system resistance (Rrs) and elastance (Ers) and airway responsiveness to methacholine were measured using a small animal ventilator (FlexiVent). **Results:** Mice exposed to Cl\textsubscript{2} showed a time-dependent increase in total inflammatory cells. Eosinophils, lymphocytes, neutrophils and epithelial cells
were elevated following Cl₂ compared to air-exposed mice. Anti-Gr1 completely abolished neutrophilia in BAL following Cl₂ at 6 and 24 hours. Macrophages were also slightly decreased at baseline and 24 hours, but increased at 6 hours following Cl₂. Cl₂ induced a robust increase in both respiratory resistance and elastance in response to methacholine at 24 hours. Anti-Gr1 abrogated Cl₂-induced airway hyperresponsiveness as assessed with Rrs. Increases in elastance were not affected by neutrophil depletion. Anti-Gr1 did not affect airway responsiveness in air-exposed mice. Neutrophil depletion prevented increases in mRNA levels of NRF2 and SOD1, and prevented the Cl₂-induced downregulation of GPX2. Furthermore, Cl₂ exposure induced nuclear translocation of NRF2 in bronchial epithelial cells, an effect prevented by anti-Gr1 treatment. Neither depletion of eosinophils nor macrophages affected airway hyperresponsiveness or other inflammatory cell populations.

**Conclusion:** Airway neutrophilia following Cl₂ results in airway hyperresponsiveness, increased NRF2 and SOD expression and nuclear translocation of NRF2 in bronchial epithelial cells, effects prevented by administration of anti-Gr1. These results suggest that neutrophils may directly affect mechanisms that mediate oxidant damage in the airways resulting in hyperresponsiveness.
4.3 Introduction

Irritant induced asthma (IIA) is a form of occupational asthma that develops without latency. IIA is characterized by the development of asthma symptoms following either a single (acute) or multiple (chronic) exposures to an irritant substance. Clinical findings of those afflicted by IIA often include bronchial hyperresponsiveness, airway inflammation, edema and airway damage. There are a number of causative agents induce IIA, including diesel exhaust, disinfectants, preservatives such as formaldehyde and cleaning products like ammonium and amine compounds. Among irritants, exposure to Cl₂ gas and Cl₂-related compounds remains the most reported cause of inhalational accidents resulting in IIA. Those at risk include workers in industry (pulp and paper mills and transport as Cl₂ is moved in quantities that exceed 15 million tons annually, putting it among the top ten most manufactured chemicals, and increasing the risk of Cl₂ exposure and spillages from trucks or trail derailments. Accidental exposure may also occur in swimmers and from the improper mixing of cleaning agents.

Human exposure to Cl₂ has been studied and reveals both acute and long term effects, even following a single inhalation. Pathological findings include goblet cell hyperplasia, increased collagen deposition, ASM proliferation and persistent AHR. A particularly interesting case study followed a patient from 60h post Cl₂ exposure up to 5 months, taking bronchial biopsies at several time points. This study revealed that epithelial shedding
persisted for several weeks, and regeneration of airway epithelial cells occurred for months and was accompanied by persistent bronchial hyperresponsiveness. (14,15) Investigators using rodents to evaluate the damage induced by Cl\textsubscript{2} inhalation have found similar pathologies to those observed in human studies. Single exposures to Cl\textsubscript{2} in mice induced persistent bronchial hyperresponsiveness, airway inflammation, increases in ASM mass and epithelial cell proliferation. (15) Mice also demonstrate epithelial cell shedding, increased plasma protein leak, a high degree of neutrophilia and elevated levels of KC (the murine ortholog of IL-8) in the hours immediately following Cl\textsubscript{2} exposure, and lasting 48 hours. (16)

While the precise mechanism of action remain unknown, substantial evidence points to the majority of Cl\textsubscript{2}-induced damage originating from its by-products which include chloramines, hydrochloric acid, chlorine dioxide and hypochlorous acid (HOCl). Cl\textsubscript{2} is highly soluble in water and a potent oxidant, having greater toxicity than nitrogen dioxide (NO\textsubscript{2}), oxygen (O\textsubscript{2}), ozone (O\textsubscript{3}) and hydrochloric acid (HCl). (17) Increased oxidant production has been associated with Cl\textsubscript{2} inhalation including elevated peroxynitrite in the airway tissues and increased carbonylation of proteins. (18) While oxidant damage from Cl\textsubscript{2} gas can be direct, it is important to note that Cl\textsubscript{2} is scrubbed from the airway fairly rapidly (19) and previous work has shown evidence of on-going oxidant stress and airway damage, suggesting additional mechanisms for Cl\textsubscript{2}-induced airway injury. (20,21) These indirect effects likely include damage
caused by increased oxidative-stress, the source of which may be activated neutrophils.(22)

Neutrophils have been associated with severe asthma and asthma exacerbations (23) and previous work has shown that inhalation of irritant, oxidizing substances such as ozone (24, 25) and phosgene (26) rapidly induce the influx of neutrophils to the airways. Neutrophils may contribute to increased oxidant burden through the production of reactive oxygen species and in particular, through the release of myeloperoxidase, which catalyzes the formation of HOCl from hydrogen peroxide.(27) Following Cl₂ inhalation, studies in rodents (28) and case studies in humans (29) have shown striking neutrophilia, with modest changes in recruitment of other inflammatory cell types, suggesting neutrophils play an important role in the development of Cl₂-induced injury.(30) Additionally, in vitro studies have shown that in the presence of reactive oxygen species (ROS) bronchial epithelial cells release large amounts of the neutrophil chemoattractant IL-8(31), while direct application of HOCl induced up-regulation of antioxidant genes, glutathione activity, and nuclear factor-erythroid 2-related factor (NRF2), a critical transcription factor in the endogenous antioxidant response.(32)

In the current study, we wished to examine the role of neutrophils in a Cl₂-induced model of airway injury, the endogenous anti-oxidant response and AHR. For this, mice were administered an antibody directed against granulocyte receptor-1 (Gr-1), which has been used extensively to deplete neutrophils in vivo.(33-39) Following Cl₂ exposure, we measured respiratory
system mechanics in response to inhaled methacholine, assessed airway inflammatory cell profiles, antioxidant gene expression and NRF-2 epithelial cell nuclear translocation. We also assessed the independent contribution of eosinophils and macrophages in the induction of Cl₂ induced AHR and inflammation using an anti-IL5 antibody or clodronate liposomes, respectively.
4.4 Materials and Methods

Mice

Balb/C wild-type male mice (8 to 10 weks) were purchased from Charles River (Wilmington, MA, USA) and housed in a conventional animal facility at McGill University. At the time of experimentation, mice weighed 22.4g (+/- 0.6g). All mice were treated in accordance with the guidelines of the Canadian Council for Animal Care and protocols were approved by the Animal Care Committee of McGill University. Animals were provided with water and food ad libitum throughout the experiment.

Chlorine Exposure

Mice were exposed to Cl₂ gas for 5 minutes using a nose-only exposure device at a concentration of 100ppm. Cl₂ gas was mixed with room air using a standardized calibrator (VICI Metronics, Dynacalibrator, Model 230-28A). The concentration of Cl₂ delivered during the exposure is established by the manufacturer to be within 1-3% of the desired concentration. Briefly, the delivery system has two main components, a gas generator, which includes a heated permeation chamber, and an air-flow generator. Dynacal Teflon permeation tubes contain Cl₂ in both gas and liquid phases. When the tubes are heated to 30 °C, the Cl₂ reaches a constant vapor pressure and it permeates the tube at a constant rate. Following each exposure, the chamber is recalibrated to ensure that the desired concentration of Cl₂ was maintained between mouse exposures.
Measurements of respiratory system mechanics and airway responsiveness to methacholine

Twenty-four hours following Cl₂ exposure, airway function was measured using a small animal ventilator (FlexiVent; Scireq, Montreal, QC, Canada). Prior to measurements, mice were sedated (xylazine hydrochloride, 8 mg/kg, ip) and anesthetized (pentobarbital, 30 mg/kg, i.p.). Once anesthetized, mice were tracheotomized using an 18-gauge cannula and connected to the ventilator. Muscle paralysis was induced with pancuronium bromide (0.2 mg/kg ip). The mice were ventilated in a quasi-sinusoidal fashion with the following settings: a tidal volume of 10 ml/kg, maximum inflation pressure of 30 cm H₂O, a positive end expiratory pressure of 3 cmH₂O, and a frequency of 150/min. After an equilibration period of 3 min of tidal ventilation, two lung inflations to a transrespiratory pressure of 25 cm H₂O were performed and baseline measurements were taken. Airway responsiveness was evaluated using two distinct forced oscillation maneuvers. The first was a 1.5s, 2.5Hz maneuver which is used to estimate respiratory system resistance (Rrs) and respiratory system elastance (Ers). The second, a 3s, broadband low frequency forced oscillation maneuver containing 13 mutually prime frequencies between 1 and 20.5 Hz which is used to estimate Newtonian resistance (Rn), tissue damping (G) and tissue elastance (H).

Baseline was established as the average of three perturbations. Methacholine (MCh) was administered using an in-line nebulizer (Aeroneb
Lab, standard mist model; Aerogen Ltd., Galway, Ireland), and progressively doubling concentrations ranging from 6.25 to 25 mg/ml were administered over ten seconds and synchronous with inspiration. Six measurements were made at each dose of MCh to establish the peak response. The highest value was kept for analysis subject to a coefficient of determination above 0.85.

**Granulocyte depletion**

To establish the lowest possible effective dose required to deplete granulocytes, mice were injected intraperitoneally (i.p.) 100µg/mouse or 200µg/mouse of anti-GR1 (Hycult Biotechnology, King of Prussia, PA, USA) in 100µl sterile phosphate buffered saline (PBS) 6 hours prior to Cl\(_2\) exposure. Mice were exposed to Cl\(_2\) and allowed to recover for either 6 or 24 hours following exposure. Bronchoalveolar lavage (BAL) was performed to determine numbers of total inflammatory cells present following Cl\(_2\) as well as efficacy of the depletion antibody. Total cell counts were determined using a hemacytometer followed by analysis of cell differentials by performing cytospins and staining with the Diff-Quik® method (Medical Diagnostics, Düdingen, Germany). We determined 100µg/mouse to be the lowest effective dose required to completely deplete neutrophils from BAL and lung tissue and all subsequent experiments were performed with this dose. Mouse IgG2b was used as an isotype control and injected at the same time points as anti-GR1.

Although neutrophils are the predominant GR-1 expressing cells, low levels of Gr-1 are found on eosinophils and macrophages. To address the effect
of anti-GR1 on eosinophil and macrophage numbers in BAL, selective eosinophil and macrophage depletions were performed. Eosinophil depletion was achieved using 100μg/mouse (i.p.) of TRFK5 (anti-IL-5) (BD Biosciences, Mississauga, ON, Canada) in 100μL PBS containing ≤0.09% sodium azide. Anti-IL-5 was injected one hour prior to Cl₂ exposure and mice were allowed to recover for 24 hours following Cl₂. Airway function measurements were taken followed by BAL.

Macrophage depletion was achieved using clodronate (Encapsula NanoSciences, Nashville, TN, USA) encapsulated into liposomes. Clodronate was administered intra-tracheally in a single, 50μL instillation at a concentration of 250μg 48 hours prior to Cl₂ exposure. Empty liposomes (Encapsula NanoSciences, Nashville, TN, USA) were used as a control and administered in the same fashion. Twenty-four hours following Cl₂ exposure, airway function measurements were taken followed by BAL.

**Bronchoalveolar Lavage**

Six or 24 hours after Cl₂ exposure mice were euthanized with an excess of sodium pentobarbital (60 mg/kg, i.p.). Four aliquots of sterile saline of 1mL each were instilled into the lungs via the tracheal cannula and the fluid recovered was placed in a 15-ml tube (BD Biosciences, Mississauga, ON, Canada) kept on ice. The volume recovered did not differ significantly among the groups. BAL fluid was centrifuged at 1500 rpm for 5 min at 4°C and the supernatant was discarded. The cell pellet was re-suspended in 500μL sterile
saline. Total live and dead cells were counted by hemacytometer and trypan blue exclusion. Cytospin slides were prepared using a cytocentrifuge (Shandon, Pittsburgh, PA, USA) and stained with DiffQuik. Differential cell counts were determined based on a count of 300 cells/slide.

**RNA Isolation**

At either 6 or 24 hours following Cl₂ exposure, lungs were inflated with RNAlater (Qiagen, Mississauga, ON, Canada) and placed in 1.5mL tubes (Eppendorf) containing 1.0mL RNAlater. Lungs were stored at 4C overnight. The next day, lung tissue was separated into parahilar and peripheral areas to evaluate differences in mRNA expression in large and peripheral airways, respectively. RNA was extracted using RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's instructions.

**Reverse Transcription and Real-Time quantitative PCR**

Following RNA isolation, cDNA was synthesized with Oligo (dT) primers and Super-Script™ II reverse transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. A total of 250 ng total RNA was reverse-transcribed into cDNA.

For quantitative real-time PCR, SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) using the Applied Biosystems StepOne Plus Real-Time PCR System. Primer sequences were used to determine NRF2 expression, SOD-1, and GPX-2. NRF2 forward;
TCTCCTCGCTGGAAAAAGAA, reverse; AATGTGCTGGCTGTGCTTTA.

SOD-1: forward; CAGGACCTCATTTTAATCCTCAC, reverse;
TGCCCAGGTCTCTCAACAT. GPX2: forward;
CTGTGTAACTCTGCTGTTCC, reverse;
CCACACTACCTGAGTCTACC. SOD-1 and GPX-2 primer sequences were
obtained using Roche primer design software. PCR samples contained 9 μl of
Power SYBR® Green PCR Master Mix and 1 μl of cDNA with a final primer
concentration of 0.5 μM. The run method included a holding stage (95 °C for
10 min), a cycling stage (45 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for
25s) and a melting curve stage (95 °C for 15 s, 60 °C for 45 s, 95 °C for 30 s).
Analysis was performed using StepOne software (version 2.1) and the
comparative ΔΔCt method, with S9 as the endogenous control gene.

Tissue neutrophil quantification

Six or 24 hours following Cl₂ exposure, the pulmonary circulation was flushed
with sterile saline via the right ventricle until the effluent was clear. After
removal, the lungs were fixed by intra-tracheal perfusion with 10% buffered
formalin at a constant pressure of 25 cm H₂O for a period of 24 h. 5-μm sections
were cut from paraffin-embedded lung tissue using a microtome. To determine
if anti-Gr1 was effective at depleting neutrophils within lung tissue, sections
were stained using the anti-neutrophil antibody NIMP-R14 (Abcam,
Cambridge, MA, USA). Briefly, following deparaffination of sections, slides
were immersed in antigen unmasking solution (Vector Laboratories,
Burlington, ON, Canada) for 8 min. Sections were washed for 5 min twice with Tris-buffered saline (TBS; 0.5 M Tris–HCl, 1.5 M NaCl, pH 7.6). Lung sections were permeabilized using 0.2% Triton X-100 for 20 min. Nonspecific binding sites were saturated with Universal Blocking Solution (Dako Canada, Burlington, ON, Canada) for 20 min. Next, endogenous peroxidase activity was blocked by incubating slides in a 3% hydrogen peroxide solution for 10 minutes followed by a 5 minute wash in TBS. Neutrophil specific primary antibody, anti-NIMP-R14 (GeneTex, Irvine, CA, USA) was prepared in Universal Antibody Diluent (Dako Canada, Burlington, ON, Canada) at a dilution of 1:150 and applied to tissue sections for incubation in a humidified chamber at room temperature for 1 hour. IgG2b was used as an isotype control. Sections were then washed with TBS twice for 5 minutes, incubated with a Signal Enhancer for 20 minutes (GeneTex, Irvine, CA, USA) followed by anti-mouse rat and rabbit IgG (H + L) secondary antibody conjugated to HRP (GeneTex, Irvine, CA, USA). Last, Nova Red (Vector Laboratories) for 15 minutes and counterstained with hematoxylin. Sections were dehydrated by moving slides through three baths of xylene and mounted with Vectamount mounting medium (Vector Laboratories). Neutrophils were identified in tissue using light microscopy using an Olympus BX51 microscope. Images were taken using QImaging Retina 2000r camera and Image Pro-Plus.

NRF2 Nuclear Translocation
Tissue sections were prepared as above. Following non-specific blocking, tissue sections were incubated with anti-NRF2 (Abcam, Cambridge, MA, USA) at a concentration of 1:300 overnight at 4°C. Following a 10 minute wash in TBS, Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Invitrogen, Burlington, ON, Canada) was used at a concentration of 1:1000 and sections were incubated for 45 minutes at room temp. Following a 10 minute wash in TBS, nuclei were stained with Hoechst at a concentration of 1:10,000 for 7 minutes. Slides were washed in water for 10 minutes and mounted (Fluoromount Aqueous Mounting Medium, Sigma-Aldrich, Oakville, ON, Canada). Following NRF2 immunofluorescent staining, tissue sections were visualized using an Olympus BX51 microscope. Images were taken using QImaging Retina 2000r camera.

NRF2 nuclear translocation corrected for total NRF2 immunofluorescence was determined using nuclear co-localization commercial software (Image-Pro Plus). Briefly, two single wavelength images were taken of each airway; either green (NRF2) or blue (Hoechst) channels. Next, a scatter plot of the individual pixels from the paired images were generated and an area of interest (AOI) was chosen that indicated areas of overlap between green and blue, allowing exclusion of any background or non-specific signal(40). Following AOI selection, a Pearson’s correlation was calculated to determine the extent of overlap between the two images using the following formula: where, S1 is signal intensity of pixels in the first channel
and S2 is signal intensity of pixels in the second channel, \( S_1 \text{ aver} \) and \( S_2 \text{ aver} \) - average intensity of first channel and second channels respectively.

Next, co-localization coefficients were calculated to estimate the contribution of one color channel in the co-localized areas of the image to the overall co-localized fluorescence in the image. These coefficients, \( M_1 \) and \( M_2 \), are proportional to the amount of fluorescence of co-localizing objects in each component of the image, relative to the total fluorescence in that component. The components are described as the green and blue images, respectively. \( M_1 \) is used to describe the contribution of NRF2 (green) to the co-localized area while \( M_2 \) is used to describe the contribution of blue. These calculations were made based on the following formula: where, \( S_{1i, coloc} = S_{1i} \) if \( S_{2i} \) is within thresholds defined by AOI \( S_{1i, coloc} = 0 \) if \( S_{2i} \) is outside the threshold levels. \( S_{2i, coloc} = S_{2i} \) if \( S_{1i} \) is within thresholds \( S_{2i, coloc} = 0 \) if \( S_{1i} \) is outside the AOI. Six animals per group were assessed with a minimum of 6 airways per animal. Only airways that showed a Pearson coefficient of <0.95 was used. Results shown are for \( M_1 \), as \( M_2 \) was of little interest.

**Statistical analysis**

Data for respiratory system mechanics were analyzed using a repeated measures ANOVA. Comparisons among several means was performed using ANOVA and the *post hoc* analysis with Newman Keuls test. A *p* value < 0.05 was considered significant.
4.5 Results

*Partitioning of changes in respiratory mechanics following chlorine exposure*

Twenty-four hours following an exposure of mice to 100ppm Cl$_2$ for 5 minutes, respiratory system mechanics were evaluated at baseline and in response to increasing doses of inhaled MCh. Cl$_2$ exposure resulted in no change in baseline measurements but increases in Newtonian resistance, tissue damping and tissue elastance (Figure 4.1A-C). All three of these parameters were increased in response to MCh following Cl$_2$ exposure compared to control groups.

*Changes in BAL of Chlorine-Exposed Mice*

Cl$_2$-induced airway inflammation and epithelial cell shedding were assessed by bronchoalveolar lavage 6 hours and 24 hours following Cl$_2$ exposure. Total cell counts were significantly increased 6 hours and 24 hours after exposure to Cl$_2$ (Figure 4.2A) compared to air-exposed animals. Macrophages were not significantly altered in number at either time point (Figure 4.2B). Neutrophils were significantly increased at both 6 and 24 hours compared to control animals but were higher at 6 than 24 hours post-exposure (Figure 4.2C). Eosinophils were modestly increased in number at 6 hours but not at 24 hours (Figure 4.2D). Lymphocyte numbers were increased by 6 hours and remained elevated at 24 hours (Figure 4.2E). Epithelial cells were highest at 6 hours, but significant numbers of epithelial cells were still found in the BAL at 24 hours post-Cl$_2$ (Figure 4.2F).
Anti-Gr1 depletes granulocytes from BAL

Six hours following i.p. administration of 100\(\mu\)g anti-GR-1 antibody, mice were exposed to Cl\(_2\) and allowed to recover for either 6 or 24 hours. Following recovery, BAL was performed and inflammatory cell profiles were determined. Illustrative photomicrographs of BAL cells are shown in Figure 4.3G-I. Normal BAL fluid shows a predominantly macrophage population of cells. Following Cl\(_2\) exposure there is a marked neutrophilia which was absent from the BAL at 6 and 24 hours in anti-GR1 treated mice. At 6 hours, the total number of cells in the BAL fluid was not different between groups that received anti-GR1 and those that did not but by 24 hours the total cells were fewer in anti-GR1 treated mice compared to untreated animals (Figure 4.3A). We observed a smaller number of macrophages between air-exposed anti-GR1 treated and non-treated mice at baseline, which then increased slightly at 6 hours in Cl\(_2\) exposed groups and decreased again at 24 hours in Cl\(_2\) exposed groups (Figure 4.3B). Anti-Gr1 was highly effective at inhibiting BAL neutrophilia at 6 hours following Cl\(_2\) exposure (Figure 4.3C) but it also caused a decrease in the number of eosinophils at the same time point (Figure 4.3D). There were no changes in the number of lymphocytes or shed epithelial cells among any groups (Figure 4.3E & F).

Anti-Gr1 depletes neutrophils from lung tissue

In addition to evaluation of the efficacy of anti-GR1 in reducing BAL neutrophils, lung tissues were stained for a neutrophil specific marker, NIMP-
r14 to ensure depletion was complete from lung tissue as well as the airways. No neutrophils were present in air-treated conditions (Figure 4.4A & B). By 6 hours post-Cl₂, neutrophils could be clearly visualized in the airways and frequently took the form of adherent clumps of neutrophils (Figure 4.4C). In contrast, very few neutrophils could be identified in anti-GR1 treated lungs (Figure 4.4D). By 24h, the neutrophil clumps appeared less frequent in Cl₂ treated mice but abundant neutrophils were still present (Figure 4.4E). This finding was not seen in anti-GR1 treated mice, where no neutrophils were seen in either airways or lung parenchymal tissues at 24h post-Cl₂ (Figure 4.4F). No neutrophils were seen in the air-exposed isotype control mice (Figure 4.4G).

*Anti-GR1 treatment reduces chlorine-induced AHR*

Mice given anti-GR1 6 hours prior to Cl₂ exposure had significantly decreased Rn and G (Figure 4.5A & B) compared to Cl₂-exposed mice given PBS alone at the highest dose of MCh. Anti-Gr1 did not reduce tissue elastance H (Figure 4.5C). Isotype control antibody for anti-Gr1 (IgG2a) had no effect on AHR for any parameter (Figure 4.5A-C).

*Anti-GR1 prevents increases in chlorine-induced oxidant gene upregulation*

At 6 and 24 hours following Cl₂ exposure, the antioxidant gene expression in the central lung compartment of mice either treated with or without anti-GR1 was assessed. Cl₂ induced an increase in NRF2 at 24 hours (Figure 4.6A). Treatment with anti-Gr1 significantly reduced NRF2 mRNA levels (Figure 4.6A). SOD-1 was significantly increased at 24 hours post Cl₂
and this increase was prevented with anti-GR1 treatment (Figure 4.6B). GPX-2 was significantly decreased in mice exposed to Cl\textsubscript{2} compared to those treated with anti-Gr1 and Cl\textsubscript{2} (Figure 4.6C).

**Anti-Gr1 prevents NRF2 nuclear translocation**

Six hours prior to Cl\textsubscript{2} exposure, mice were injected with 100µg anti-Gr1. At 24 hours following Cl\textsubscript{2} exposure lungs were fixed and stained for NRF2 to determine if nuclear translocation had taken place in response to Cl\textsubscript{2}. Mice that were exposed to air showed clear NRF2 positivity in the cytoplasm and not in the nuclear region (Figure 4.7A-C). Visualization of NRF2 for anti-GR1 treated mice demonstrated similar patterns (Figure 4.7D-F). However, in Cl\textsubscript{2} exposed mice NRF2 nuclear translocation was apparent. (Figure 4.7G-I). In mice treated with anti-GR1, less nuclear translocation took place despite being exposed to Cl\textsubscript{2} (Figure 4.7J-L). Quantification of NRF2 immunoreactivity confirmed that following Cl\textsubscript{2}, NRF2 translocated to the nucleus, an effect prevented by anti-Gr1 (Figure 4.7M).

**Anti-IL-5 prevented eosinophilia selectively following chlorine-exposure**

To determine if eosinophils, an important inflammatory cell that may influence AHR, played a role in the changes in airway responsiveness observed in Cl\textsubscript{2}-exposed mice we depleted eosinophils with anti-IL5. Also, in addition to depleting neutrophils, anti-GR1 also caused a reduction in BAL fluid eosinophilia. Therefore to ensure that the results observed using anti-GR1 were
specific to the depletion of neutrophils, eosinophil-specific depletion was critical. Anti-IL5 did not affect total inflammatory cells found in BAL following Cl₂ exposure (Figure 4.8A). It did not influence any of the cell types evaluated with the exception of eosinophils (Figure 4.8B-F). IgG2a was used as an isotype control and it had no effect on eosinophils or any other cell type (data not shown).

_Eosinophil depletion does not affect chlorine-induced AHR_

Absence of eosinophils in the airways did not prevent the Cl₂-induced increase in Rrs, Ers, Rn, G or H (Figure 4.9A-E). Similarly anti-IL-5 did not affect the airway responsiveness of mice exposed only to air.

_Depletion of macrophages using clodronate liposomes does not prevent chlorine-induced AHR_

Like neutrophils and eosinophils, macrophages are an important inflammatory cell type that may influence AHR. Therefore, we depleted macrophages using clodronate liposomes administered by intratracheal instillation (i.t.) 48 hours prior to Cl₂ exposure. Clodronate administration did not affect total cell counts (Figure 4.10A) but was effective in reducing significantly the proportion of macrophages in BAL fluid (Figure 4.10B). No other cell type was significantly affected by clodronate (Figure 4.10C-F). No cell type was depleted by i.t. instillation of encapsosomes.
Depletion of macrophages was unable to reduce Cl\(_2\) induced AHR. Rrs, Ers, Newtonian resistance, tissue damping or tissue elastance were not reduced following macrophage depletion (Figure 4.11A-E). Encapsosomes did not have an effect on airway responsiveness.
4.5 Discussion

Chlorine exposure causes an oxidative injury that results in a predominantly neutrophilic airway inflammation, airway hyperresponsiveness to inhaled aerosolized methacholine and oxidative damage. In the current study we wished to assess the role of the neutrophil in Cl2-induced airway hyperresponsiveness and its contribution to oxidative stress. Using an anti-GR1 antibody to prevent neutrophil migration into the lung and airway compartments we demonstrated that granulocytes accounted for increased airway responsiveness measured by respiratory system mechanical parameters. Upregulation of mRNA associated with anti-oxidants occurred following Cl2 exposure and was prevented by anti-GR1 treatment. Likewise translocation of NRF2 to the nucleus which followed Cl2 exposure was also prevented by neutrophil depletion.

Chlorine is a highly reactive gas and when hydrated forms hypochlorous acid that is also a strong oxidant. There is a substantial body of literature addressing the role of oxidant injury from chlorine (6) and documenting the efficacy of anti-oxidant treatments.(18,19) Of particular interest is the finding that administration of anti-oxidants after chlorine exposure also attenuates airway injury and dysfunction.(18) These data suggest that oxidative injury is not limited to the acute effects of Cl2 but is also related to the ongoing production of oxidant species post-exposure, likely resulting from epithelial activation or from the influx of activated leukocytes as evidenced by the strong NRF2 nuclear translocation observed in Cl2 exposed
mice. NRF2 mRNA was increased following Cl\textsubscript{2} exposure, as was SOD-1 mRNA, an NRF2-dependent antioxidant. The epithelial antioxidant glutathione peroxidase (GPX-2) was downregulated, not a unique observation as others have shown downregulation of the GPX family of antioxidants following a severe, acute oxidative stress.(36) Unlike SOD-1, GPX-2 transcription is not dependent on NRF2 although treatment with anti-Gr1 prevented changes in NRF2, SOD-1 and GPX-2 maintaining all three at baseline levels despite Cl\textsubscript{2} exposure.

NRF2 translocation to the nucleus occurred in the airway epithelial cells after Cl\textsubscript{2}, indicative of an altered redox state. Depletion of neutrophils prevented NRF2 translocation suggesting their presence increases the level of oxidative stress such that epithelial cells become overwhelmed and engagement of the battery of NRF2 dependent antioxidants is necessitated. This finding further suggests that acute exposure to Cl\textsubscript{2} does not induce excessive oxidative stress, as previously demonstrated by the efficacy of antioxidant administration post-Cl\textsubscript{2} exposure (18) and that oxidative stress induced by neutrophils is sufficient to engage the NRF2 antioxidant system. However, further confirmation of NRF2 nuclear translocation would be prudent to ensure the robustness of results. ChIp, EMSA or NRF2 reporter activity assays would ideally be used to validate our current results.

AHR following Cl\textsubscript{2} is reflected as exaggerated changes in respiratory system resistance and elastance after inhaled methacholine measured at 24 hours after exposure. To assess the relationship between the inflammatory cell
influx and AHR we targeted cells expressing GR1. GR1 is expressed in high levels on the surface of granulocytes (22) but also is expressed, albeit at lower levels, on monocytes/macrophages and dendritic cells. Eosinophils rose in the BAL fluid after Cl₂ although to levels that were about 1/20th of the neutrophils and were also significantly reduced by anti-GR1 treatment. Alveolar macrophages were only reduced under baseline conditions by anti-GR1 antibody and increased to comparable levels following Cl₂ exposure. Lymphocytes were not significantly affected. Anti-IL-5 antibody prevented the increase in eosinophils and macrophages were reduced by about 60% after clodronate liposomes but neither intervention affected AHR significantly. It therefore seems probable that the observed effects of anti-GR1 were indeed mediated by reduction in neutrophils.

The protective effect of granulocyte depletion was most marked against the changes in resistance and less so against the changes in elastance. A further analysis of respiratory system mechanics using the constant phase model which captures changes in large airway mechanics (Newtonian resistance, RN), peripheral lung resistance (G) and tissue elastance (H)(42) indicates that the protective effect of anti-GR1 is predominantly seen in the large airways, as neutrophil depletion was able to reduce Newtonian resistance but not other parameters. These findings suggest potentially different mechanisms of alterations in mechanics in large versus peripheral airways. The peripheral airways, populated in part by Clara cells, rely on surfactant to maintain low surface tension. Surfactant production can be reduced in the
setting of oxidative stress via apoptosis of surfactant producing cells or through oxidation of surfactant proteins. (43) A reduction in surfactant levels may lead to airway closure and it has been associated with increased elastance as demonstrated in models of lung injury. (45) Airway mechanic measurements reflect that neutrophil depletion did not prevent AHR in the parameter for tissue damping (G), a reflection of tissue stiffness, a property associated with surfactant function. (46) Previous work has shown increased tissue damping in surfactant knock-out mice (47), and a reduction of tissue damping levels in surfactant overexpressing mice following oxidative stress-induced lung injury. (48) While neutrophils are capable of producing high levels of HOCl, and causing oxidative stress induced damage to the airways, they also serve in the reparative process of inducing surfactant production through the release of vascular endothelial growth factor (VEGF). (50,51) In the presence of neutrophils, elastance may be increased due to the release of HOCl and subsequent damage to surfactant-producing cells, resulting in increased elastance and tissue damping. Under neutrophil depletion circumstances, the initial Cl₂ exposure may be sufficient to oxidize surfactant proteins such that neutrophil derived VEGF may be needed to restore surfactant production. Measurements of large airway parameters clearly showed that neutrophil depletion induced a reduction in AHR. One potential explanation for these observations may be due to the mechanical effect of excess cellular influx within the airways. Histological images demonstrated neutrophil accumulation on the airway walls and it is possible that these areas
that encroach on the airway lumen sufficiently to contribute to the alterations in mechanics as any space occupying elements inside the airway lumen may also augment the increases in resistance evoked by MCh. Simply preventing the influx of those encroaching cells may be sufficient to prevent increased airway resistance in large airways.

In conclusion we have found that Cl₂ exposure upregulates NRF2 and SOD-1, augments airway responses to MCh, increases airway inflammation, and induces nuclear translocation of NRF2. Anti-Gr1 monoclonal antibody treatment reduced all of these effects while reducing BAL fluid and lung neutrophilia. Inhibition of eosinophilia and reduction of alveolar macrophages did not affect AHR, suggesting that oxidant stress generated by neutrophilia is responsible for a substantial portion of the observed airway dysfunction.
Figure 4.1

Chlorine induces an increase in AHR

Twenty-four hours following Cl₂ exposure, airway physiology measurements were evaluated in response to inhaled methacholine. Panel A shows Cl₂-exposed mice demonstrate increases in respiratory system resistance in response to methacholine inhalation at the 12.5, 25 and 50 mg/ml compared to air-exposed mice. Panel B shows respiratory system elastance is increased in Cl₂-exposed mice at 6.25, 12.5, 25 and 50 mg/ml in response to inhaled methacholine compared to air-exposed mice. Panel C shows Newtonian resistance increased at the highest dose of inhaled methacholine following Cl₂ exposure. Panel D shows tissue damping increased at the highest dose of methacholine in response to methacholine in Cl₂-exposed mice. Panel E shows tissue elastance is increased at the highest dose of methacholine in mice exposed to Cl₂. *p<0.05, **p<0.01, ***p<0.001; n=6-10 per group.
Changes in BAL of Chlorine-Exposed Mice

At either 6 or 24 hours following Cl₂ exposure, BAL was collected for total and differential cell count analysis. Panel A shows total cell counts were increased at 6 and 24 hours in Cl₂-exposed mice compared to air-exposed mice. Panel B shows Cl₂ exposure did not induce a significant change in macrophage numbers between the conditions. Panel C shows the increase in neutrophil numbers at both 6 and 24 hours following Cl₂ exposure compared to air-exposed mice. Cl₂ exposure resulted in significantly higher neutrophil numbers at 6 hours compared to 24 hours. Panel D shows Cl₂ exposure resulted in a modest increase in eosinophil numbers 24 hours after chlorine exposure compared to air-exposed mice. Panel E shows lymphocyte numbers increased at 6 hours and remained elevated at 24 hours in Cl₂ exposed mice. Panel F shows Cl₂ exposure resulted in increased epithelial cell shedding at 6 and 24 hours following Cl₂. Epithelial cell shedding peaked at 6 hours. *p<0.05, **p<0.01***p<0.001; n=6-10 per group.
Figure 4.3

Anti-Gr1 depletes granulocytes from BAL

100µg of anti-Gr-1 was injected i.p. 6 hours prior to Cl₂ exposure. At either 6 or 24 hours following Cl₂, BAL was collected and evaluated for inflammatory cells. (A) Treatment with anti-Gr1 reduced total inflammatory cells at 24 hours compared to mice that did not receive anti-Gr1. (B) Anti-G1 reduced macrophage numbers in air-exposed mice as well as in cl2 mice at 24 hours. (C) Treatment with anti-Gr-1 completely attenuated increased neutrophil numbers seen the BAL of untreated mice. (D) Anti-Gr1 reduced eosinophil numbers at 6 hours, but not in air-exposed mice, or at 24 hours following Cl₂ exposure. (E) Lymphocyte numbers were decreased at 6 hours by anti-Gr1 treatment but not in air-exposed mice, or at 24 hours following Cl₂. (F) Epithelial cell shedding was not affected by anti-Gr1 treatment. (G-L) Cytospins collected from mice treated with or without anti-Gr1 and exposed to Cl₂ or air to confirm the absence of neutrophils. Cells were stained with H & E. *p<0.05, **p<0.01, ***p<0.001; n=6-10 per group.
Figure 4.4

Anti-Gr1 depletes neutrophils from lung tissue

Lung tissue was sectioned at either 6 or 24 hours following chlorine exposure and with or without pre-treatment with 100μg of anti-GR1. Presence of neutrophils was identified using the neutrophil specific antibody Nimp-R14. (A-)B Few neutrophils are seen in the airways or surrounding tissue in air-exposed mice regardless of anti-GR1 treatment. (C) Six hours following Cl₂ exposure, neutrophils can be easily visualized in the airway lumen. (D) Compared to Cl₂ exposed mice not treated with anti-Gr1, there are very few neutrophils in the airways and surrounding tissue following anti-Gr1 treatment. (E) At 24 hours, neutrophils were still easily identified in the airways and surrounding tissue in Cl₂ exposed mice. (F) Anti-Gr1 treatment prevented neutrophil influx to the airways and tissue, and very few neutrophils were visible. *p<0.05, **p<0.01, ***p<0.001; n=6-10 per group.
Figure 4.5

Anti-GR1 treatment reduces chlorine-induced AHR

Mice were injected with 100μg anti-Gr-1 6 hours prior to Cl₂ inhalation. Twenty-four hours later airway function measurements were taken in response to inhaled methacholine. Mouse IgG2b was used as an isotype control for Air or Cl₂ groups. (A) Neutrophil depletion completely attenuated Cl₂ induced increases in respiratory system resistance. (B) Anti-Gr1 did not reduce respiratory system elastance. (C) Newtonian resistance was attenuated by anti-Gr1 treatment in Cl₂ treated mice. (D-E) Anti-Gr1 had no effect on either tissue damping or tissue elastance. Mice given control antibody, IgG2a, did not show any differences, in any parameter, from mice given PBS only (data not shown) **p<0.01, ***p<0.001; n=6-9 per group.
Figure 4.6

Anti-GR1 prevents increases in chlorine-induced oxidant gene upregulation

Six hours prior to Cl₂ exposure, 100μg of anti-Gr1 was administered i.p. mRNA was isolated from the central portion of mouse lungs 6 or 24 hours following Cl₂ exposure. Mouse IgG2b was used as an isotype control. (A) Treatment with anti-Gr1 prevented Cl₂-induced increases in NRF2, a critical transcription factor involved the endogenous antioxidant response at 24 hours. (B) Anti-Gr-1 prevented increases in superoxide dismutase 1 (SOD-1) at 24 hours. (C) Cl₂ exposure led to a decrease in glutathione peroxidase 2 (GPX-2) mRNA at 24 hours, an effect prevented by anti-Gr1 treatment. *p<0.05, **p<0.01; n=6-10 per group.
Figure 4.7

Anti-Gr1 prevents NRF2 nuclear translocation

The first panel shows quantification of NRF2 translocation using Image ProPlus software. NRF2 translocation occurred after Cl₂ exposure and this translocation was abrogated by anti-Gr1 antibody treatment of animals prior to exposure. Immunohistochemistry for NRF2. Upper row shows nuclear staining with Hoechst stain. Middle row shows NRF2. Lower row shows merged images. At baseline distinct green and blue staining is seen, reflecting images of cytoplasmic NRF2 and nuclear staining. After Cl₂ there is a reduction of cytoplasmic staining for NRF2 (reduced green). However after anti-Gr1 there is no loss of cytoplasmic staining, consistent with lack of nuclear translocation.
M1- intensity of green to colocalized area

Relative Units

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<th>Condition</th>
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</tr>
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<tr>
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</tbody>
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Air-exposed Anti-Gr1 + Air-exposed Cl₂ Exposed Anti-Gr1 + Cl₂ Exposed

A  D  G  J
B  E  H  K
C  F  I  L
Figure 4.8

Anti-IL-5 prevented eosinophilia selectively following chlorine-exposure

Mice were treated with 100μg anti-IL-5 1 hour prior to Cl₂ inhalation. Mouse gG2b was used as an isotype control. (A) Anti-IL5 had no effect on total inflammatory cells found in BAL. (B-C) Anti-IL-5 did not affect macrophage or neutrophil numbers in either air or Cl₂ exposed mice. (D) Anti-IL-5 was effective at reducing eosinophil numbers following Cl₂ exposure. (E-F) Anti-IL-5 did not affect epithelial cell shedding or lymphocyte numbers in BAL. *p<0.05, n=6-7 per group.
Figure 4.9

**Eosinophil depletion does not affect chlorine-induced AHR**

Mice were injected i.p. with 100µg of anti-IL5 one hour prior to Cl₂ exposure. Mouse IgG2a was used as an isotype control. Twenty four hours later airway physiology measurements were taken in response to inhaled methacholine. (A-E) Anti-IL-5 had no effect on respiratory system mechanics in any of the parameters examined. n=6-7 per group.
Figure 4.10

Clodronate liposomes depletion of BAL macrophages.

In order to assess the effect of clodronate on macrophage depletion and airway inflammation following Cl₂, mice were given intratracheal instillations of liposomes either loaded with clodronate or liposomes alone (encapsosomes) 72 hours prior to Cl₂ exposure. Twenty-four hours following Cl₂ exposure, total and differential cell counts were evaluated for efficacy of macrophage depletion. (A) No significant difference in the total cell count in untreated, encapsosome and clodronate treated mice were detected. (B) Clodronate liposomes were effective at depletion macrophages following Cl₂ exposure. (C-F) Clodronate liposomes had no effect on neutrophils, eosinophils lymphocytes or epithelial cells. *p<0.05, n=6 per group.
Macrophage Depletion does not affect Chlorine-Induced Airway AHR

Following macrophage depletion using clodronate liposomes, airway responsivness to inhaled methacholine was assessed. Mice were either exposed to air or Cl₂ and respiratory function measurements taken 24 hours later. (A-E) Macrophage depletion did not produce any significant differences in respiratory system resistance, respiratory system elastance, Newtonian resistance, tissue damping or tissue elastance in response to methacholine between clodronate treated and control mice. n=6 per group.
4.7 References


Chapter 5

Absence of cysLTr1 augments airway damage from chlorine and impairs antioxidant responses
5.1 Prologue

In Chapter 5 we begin to explore the role of cysteiny1-leukotrienes (cysLTs) and the cysteiny1-leukotriene receptor 1 (cysLTr1) in our model of Cl₂ induced asthma. Because cysLTs are potent bronchoconstrictors and known to be pro-inflammatory in allergic asthma, we reasoned that they may play a role in oxidative stress induced asthma. We first established that cysLTs are released in the lung response to Cl₂ exposure by measuring cysLTs in the BAL. We also found that cysLT related biosynthetic genes were upregulated following Cl₂. Importantly, we observed that cysLT release is attenuated by pre-Cl₂ exposure treatment with the antioxidant DMTU, suggesting that cysLTs are released in response to oxidative stress. To test the role of cysLTr1, we utilized cysLTr1 deleted mice (cysLTr1⁻/⁻). To our surprise, these mice demonstrated increased AHR and profound neutrophilia. Furthermore, we found that cysLTr1⁻/⁻ are particularly susceptible to oxidative stress as they do not translocate NRF2 to the nuclear region in bronchial epithelial cells following Cl₂. We measured antioxidants, and found that cysLTr1⁻/⁻ mice do not upregulate mRNA for NRF2 or SOD-1, unlike wild-type mice. We observed that cysLTr1⁻/⁻ mice have an innate overexpression of e-cadherin in the airways, and propose a mechanism by which lack of cysLTr1 predisposes these mice to increased airway damage and airway hyperresponsiveness by increasing oxidative stress burden.
5.2 Abstract

**Rationale:** Inhalation of Cl₂ gas is one of the more common toxic substances that may lead to the development of irritant induced asthma through mechanisms that involve direct and indirect consequences of oxidative stress. Pulmonary production of cysteinyl-leukotrienes (cysLTs) is associated with bronchoconstriction and pro-inflammatory effects. However, recent work has shown exaggerated injury following exposure of cysLTr1 deficient (cysLTr1-/-) mice to Cl₂. We hypothesized that increased lung injury observed in cysLTr1-/- mice following Cl₂ exposure is due to an inability of bronchial epithelial cells to mount an appropriate antioxidant response through NRF-2-related mechanisms. **Methods:** Methods: Balb/C or cysLTr1-/- mice were exposed to Cl₂ at a concentration of 100ppm for 5 minutes and sacrificed 6 or 24 hours later. At 24 hours airway responsiveness to methacholine was assessed using a small animal ventilator (FlexiVent). At 6 and 24 hours, bronchoalveolar lavage fluid was collected to assess inflammatory cells and cysLT levels by ELISA. Lung tissue was harvested for mRNA analysis and lungs were fixed for histological analysis. **Results:** CysLTr1-/- mice exposed to Cl₂ demonstrated increased AHR, epithelial cell apoptosis and marked neutrophilia compared to controls. Wild-type mice showed increased NRF-2 gene expression and NRF-2 translocation to the nucleus (immunofluorescence microscopy) in bronchial epithelial cells following Cl₂. However, CysLTr1 deleted mice did not demonstrate an increase in NRF-2 mRNA nor did we observe nuclear translocation of NRF-2. CysLTr1-/- mice demonstrated...
increased e-cadherin (immunofluorescence microscopy) in bronchial epithelial cells at both baseline and following Cl₂ exposure compared to wild-type mice. **Conclusion**: CysLTr1 deletion results in increased lung damage, particularly to bronchial epithelial cells following Cl₂ exposure. Our data suggests this damage may be due to inability to mount an appropriate response to oxidative stress, leading to excessive neutrophil influx. Over-expression of e-cadherin has been reported to impair NRF-2 function and nuclear translocation. High levels of e-cadherin found in cysLTr1/- mice coupled with low NRF-2 expression and lack of NRF-2 nuclear translocation suggests that the disruption of cysLTr1 functionality results in aberrant endogenous antioxidant response and neutrophilia, ultimately causing excessive airway damage in response to Cl₂ exposure.
5.3 Introduction

Irritant-induced asthma (IIA) caused by single, high exposures to noxious inhalants is a significant clinical problem and there is increasing evidence to support the role of irritants in the triggering of asthma.[1] Cl₂ gas is one of the more common irritants causing toxic exposure and leads to the development of IIA. Cl₂ is synthesized in large quantities for use in a variety of industries including pulp bleaching, waste sanitation, organic compound, pharmaceutical manufacturing, water treatment, and maintenance of swimming pools. Airway exposure to Cl₂ gas may occur through terrorist attacks, major spillages during transport [2], industrial accidents [3-5], the inappropriate mixing of cleaning products [6] and accidents at swimming pools.[7, 8] High concentrations of Cl₂ cause predominantly airway injury characterized by bronchospasm with cough, wheezing and airway hyperresponsiveness (AHR), airway epithelial shedding, and a predominantly neutrophilic inflammation although with sufficient exposure alveolar damage with acute lung injury may also result.[9-11]

Cl₂ induces IIA through oxidative stress driven mechanisms and we have demonstrated an important role for oxidant damage following inhaled Cl₂ gas in the mouse.[12-14] Respiratory toxicity may result from Cl₂, hypochlorous acid (HOCl), chlorine dioxide and chloramine. Cl₂ gas forms HOCl and hydrochloric acid on contact with moist mucosal surfaces and airways. Further oxidative injury results from Cl₂ gas combining with reactive oxygen species to form a variety of highly reactive oxidants including
peroxynitrite. Acute effects of Cl₂ causing airway dysfunction are mediated to a substantial extent through formation of oxidants such as HOCl, as evidenced by the immediate release of glutathione from airway epithelial cells following Cl₂ exposure. Migration and activation of inflammatory cells such as neutrophils within the airways and subsequent release of oxidants and proteolytic enzyme compounds worsen injury as HOCl is a major product of activated neutrophils and neutrophil-derived HOCl likely contributes to oxidative damage following Cl₂ exposure.

Cysteinyl-leukotrienes (cysLTs) are potent lipid mediators derived from the arachidonic acid pathway that comprise leukotriene (LT) C₄, LTD₄, and LTE₄. In the lung, cysLTs are mainly synthesized by mast cells, basophils, eosinophils, macrophages, neutrophils, and epithelial cells in response to a variety of stimuli. Among the cysLT receptors, cysLTr1 is found to be most prevalent in the lung. cysLTs are involved in the pathophysiology of asthma and contribute by causing bronchoconstriction, edema, mucus secretion, and increased inflammatory cell recruitment to the airways. High concentrations of cysLTs are present in the sputum of patients that have moderate to severe asthma and immediately following acute exacerbations. Several studies examining humans and animal models have found strong correlative evidence between increased cysLT production, cysLTr1 receptor expression and oxidative stress in the lung.

CysLTs and their receptors have historically been viewed as pro-inflammatory and involved exclusively in pathological processes. Exhaustive
laboratory and clinical research efforts have characterized these mediators as major players contributing to airway dysfunction associated with allergen-driven disease. In the following study, we sought to clarify the role of cysLTs and the cysLTr1 in airway inflammation and dysfunction induced by Cl$_2$ gas inhalation. We first established a robust synthesis and release of cysLTs in the lungs of wild-type mice following Cl$_2$ exposure. Next, we evaluated cysLT-related biosynthetic enzyme expression, AHR, airway inflammation, epithelial cell apoptosis, antioxidant gene activity, endogenous antioxidant response and oxidative damage in lung tissue in both wild-type and cysLTr1 deleted mice following Cl$_2$ exposure. Given the historical role of cysLTs, we initially hypothesized that a mouse strain carrying a deletion of cysLTr1 would have less airway damage and inflammation following Cl$_2$ exposure than a wild-type mouse following Cl$_2$ exposure. However our results demonstrate the contrary; the absence of cysLTr1 was associated with an exaggerated inflammatory response and airway hyperresponsiveness. To address the high probability that neutrophils exacerbate Cl$_2$-induced airway injury due to their high levels of recruitment and HOCl production upon activation, we depleted neutrophils in Cl$_2$-exposed cysLTr1$^{-/-}$ and wild-type mice and measured AHR and inflammation. Lastly, we showed the necessity of functional cysLTr1 to confer protection against oxidative stress through demonstration of an aberrant endogenous antioxidant response in the bronchial epithelial cells of mice carrying a cysLTr1 deletion.
5.4 Materials and Methods

Mice

Wild-type (WT) Balb/C mice (18–22g) were purchased from Charles River (Wilmington, MA, USA) and housed in a conventional animal facility at McGill University. CysLTr1 gene deleted mice (CysLTr1-/-) in a Balb/C background were generated [29] and given as a generous gift from Dr. Yoshihide Kanaoka at Harvard Medical School. All cysLTr1-/- mice were age and sex-matched to WT controls. Animals were treated according to the guidelines of the Canadian Council for Animal Care and protocols were approved by the Animal Care Committee of McGill University. Animals were given with water and food ad libitum throughout the experiment.

Experimental Groups

Mice were evaluated 24 hours post-Cl₂ exposure for airway function measurements, lung inflammatory cell counts, mRNA analysis, bronchial epithelial cell apoptosis and E-cadherin protein expression. Groups for these experiments were: WT Cl₂ exposed (n=10), WT air exposed Cl₂ (n=10), cysLTr1-/- Cl₂ exposed (n=12), and cysLTr1-/- air exposed (n=14). In separate experiments mice were evaluated at 6 hours post-Cl₂ exposure for lung inflammatory cell counts, mRNA analysis and bronchial epithelial cell apoptosis. Groups for these experiments were: WT Cl₂ exposed (n=8), WT air exposed Cl₂ (n=9), cysLTr1-/- Cl₂ exposed (n=8), and cysLTr1-/- air exposed (n=8).
To evaluate cysLT levels in bronchoalveolar lavage fluid following Cl\(_2\) exposure, WT mice were exposed to Cl\(_2\) and bronchoalveolar lavage fluid was collected at several times points; 30 (n=6) minutes, 1 hour (n=6), 2 hours (n=5), 4 hours (n=6), 6 hours (n=6), 8 hours (n=6), 24 hours (n=6), and 4 hours (n=6). Air exposed mice were used as controls (n=10).

To test the effect on cysLT levels and cysLT associated biosynthetic gene expression WT mice were given an i.p injection of the antioxidant DMTU (100mg/kg) dissolved in phosphate buffered saline (PBS) 1 hour prior to Cl\(_2\) exposure. Groups for these experiments were: PBS Cl\(_2\) exposed (n=6), PBS air exposed (n=6), DMTU Cl\(_2\) exposed (n=8), DMTU air exposed (n=8).

To test the effect of neutrophil depletion on WT and CysLTr1-/- mice, an injection of the neutrophil depletion antibody anti-GR1 (100μg/mouse) was given 6 hours prior to chlorine exposure. Mice were evaluated at 6 and 24 hours post-Cl\(_2\) exposure for neutrophil numbers in bronchoalveolar lavage fluid and neutrophil presence in lung tissue, Mice that were used for neutrophil depletion were given an i.p injection of anti-GR1 antibodies as follows: WT Cl\(_2\) exposed (n=10), WT air exposed Cl\(_2\) (n=10), CysLTr1-/- Cl\(_2\) exposed (n=10), and CysLTr1-/- air exposed (n=10).

*Exposed to Cl\(_2\)*

Mice were restrained and exposed to Cl\(_2\) gas for 5 min using a nose-only exposure device as previously described [13]. Cl\(_2\) gas was mixed with room air using a standardized calibrator (VICI Metronics, Dynacalibrator, Model
230-28A). The Cl₂ delivery system controls the accuracy of the Cl₂ generated to within 1–3% of the desired concentration according to the manufacturer's specifications. The device is attached to the exposure chamber and allowed to calibrate for 30 min until the optimum temperature of 30 °C is reached and the Cl₂ flow is constant.

*Measurements of respiratory system mechanics and airway responsiveness to methacholine*

Twenty-four hours following Cl₂ exposure, airway function was measured using a small animal ventilator (FlexiVent; Scireq, Montreal, QC, Canada) designed to estimate respiratory system resistance and respiratory system elastance in response to inhaled methacholine. Prior to measurements, mice were sedated (xylazine hydrochloride, 8 mg/kg, ip) and anesthetized (pentobarbital, 30 mg/kg, i.p.). Once anesthetized, mice were tracheotomized using an 18-gauge cannula, connected to the ventilator and ventilated in a quasi-sinusoidal fashion with the following settings: a tidal volume of 10 ml/kg, maximum inflation pressure of 30 cm H₂O, a positive end expiratory pressure of 3 cm H₂O, and a frequency of 150/min. Muscle paralysis was induced with pancuronium bromide (0.2 mg/kg ip). After an equilibration period of 3 min of tidal ventilation, two lung inflations to a transrespiratory pressure of 25 cm H₂O were performed and baseline measurements were taken. Respiratory mechanics were calculated also using the constant phase model. Baseline was established as the average of three perturbations. Methacholine was administered using an in-line nebulizer (Aeroneb Lab, standard mist model; Aerogen Ltd., Galway, Ireland), and progressively
doubling concentrations ranging from 6.25 to 25 mg/ml were administered over ten seconds and synchronous with inspiration. Six measurements were made at each dose of MCh to establish the peak response. The highest value was kept for analysis subject to a coefficient of determination above 0.85.

**Bronchoalveolar Lavage**

Six or 24 hours after Cl₂ exposure mice were euthanized with an excess of sodium pentobarbital (60 mg/kg, i.p.). Four aliquots of sterile saline of 1mL were instilled into the lungs *via* a tracheal cannula and the fluid recovered was placed in a 15-ml tube (BD Biosciences, Mississauga, ON, Canada) kept on ice. The volume recovered averaged 80% of the instilled volume and did not differ significantly among the groups. BAL fluid was centrifuged at 1500 rpm for 5 min at 4°C and the supernatant was discarded. The cell pellet was resuspended in 500μL sterile saline. Total live and dead cells were counted using with a hemacytometer and trypan blue exclusion. Cytospin slides were prepared using a cytocentrifuge (Shandon, Pittsburgh, PA, USA) and stained with DiffQuik. Differential cell counts were determined based on a count of 300 cells/slide.

**RNA Isolation**

At either 6 or 24 hours following Cl₂ exposure, lungs were inflated with RNAlater (Qiagen, Mississauga, ON, Canada) and placed in 1.5mL tubes (Eppendorf) containing 1.0mL RNAlater. Lungs were stored at 4C overnight. The next day, RNA was extracted from a parahilar sample of lung tissue using RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) according to manufacturer’s instructions.
Reverse Transcription and Real-Time quantitative PCR

Following RNA isolation, cDNA was synthesized with Oligo (dT) primers and Super-Script™ II reverse transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. A total of 250 ng total RNA was reverse-transcribed into cDNA.

For quantitative real-time PCR, SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) using the Applied Biosystems StepOne Plus Real-Time PCR System. Primer sequences were used to determine NRF-2 expression, SOD-1, GPX-2, 5 Lipooxygenase (5-LO), Five-Lipooxygenase Activating Protein (FLAP), LTC₄ synthase, CysLTr1, and LTA₄ hydrolase. NRF-2 forward: TCTCCTCGCTGGAAAAAGAA, reverse: AATGTGCTGGCTGTGCTTTA.

SOD-1: forward; CAGGACCTCATTAAAATCTC, reverse; TGCCCAGGTCTCCAACAT.

GPX2: forward; CTGTGTAACCTCTGCTGTTCC, reverse; CCACACTACCTGAGTCTACC. Primer sequences were obtained using Roche primer design software. PCR samples contained 9 μl of Power SYBR® Green PCR Master Mix and 1 μl of cDNA with a final primer concentration of 0.5 μM. The run method included a holding stage (95 °C for 10 min), a cycling stage (45 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 25s) and a melting curve stage (95 °C for 15 s, 60 °C for 45 s, 95 °C for 30 s). Analysis was performed using StepOne software (version 2.1) and the comparative ΔΔCt method, with S9 as the endogenous control gene.
Cysteinyl-Leukotriene Levels in Bronchoaveolar Lavage Fluid

Following Cl$_2$ exposure, 1mL of sterile PBS was instilled into the cannulated trachea and placed in a 1.5mL Eppendorf tube on ice. Immediately following collection, samples were spun at 1500rpm for 5 minutes. Supernatants were retained and kept at -80°C. To evaluate cysLT levels, we used a Cysteinyl Leukotriene Express EIA Kit (Caymen Chemical, Ann Arbor, MI).

Tissue preparation for Immunohistochemical Analysis

Mice were allowed to recover for 6 or 24 hours in separate groups after Cl$_2$ exposure before being euthanized using an overdose of sodium pentobarbital (60mg/kg, i.p.). The pulmonary circulation was flushed with sterile saline via the right ventricle until the effluent was clear. After removal the lungs were fixed by intratracheal perfusion with 10% buffered formalin at a constant pressure of 25 cm H$_2$O for a period of 24 hours. Lung tissue was processed through standard tissue processing protocols and embedded in paraffin. Immunohistochemistry was performed using a microtome to cut 5-μm thick sections that were mounted on SuperFrost slides.

NRF-2 Nuclear Translocation

Tissue sections were prepared as above. Following non-specific blocking, tissue sections were incubated with an anti-NRF-2 antibody (Abcam, Cambridge, MA, USA) at a dilution of 1:300 overnight at 4°C. Following a 10 minute wash in TBS, Alexa Fluor® 488 goat anti-rabbit IgG (H+L)
(Invitrogen, Burlington, ON, Canada) was used at a concentration of 1:1000 and sections were incubated for 45 minutes at room temp. Following a 10 minutes wash in TBS, nuclei were stained with Hoechst at a dilution of 1:10,000 for 7 minutes. Slides were washed in water for 10 minutes and mounted using Fluoromount Aqueous Mounting Medium (Sigma-Aldrich, Oakville, ON, Canada). Following NRF-2 immunofluorescent staining, tissue sections were visualized using an Olympus BX51 microscope. Images were taken using QImaging Retina 2000r camera.

Assessment of NRF-2 nuclear translocation corrected for total NRF-2 signal was determined by nuclear co-localization using commercial software (Image-Pro Plus).[30, 31] Briefly, two single wavelength images were taken of each airway; either green (NRF-2) or blue (Hoechst) channels. Next, a scatter plot of the individual pixels from the paired images were generated and an area of interest (AOI) was chosen that indicated areas of overlap between green and blue, allowing exclusion of any background or non-specific signal. Following AOI selection, a Pearson’s Correlation was calculated to determine the extent of overlap between the two images using the following formula:

$$R_r = \frac{\sum_i (S_{1i} - S_{1\text{aver}}) \cdot (S_{2i} - S_{2\text{aver}})}{\sqrt{\sum_i (S_{1i} - S_{1\text{aver}})^2 \cdot \sum_i (S_{2i} - S_{2\text{aver}})^2}}$$

where, $S_1$ is signal intensity of pixels in the first channel and $S_2$ is signal intensity of pixels in the second channel, $S_1 \text{ aver}$ and $S_2 \text{ aver}$ - average intensity of first channel and second channels respectively.
Next, co-localization coefficients were calculated to estimate the contribution of one color channel in the co-localized areas of the image to the overall co-localized fluorescence in the image. These coefficients, \( M_1 \) and \( M_2 \), are proportional to the amount of fluorescence of co-localizing objects in each component of the image, relative to the total fluorescence in that component. The components are described as the green and blue images, respectively. \( M_1 \) is used to describe the contribution of NRF-2 (green) to the co-localized area while \( M_2 \) is used to describe the contribution of blue. These calculations were made based on the following formula:

\[
M_1 = \frac{\sum S_{1_i, coloc}}{\sum S_{1_i}} \quad \text{and} \quad M_2 = \frac{\sum S_{2_i, coloc}}{\sum S_{2_i}}
\]

where, \( S_{1_i, coloc} = S_{1_i} \) if \( S_{2_i} \) is within thresholds defined by AOI. \( S_{1_i, coloc} = 0 \) if \( S_{2_i} \) is outside the threshold levels. \( S_{2_i, coloc} = S_{2_i} \) if \( S_{1_i} \) is within thresholds. \( S_{2_i, coloc} = 0 \) if \( S_{1_i} \) is outside the AOI.

Six animals per group were assessed with a minimum of 6 airways per animal. Only airways that showed a Pearson coefficient of <.95 was used. Results shown are for \( M_1 \), as \( M_2 \) was of little interest.

**Evaluation of Bronchial Epithelial Cell Apoptosis**

Determination of apoptotic cells was done using TUNEL method. For this we used ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA) according to manufacturer’s instructions.

**Quantitative morphology for Apoptotic Bronchial Epithelial Cells**
Numbers of TUNEL-positive cells were quantified after staining. Only airways with a major/minor (long axis/short axis) diameter ratio of >2.5 in cross section were selected for analysis. Airways analyzed ranged in size from 0.5 to 3 mm in diameter. The number of TUNEL-positive cells in the epithelium was quantified by light microscopy using a 40× objective. The airway basement membrane length was measured by superimposing the image of the airway onto a calibrated digitizing tablet (Jandel Scientific, Chicago, IL, USA), with a microscope equipped with a camera lucida projection system (Leica Microsystems, Richmond Hill, ON, Canada). The numbers of TUNEL positive cells was corrected for airway size and expressed as the number of apoptotic cells/mm of basement membrane perimeter (PBM).

**E-Cadherin Expression in Lung Tissue**

Visualization of e-cadherin expression was performed using E-Cadherin Rabbit mAb conjugated to Alexa Fluor® 488 (Cell Signaling, Danvers, MA, USA). Briefly, following de-paraffinization, nonspecific binding sites were saturated with Universal Blocking Solution (Dako Canada, Burlington, ON, Canada) for 60 minutes. Next, primary antibody was applied at a concentration of 1:400 and incubated overnight in a humidified chamber at 4°C. Following incubation tissue sections were washed three times in PBS for 5 minutes each. Nuclear staining was done using Hoechst (Life Technologies, Burlington, ON, Canada) at a concentration of 1μg/mL for 7 minutes. Following incubation sections were washed three times in PBS for 5 minutes each. Slides were then
cover-slipped with Prolong® Gold Anti-Fade Reagent (Cell Signaling, Danvers, MA, USA).

*Granulocyte depletion*

To establish the lowest possible effective dose required to deplete granulocytes, mice were injected intraperitoneally (i.p.) 100μg/mouse of anti-GR1 (Hycult Biotechnology, King of Prussia, PA, USA) in 100μl sterile phosphate buffered saline (PBS) 6 hours prior to Cl₂ exposure. IgG2b was used as an isotype control and injected at the same time points as anti-Gr1. Mice were exposed to Cl₂ and allowed to recover for either 6 or 24 hours following exposure. At 6 and 24 hours, lung tissue was collected for histological assessment. In separate mice, at 24 hours, bronchoalveolar lavage (BAL) was performed to determine numbers of total inflammatory cells present following Cl₂ as well as efficacy of the depletion antibody. Total cell counts were determined using a hemacytometer followed by analysis of specific cell populations evaluated by performing cytopsins and staining with the Diff-Quik® method (Medical Diagnostics, Düdingen, Germany).

*Determination of Neutrophils in Lung Tissue*

To determine if anti-Gr1 was effective at depleting neutrophils within lung tissue, sections were stained using the anti-neutrophil antibody NIMP-R14 (Abcam, Cambridge, MA, USA). Briefly, following de-paraffinization of sections, slides were immersed in antigen unmasking solution (Vector
Laboratories, Burlington, ON, Canada) for 8 min. Sections were washed with Tris-buffered saline (TBS; 0.5 M Tris–HCl, 1.5 M NaCl, pH 7.6). Lung sections were permeabilized using 0.2% Triton X-100 for 20 min. Nonspecific binding sites were saturated with Universal Blocking Solution (Dako Canada, Burlington, ON, Canada) for 20 min. Next, endogenous peroxidase activity was blocked by incubating slides in a 3% hydrogen peroxide solution for 10 minutes followed by a 5 minute wash in TBS. Primary antibodies against rat anti-NIMP-R14 (GeneTex, Irvine, CA, USA) were prepared in Universal Antibody Diluent (Dako Canada, Burlington, ON, Canada) at a dilution of 1:150 and applied to tissue sections for incubation in a humidified chamber at room temperature for 1 h. IgG2b was used as an isotype control. Sections were then washed with TBS twice for 5 min. Sections were then incubated with a Signal Enhancer for 20 minutes (GeneTex, Irvine, CA, USA) followed by anti-mouse rat and rabbit IgG (H + L) secondary antibody conjugated to HRP (GeneTex, Irvine, CA, USA). Last, Nova Red (Vector Laboratories) was used to develop the sections for 15 min and counterstained with hematoxylin and washed in ddH2O for 10 minutes. Sections were dehydrated by moving slides through three baths of xylene and mounted with Vectamount mounting medium (Vector Laboratories). Neutrophils were identified in tissue by light microscopy (Olympus BX51 microscope). Images were taken using Q-Imaging Retina 2000r camera.
5.5 Results

*Cl₂ Exposure induces Airway Hyperresponsiveness*

Mice were exposed to 100ppm Cl₂ for 5 minutes. Twenty-four hours later, the animals were challenged with doubling doses of MCh ranging from 6.25 to 50 mg/ml. Respiratory system resistance and elastance were evaluated. There was a dose-dependent increase in responsiveness to MCh. Cl₂ exposed mice showed significantly higher responsiveness at the three highest doses of MCh for respiratory system resistance (Figure 5.1A) and at the four highest doses of MCh for respiratory system elastance (Figure 5.1B).

*Changes in bronchoalveolar lavage cells after Cl₂ gas exposure*

The effects of Cl₂ exposure on airway inflammation and epithelial cell shedding were assessed by bronchoalveolar lavage (BAL) at 6 and 24 hours after Cl₂ exposure. Total cell counts were greater at 6 and 24 hours in Cl₂ exposed than in air exposed mice. There was no difference in total cell numbers in Cl₂ exposed mice between 6 and 24 hours (Figure 5.2A). There were no differences in macrophage numbers among any group (Figure 5.2B). In contrast there was a marked increase in neutrophil numbers at 6 hours which was maintained through 24 hour in mice exposed to Cl₂ as compared to air exposed mice (Figure 5.2C). Increases in eosinophils were significant at 24 hours following exposure only (Figure 5.2D). At 6 and 24 hours increases in lymphocyte numbers were noted in Cl₂ exposed animals compared to air exposed (Figure 5.2E). Large numbers of epithelial cells were shed at both 6
and 24 hours following Cl₂ exposure. The number of epithelial cells detected in the BAL was fell between 6 and 24 hours (Figure 5.2F).

_Evaluation of cysteinyll-leukotrienes in BAL following Cl₂ exposure_

Mice were exposed to Cl₂ and BAL was collected at several time points to determine if Cl₂ triggered synthesis of cysLTs in the lung by ELISA. Collection times were 30 minutes, 1, 2, 4, 6, 8, 24 and 48 hours post- Cl₂ exposure. The levels of cysLTs in BAL were lower than baseline at 30 minutes but were significantly increased 24 hours following Cl₂ exposure, returning to baseline levels by 48 hours (Figure 5.3A).

_CysLT biosynthetic enzyme gene expression following Cl₂ exposure_

At 6 and 24 hours following Cl₂ exposure, lung tissue was collected for gene expression analysis of cysLT related biosynthetic genes and cysLTr1. At 6 hours, 5-lipoxygenase (5-LO) (Figure 5.4A), 5-lipoxygenase activating protein (FLAP) (Figure 5.4B), and LTC₄ synthase ( LTC₄s) (Figure 5.4C) were increased compared to air exposed mice only in the central lung. Samples of peripheral lung were taken separately and no changes in any biosynthetic enzyme mRNA levels were detected. At 6 hours, only LTC₄s was increased compared to 24 hours (Figure 5.4C). CysLTr1 was increased 24 hours following Cl₂ exposure (Figure 5.4D). No increase in LTA₄ hydrolase was detected (Figure 5.4E).


Antioxidant gene expression following Cl\(_2\) exposure

To determine if Cl\(_2\) exposure induces oxidative stress related gene expression, NRF-2, SOD-1 and GPX-2 expression levels were evaluated by PCR. NRF-2 was increased in Cl\(_2\) exposed WT mice, but was not elevated in cysLTr1-/-mice (Figure 5.5A). SOD-1 levels increased in WT mice by 24 hours but were significantly lower at baseline, 6 hours and 24 hours in cysLTr1-/-mice compared to WT (Figure 5.5 B). GPX-2 levels were reduced in cysLTr1-/-mice at baseline, but rose at 6 and 24 hours. There were no significant differences in GPX-2 levels at any time point for WT mice (Figure 5.5C).

Effects of DMTU on cysLT related gene expression and cysLT production

To relate the increase in oxidative stress following Cl\(_2\) to the increased gene expression of cysLT related genes and biosynthesis of cysLTs, the antioxidant DMTU was administered 1 hour prior to Cl\(_2\) exposure. Lung tissue was collected 6 hours following Cl\(_2\) exposure. DMTU prevented increases in 5-LO (Figure 5.6A) and LTC\(_{4}\)s (Figure 5.6B), but had no effect on LTA\(_4\) hydrolase (Figure 5.6C). At 24 hours following Cl\(_2\) exposure, DMTU prevented increases in cysLTs found in BAL (Figure 5.6D).

Deletion of CysLTr1 results in increased inflammatory cell influx

To evaluate if cysLTr1 played a role in the development of airway inflammation following Cl\(_2\) exposure, we assessed the lung inflammatory profiles of mice carrying a cysLTr1 deletion (cysLTr1-/-). WT and cysLTr-/-
mice were exposed to Cl$_2$ and BAL was collected at 6 or 24 hours later. Compared to WT mice, cysLTr1/-/- did not show increases in total cells at either time point (Figure 5.7a). Macrophage and eosinophil numbers did not differ between WT and cysLTr1/-/- (Figure 5.7B & C). There was a striking increase in the number of neutrophils found in BAL of cysLTr1/-/- compared to WT at 6 hours and this increase was retained through 24 hours (Figure 5.7D). CysLTr1/-/- had more BAL lymphocytes at both 6 and 24 hours following Cl$_2$ compared to WT (Figure 5.7E). The number of shed epithelial cells was lower in cysLTr1/-/- compared to WT at 6 hours, with no difference between strains at 24 hours (Figure 5.7F).

*Cl$_2$ exposure induces exaggerated airway hyperresponsiveness in CysLTr1/-/- mice*

CysLTr1/-/- and WT mice were exposed to Cl$_2$ and 24 hours later airway responsiveness to inhaled MCh was evaluated. CysLTr1/-/- mice demonstrated significantly increased respiratory system resistance compared to WT mice at the highest dose of MCh and no differences were seem between strains exposed only to air (Figure 5.8A). Respiratory system elastance was not significantly increased in among cysLTr1/-/- mice exposed to Cl$_2$, despite an overall trend in this group showing greater responsiveness (Figure 5.8B).

*Deletion of CysLTr1 prevents NRF-2 translocation and transcription*
To determine if the increases in respiratory system resistance among cysLTr1-/- mice was related to the endogenous anti-oxidant response, we evaluated nuclear translocation of NRF-2 following Cl₂. Under conditions of oxidative stress, NRF-2 translocates to the nucleus where it acts as a transcription factor, ultimately initiating the synthesis of several critical antioxidants. Following Cl₂, WT mice initiate an antioxidant response and NRF-2 is translocated to the nucleus of bronchial epithelial cells by 24 hours compared to air exposed mice (Figure 5.9A). In contrasting, quantification of NRF-2 translocation in cysLTr1-/- mice showed no detectable translocation of NRF-2 to the nuclear region at either 6 or 24 hours (Figure 5.9A). Furthermore, and in contrast to WT (Figure 5.6A), NRF-2 mRNA levels were not increased above baseline in cysLTr1-/- following Cl₂ (Figure 5.9B).

**CysLTr1-/- have increased apoptosis following Cl₂ exposure**

In order to address a potential consequence of the failure cysLTr1-/- mice to mount an NRF-2-based antioxidant response, we evaluated bronchial epithelial cell apoptosis. Using TUNEL staining at 6 and 24 hours following Cl₂ exposure in cysLTr1-/- and WT mice we quantified the number of apoptotic cells for each airway corrected for the perimeter of the basement membrane. The number of apoptotic bronchial epithelial cells was significantly increased in both WT and cysLTr1-/- mice at 6 hours (Figure 5.10A). However, by 24 hours, the number of apoptotic cells in WT mice had returned to baseline.
whereas the rates of apoptosis in cysLTr1-/ mice continued to rise (Figure 5.10A).

_E-cadherin is overexpressed in cysLTr1/-_  
CysLTs acting through cysLTr1 downregulate e-cadherin, and that downregulation plays a role in the prevention of apoptosis. Over-expression of E-cadherin alters NRF-2 mediated endogenous antioxidant responses [32]. Therefore, we evaluated the expression of e-cadherin protein in airways of WT and cysLTr1/- mice. CysLTr1/- express more e-cadherin than WT mice under basal conditions (Figure 5.11A &5.11B).

_Anti-Gr1 prevents neutrophil influx following Cl2 exposure in CysLTr1/-_  
Neutrophilia has been observed in mice with defective NRF-2 mechanisms. To address the potential role of neutrophils as contributors to airway damage and AHR, anti-Gr1 or isotype control was administered to cysLTr1/- mice 6 hours prior to Cl2 exposure. At 24 hours following Cl2, BAL was performed and lungs were fixed for histological analysis to evaluate neutrophil numbers. Compared to isotype control, anti-Gr1 effectively prevented neutrophil recruitment to the lungs as reflected in BAL did not affect total cells, macrophages, eosinophils, lymphocytes or epithelial cells and (Figure 5.12A-12F). In lung tissue, neutrophils both in air exposed and Cl2 exposed mice were depleted and minimal neutrophil influx was detected in lung tissue sections (Figure 5.12G-12J).
Neutrophil depletion prevents Cl2-induced AHR in cysLTR1-/-

Mice treated with anti-Gr1 6 hours prior to Cl2 exposure were evaluated for AHR in response to inhaled MCh 24 hours following Cl2. Depletion of neutrophils significantly reduced respiratory system resistance at the two highest dose of MCh compared to mice injected with isotype control. (Figure 5.13A). Depletion of neutrophils was unable to attenuate the Cl2 -induced increase in respiratory system elastance (Figure 5.13B).
5.6 Discussion

In the current study, we have shown that cysLTr1 deleted mice have a greater susceptibility to Cl₂-induced airway damage compared to wild type mice. To show this, we first established that cysLTs were synthesized within the lung following Cl₂ exposure. In WT mice, cysLTs were detected in the BAL fluid by 6 hours following Cl₂ exposure and levels subsequently increased in a time-dependent manner until 24 hours. CysLT related biosynthetic enzymes and cysLTr1 expression increased also following Cl₂ exposure in the central lung. Cl₂ induces oxidative injury, therefore we evaluated gene expression of antioxidants NRF-2, SOD-1 and GPX-2. Cl₂ induced increases in NRF-2 and SOD-1, but not in GPX-2. CysLTr1/-/- mice demonstrated no change in mRNA levels of NRF-2 or SOD-1, but showed increases in GPX-2 levels. To relate the upregulation of cysLT biosynthetic enzyme production with Cl₂ induced oxidative stress, we showed that pre-treatment with the antioxidant DMTU prevented Cl₂-induced increases in these enzymes. DMTU also prevented production of cysLTs detected in BAL following Cl₂.

Next, we sought to determine the effects of cysLTr1 deletion on airway injury and recovery. We found that cysLTr1/-/- mice develop greater AHR in response to MCh following Cl₂ and have profound neutrophilia in comparison to WT mice. CysLTr1/-/- demonstrate prolonged airway cell apoptosis following Cl₂ exposure compared to WT mice. The location of these cells within the airway suggests that these may be bronchial epithelial cells, however, co-localization experiments staining for epithelial cell specific
markers in addition to TUNEL staining would be needed to confirm these conclusions. We postulated that this observation might be explained by aberrant antioxidant response as excessive oxidative stress may induce apoptosis.[33, 34] Consistent with this idea we observed that, unlike WT mice, cysLTr1−/− mice did not upregulate NRF-2 gene expression in response to Cl₂, nor did NRF-2 nuclear translocation in bronchial epithelial cells occur in response to Cl₂ exposure. Due to the high probability that neutrophils contribute to oxidative burden and neutrophils being the only inflammatory cell that was markedly increased in cysLTr1−/− compared to WT mice, we used a neutrophil depletion antibody (anti-Gr1) to deplete neutrophils in an effort to identify the physiological consequences of the excessive neutrophilia. We found that depletion of neutrophils from the airways attenuated the increased AHR observed in cysLTr1−/− mice.

Lastly, we sought to identify a mechanism that might explain our observations that cysLTr1−/− exhibit increased sensitivity to oxidative-stress induced airway damage. Given that cysLTr1−/− mice do not translocate NRF-2 to the nucleus of bronchial epithelial cells following Cl₂ exposure and this likely had severe consequences in the context of an oxidative injury we chose to examine e-cadherin. This protein is highly expressed in epithelial cells and its expression levels are known to affect NRF-2 translocation, including previous work showing over-expression of E-cadherin prevented NRF-2 translocation to the nucleus and increased oxidative burden.[32] Upon examination of e-cadherin protein expression, we observed an over-expression
of e-cadherin in cysLTr1/- mice compared to WT mice. The observation that cysLTr1/- mice demonstrate no increase in NRF-2 mRNA in response to Cl₂ further supports the notion that anti-oxidant response systems may be a consequence of cysLTr1/- deletion. Furthermore, we show that cysLTr1/- mice have very low expression of the NRF-2 dependent antioxidant SOD-1, regardless of Cl₂ exposure. Interestingly, cysLTr1/- mice show higher mRNA levels of the NRF-2 independent antioxidant GPX-2, in response to Cl₂, potentially suggesting compensatory mechanisms and further supporting the notion that cysLTr1/- mice have an aberrant anti-oxidant response.

In light of our results that demonstrate a greater susceptibility of CysLTr1/- mice to oxidative stress induced airway injury, we propose the following mechanism of action to explain these results. It has been observed that activation of CysLTr1 by LTD4 increases furin production through upregulation of the furin promoter, fur P1-Kpn1. Upregulation of furin results in increased production of the TGF-β and the metalloproteinase MT1-MMP, known for its ability to degrade extracellular matrix and E-cadherin, in particular. E-cadherin interacts with NRF-2 and acts as a docking site for NRF-2/Keap1. E-cadherin binds to the c-terminus of NRF-2, of importance as it is the c-terminus of NRF-2 that contains the nuclear localization signal. We speculate that mice lacking cysLTr1/- may not upregulate furin production, and therefore likely will not produce MT1-MMP in quantities sufficient to effectively degrade E-cadherin. Consistent with this, we observed higher E-cadherin expression in cysLTr1/- mice. The consequence of this higher E-
cadherin expression may not be revealed however, until such time as an oxidative burden, such as Cl₂ exposure, is placed on the system. Under these conditions, excessive E-cadherin will retain NRF-2 in the cytosol, preventing nuclear translocation and hindering production of endogenous antioxidants. This leads to an a relative increase in oxidative burden, likely resulting in increased airway damage including the higher apoptotic signal observed in cysLTr1/- mice. Furthermore, we observed increased neutrophilia in cysLTr1/- mice. In conjunction with increased E-cadherin playing a role in airway damage due to impeding NRF-2-dependent antioxidant production, previous studies have observed an increased neutrophil burden, myeloperoxidase activity, and neutrophil elastase activity following oxidative stress in mice with NRF-2 deficiency. These results in mice with abnormal or absent NRF-2 functionality may explain why we observe such high levels of neutrophil influx in cysLTr1/- mice in the context of increased oxidative stress.

We present evidence suggesting cysLTr1 may in fact be protective, not pathogenic, when examined in the context of oxidative-stress induced lung injury. Our results shed new light on the role of cysLTr1 and further examination in human models will serve to further characterize the role of cysLTr1 in the clinical setting.
Mice were exposed to 100ppm Cl\(_2\) for 5 minutes. Twenty-four hours later, the animals were challenged with doubling doses of MCh ranging from 6.25 to 50 mg/ml. There was a dose-dependent increase in responsiveness to MCh. (A) Cl\(_2\) exposed mice showed significantly higher responsiveness at the three highest doses of MCh for respiratory system resistance. (B) Cl\(_2\) exposed mice showed higher respiratory system elastance at the four highest doses of MCh.

*p<0.05, **p<0.01 ***p<.001; n=8 per group
Figure 5.2

Changes in bronchoalveolar lavage cells after Cl2 gas exposure

Panel A shows there was no difference in total cells in Cl2 exposed mice between 6 and 24 hours. (B) There were no differences among macrophage numbers in any group. (C) There was a marked increase in neutrophil numbers at 6 hours which was maintained through 24 hour in mice exposed to Cl2 as compared to air exposed mice. (D) Increases in eosinophil numbers was only significant at 24 hours following exposure. (E) At 6 and 24 hours increases in lymphocyte numbers were increased in Cl2 exposed animals compared to air exposed. (F) Large numbers of epithelial cells were shed at both 6 and 24 hours following Cl2 exposure. The number of epithelial cells detected in the BAL was reduced between 6 and 24 hours. *p<0.05, **p<0.01 ***p<.001; n=8 per group
Figure 5.3

Evaluation of cysteinyl-leukotrienes in BAL following Cl₂ exposure

Mice were exposed to Cl₂ and BAL was collected at several time points post-exposure to determine if Cl₂ induced production of cysLTs in the lung by ELISA. The panel shows that cysLTs in BAL were reduced at 30 minutes and were significantly increased 24 hours following Cl₂ exposure, returning to baseline levels by 48 hours. **p<0.01; n=6-10 per group
**Figure 5.4**

*CysLT biosynthetic gene expression following Cl₂ exposure*

At 6 and 24 hours following Cl₂ exposure, lung tissue was collected for gene expression analysis of cysLT related biosynthetic genes and cysLTr1. (A-C) At 6 hours, 5-Lipoxygenase, 5-Lipoxygenase activating protein, and LTC₄ synthase were increased compared to air exposed mice. At 24 hours, only LTC₄S remained elevated in comparison to 6 increases observed at 6 hours. (D) CysLTr1 was increased 24 hours following Cl₂ exposure. (E) No increase in LTA₄ hydrolase was detected. *p<0.05, **p<0.01; n=6-9 per group
Figure 5.5

Antioxidant gene expression following Cl₂ exposure

At 24 hours, NRF-2 levels increased in WT mice but not in cysLTr1-/- mice. (B) SOD-1 levels increased in WT mice at 24 hours. SOD-1 remained below WT levels at all time points. (C) GPX-2 levels did not change at any time point in WT mice. GPX-2 levels were below WT levels at baseline but increased to above WT levels at 6 and 24 hours. *p<0.05, **p<0.01, ***p<0.001; n=6-9 per group.
Figure 5.6

**Effects of DMTU on cysLT related gene expression and cysLT production**

The antioxidant DMTU was administered 1 hour prior to Cl₂ exposure. Lung tissue was collected 6 hours following Cl₂ exposure. (A-C) DMTU prevented increases in 5-LO and LTC₄ synthase, but had no effect on LTA₄ hydrolase. (D) 24 hours following Cl₂ exposure, DMTU prevented increases in cysLTs found in BAL. *p<0.05, **p<0.01; n=6-9 per group
A. S-LO
B. LTC₄ synthase
C. LTA₄ hydrolase
D. Cysteinyl Leukotrienes (pg/mL)
To evaluate if cysLTr1 played a role in the development of airway inflammation following Cl₂ exposure, we assessed the lung inflammatory profiles of mice carrying a cysLTr1 deletion (cysLTr1-/-). (A) Compared to WT mice, cysLTr1-/- did not show increases in total cells at either 6 or 24 hours following Cl₂ exposure. (B-C) Macrophage and eosinophil numbers did not differ between WT and cysLTr1-/- (D) Neutrophils were increased substantially in cysLTr1-/- compared to WT at 6 hours and this increase and remained elevated through 24 hours. (E) CysLTr1-/- had more lymphocytes at both 6 and 24 hours following Cl₂ compared to WT. (F) Shed epithelial cells were reduced in cysLTr1-/- compared to WT at 6 hours, with no difference between strains observed at 24 hours. *p<0.05, **p<0.01 ***p<.001; n=8-10 per group
Figure 5.8

Cl2 exposure induces increased airway hyperresponsiveness in CysLTr1-/- mice

(A) CysLTr1-/- mice demonstrated significantly increased respiratory system resistance compared to WT mice at the highest dose of MCh. No differences were seen between strains exposed only to air. (B) Respiratory system elastance was increased in cysLTr1-/- mice exposed to Cl2 compared to WT and cysLTr1-/- mice exposed to air, but did not reach significance in WT mice exposed to Cl2. **p<0.01 ***p<.001; n=8-10 per group.
**Figure 5.9**

**Deletion of CysLTr1 prevents NRF-2 translocation and transcription**

To determine if the increases in respiratory system resistance among cysLTr1-/- mice was related to endogenous anti-oxidant response, we evaluated nuclear translocation of NRF-2 following Cl2. (A) Quantification of NRF-2 nuclear translocation in bronchial epithelial cells showed that WT mice have more NRF-2 translocated to the nuclear region by 24 hours compared to air exposed mice. No translocation of NRF-2 occurred in cysLTr1-/- mice. (B) mRNA levels were not increased above baseline in cysLTr1-/- mice following Cl2 exposure. **p<0.01 ***p<.001; n=4-8 mice per group with a minimum of 8 airways evaluated per mouse.
**Figure 5.10**

**CysLTr1-/- have increased apoptosis following Cl₂ exposure**

We evaluated apoptotic cells using TUNEL at 6 and 24 hours following Cl₂ exposure in cysLTr1-/- and WT mice to address the potential consequences of the aberrant anti-oxidant response observed in cysLTr1-/- mice. (A-B) Very few apoptotic cells (brown color) are observed at baseline conditions in both strains. (C-D) The number of apoptotic + bronchial epithelial cells was increased in both WT and cysLTr1-/- mice at 6 hours. (E-F) At 24 hours, the number of TUNEL+ cells was reduced in WT mice, while cysLTr1-/- mice continued to demonstrate a substantial TUNEL+ signal. (G) Quantification of TUNEL+ showed that at 24 hours cysLTr1-/- mice demonstrate increased bronchial epithelial cell apoptosis. *p<.05; n=6-8 mice per group with a minimum of 9 airways evaluated per mouse.
Figure 5.11

E-cadherin is overexpressed in cysLTr1-/-

Overexpression of e-cadherin prevents NRF-2 mediated endogenous antioxidant response, potentially resulting in increased apoptosis due to excess oxidant burden. Because cysLTs are capable of acting through cysLTr1 to prevent excessive e-cadherin, we evaluated the expression of e-cadherin protein in airways of WT and cysLTr1-/- mice. Immunofluorescent staining for e-cadherin revealed that cysLTr1-/- express more e-cadherin than WT mice under basal conditions. E-cadherin is green. Nuclear staining is seen in blue. Below are representative panels demonstrating increased e-cadherin staining in cysLTr1-/- mice compared to WT mice. The upper left panel shows E-cadherin and nuclear staining and demonstrates that under basal conditions minimal e-cadherin is present in WT mice. The upper right panel is only e-cadherin, shown in black and white to show that e-cadherin is especially present in the airways. The lower left panel shows e-cadherin staining and nuclear staining. It is clear that more green is present. The lower right panel shows only e-cadherin in cysLTr1-/- mice, again demonstrating substantially more e-cadherin when compared to upper right (WT). Slides were stained at the same time and pictures were taken using identical settings and exposure times.
Figure 5.12

Anti-Gr1 prevents neutrophil influx following Cl₂ exposure in cysLTr1-/− mice

To address the potential role of neutrophils as contributors to airway damage and AHR, either isotype control antibody or 100μg of anti-Gr1 was administered to cysLTr1-/− mice 6 hours prior to Cl₂ exposure. At 6 and 24 hours lungs were fixed for histological analysis to evaluate neutrophil populations. In separate mice, 24 hours following Cl₂, BAL was performed to assess inflammatory cell numbers. Anti-Gr1 effectively prevented neutrophil recruitment to the lungs in both BAL and did not affect total cells, macrophages, eosinophils, lymphocytes or epithelial cells (Figure 5.12A-12F). In lung tissue, neutrophils both in air exposed and Cl₂ exposed mice were depleted and minimal neutrophil influx was detected in lung tissue sections (Figure 5.12G-12J). Arrows indicate neutrophils.
A. Total Cell Count

B. Macrophages

C. Eosinophils

D. Neutrophils

E. Lymphocytes

F. Epithelial Cells
Figure 5.13

Neutrophil depletion prevents Cl2-induced AHR in cysLT1-/-

Mice treated with 100ug of anti-Gr1 6 hours prior to Cl2 exposure were evaluated for AHR in response to inhaled MCh 24 hours following Cl2. (A) Depletion of neutrophils significantly reduced respiratory system resistance at the two highest doses of MCh compared to mice injected only with isotype control. (B) Depletion of neutrophils was unable to attenuate the Cl2-induced increase in respiratory system elastance.
A. 

![Graph showing resistance vs. methacholine concentration]

B. 

![Graph showing elastance vs. methacholine concentration]
5.7 References


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Chapter 6

LTD$_4$ induces HB-EGF-dependent CXCL8 release through EGFR activation in human bronchial epithelial cells
Prologue 6.1

In Chapter 6, we begin exploration of the role of bronchial epithelial cells in response to the cysteinyl-leukotriene LTD$_4$. In Chapter 5 we demonstrated the synthesis of cysLTs following Cl$_2$ exposure. Furthermore we noted the exaggerated neutrophilia and airway apoptosis in mice that were cysLTr1 deficient, indicating a protective role for cysLTs in the conditioning of the airway epithelial response to Cl$_2$. In Chapter 6 we have examined the epithelial response to LTD$_4$ with a focus on the release of the neutrophil chemokine IL-8 and the role of the epidermal growth factor receptor (EGFR) and the EGFR ligand heparin binding-EGF in mediating this release. However it is apparent that the HB-EGF may also have effects on the epithelial response to an insult through anti-apoptotic and pro-proliferative effects although these potential consequences of its release were not investigated in this paper. For this study, we explored the effect of LTD$_4$ stimulation on an epithelial cell line, BEAS2B, and on primary bronchial epithelial cells, NHBE cells. Cells were cultured in an air-liquid interface for most experiments in an attempt to mimic as closely as possible physiological conditions. First, we established that both BEAS2B and NHBE cells expressed the receptor for LTD$_4$, cysLTr1, by PCR and immunohistochemical analysis. Next, cells were stimulated with LTD$_4$ and several ligands of EGFR were evaluated including HB-EGF, TGF-$eta$ and amphiregulin. We found following LTD$_4$ stimulation, only HB-EGF was increased. We next evaluated IL-8 release following LTD$_4$ stimulation, and both cell types readily release IL-8 in response to LTD$_4$. To determine if the
IL-8 release observed was related to the EGFR pathway; we stimulated cells with exogenous HB-EGF in the presence or absence of an EGFR inhibitor AG1478 and evaluated IL-8 after four hours of stimulation. Both BEAS2B and NHBE cells released IL-8 in response to exogenous HB-EGF and this effect was completely prevented with AG1478, suggesting a direct mechanism. In an effort to understand the cascade of signals by which LTD₄ stimulation results in IL-8 release we used several inhibitors to block various steps along the proposed pathway. This included a metalloprotease inhibitor, GM6001, two EGFR inhibitors, AG1478 and PD-153035, a neutralizing antibody against HB-E, and the cysLTr₁ antagonist MK-571. Individually, each inhibitor was able to completely prevent LTD₄-induced IL-8 secretion. Finally, to solidify the conclusions, we employed the use of siRNA targeted against EGFR. Knock-down of EGFR prevented LTD₄-induced IL-8 release, further validating our findings.
6.2 Abstract

Airway epithelial cells release pro-inflammatory mediators that may contribute to airway remodeling and leukocyte recruitment. We explored the hypothesis that leukotriene D₄ (LTD₄) may trigger the release of pro-remodeling factors through activation of the EGF receptor (EGFR). We particularly focused on the effects of LTD₄ on release of heparin-binding EGF-like factor (HB-EGF) and IL-8 (CXCL8), a potent neutrophil chemoattractant that may be released downstream of EGFR activation. To address this hypothesis, both primary (NHBE) and transformed bronchial human epithelial cells (BEAS-2B) were grown on an air-liquid interface and stimulated with LTD₄. HB-EGF and CXCL8 were evaluated by ELISA in cell culture supernatants. To explore the EGFR signaling pathway, we used a broad-spectrum matrix metalloproteinase (MMP) inhibitor, GM-6001, two selective EGFR tyrosine kinase inhibitors, AG-1478 and PD-153035, an HB-EGF neutralizing antibody, and a specific small interfering RNA (siRNA) against the EGFR. Expression of the cysteinyi leukotriene receptor 1 (cysLT₁R) was demonstrated by RT-PCR and immunocytochemistry in both BEAS-2B and NHBE cells. Four hours after stimulation with LTD₄, HB-EGF and CXCL8 were significantly increased in cell culture supernatant. GM-6001 and montelukast, a specific cysLT₁R receptor antagonist, blocked the LTD₄-induced increase in HB-EGF. All inhibitors/antagonists decreased LTD₄-induced CXCL8 release. siRNA against EGFR abrogated CXCL8 release following stimulation with LTD₄ and exogenous HB-EGF. These findings suggest LTD₄ induced EGFR
transactivation through the release of HB-EGF in human bronchial epithelial cells with downstream release of CXCL8. These effects may contribute to epithelial-mediated airway remodeling in asthma and other conditions associated with cysteinyl leukotriene release.
6.3 Introduction

Cysteiny1-leukotrienes (cysLTs) are potent lipid mediators derived from the arachidonic acid pathway that comprise leukotriene (LT) C₄, LTD₄, and LTE₄.[1] In the lung, cysLTs are mainly synthesized by mast cells, basophils, eosinophils, macrophages, neutrophils, and epithelial cells in response to a variety of stimuli.[1] CysLTs are involved in the pathophysiology of asthma and contribute by causing bronchoconstriction, edema, mucus secretion, and increased inflammatory cell recruitment to the airways.[2] High concentrations of cysLTs are present in the sputum of patients that have moderate to severe asthma in the course of acute exacerbations.[3] CysLTs are also elevated in the sputum of patients with cystic fibrosis [4], a disease that is associated with a high prevalence of an asthmatic diathesis. CysLTs have been shown to be important agents in airway remodeling, and studies report that cysLT₁ receptor (cysLTr1) antagonists reduce airway smooth muscle hyperplasia in experimental asthma in rats and, in addition, subepithelial fibrosis in mice [5-7]. CysLTs exert their effects through G protein-coupled receptors, cysLTr1 and cysLTr2, although there may be additional receptors with complex interactions.[8]

CysLT receptors are highly expressed in the lung, spleen, and peripheral blood leukocytes. In the lung, the expression of the cysLTr1 predominates over the cysLTr2.[9] Inhaled LTD₄ causes recruitment of inflammatory cells including neutrophils as well as increasing hyperresponsiveness to inhaled methacholine.[10] CysLTs are weak
chemoattractants for granulocytes, suggesting the possibility that their in vivo effects are mediated indirectly through the release of other chemoattractants.

IL-8 (CXCL8) is a member of the CXC chemokine family. It is a potent neutrophil chemoattractant [11], and its levels are elevated in the sputum of patients with asthma and correlated with neutrophilia.[12] CXCL8 has properties that make it a potential contributor to airway remodeling by causing airway smooth muscle proliferation and migration.[13] LTD₄ stimulation has been shown to induce gene transcription and protein release of CXCL8 in vitro.[14] It has been reported that epithelial expression of CXCL8 is regulated by NF-κB, AP-1, and NF-IL-6, all three of which are, in turn, regulated via the EGF receptor (EGFR).[15] However, the possible role of the EGFR in mediating the effects of LTD₄ on airway epithelial cells is uncertain, and whether LTD₄ release of CXCL8 occurs through this pathway has not been clarified.

The EGFR has been shown to play a key role in airway epithelial repair following mechanical wounding in a model system.[7, 16] Innate immune responses of the airway epithelium mediated via the Toll-like receptor (TLR) 4 are modulated via activation of EGFR.[17] In vivo studies in the Brown Norway rat have shown that inhaled LTD₄ induces an increase in inflammatory cells in bronchoalveolar lavage, goblet cell hyperplasia, and epithelial and airway smooth muscle cell proliferation, effects that are prevented by pretreatment with an EGFR tyrosine kinase inhibitor.[15] LTD₄ also induces an increase in expression of heparin-binding EGF-like growth factor (HB-
EGF), a ligand of EGFR, on airway epithelial cells in the rat [18], suggesting a possible role for this ligand in mediating the effects of LTD₄ on epithelial and smooth muscle remodeling.

We reasoned therefore that LTD₄-induced CXCL8 release from human airway epithelial cells may be dependent on activation of EGFR via the proteolytic release of HB-EGF. To test this hypothesis, we examined the effects of LTD₄ on the release of three EGFR ligands [HB-EGF, amphiregulin, and transforming growth factor-α (TGF-α)] and CXCL8 by airway epithelial cells [transformed bronchial human epithelial cells (BEAS-2B) and normal human bronchial epithelial cells (NHBE)] and the effects of inhibitors of the mechanisms that lead to HB-EGF release. We also examined the effects of exogenous HB-EGF on CXCL8 release as well as an HB-EGF-specific neutralizing antibody on CXCL8 release triggered by LTD₄. Last, we used small interfering RNA (siRNA) to demonstrate that CXCL8 release via LTD₄ and HB-EGF is mediated through EGFR.
6.4 Methods

Reagents

Tyrphostin AG-1478 (10 μM) and PD-153035 (3 μM), specific inhibitors of EGFR tyrosine kinase and LTD₄, were obtained from Cayman Chemical (Ann Arbor, MI). Human recombinant HB-EGF, a ligand of EGFR, and its function-blocking antibody (2.5 μg/ml) were obtained from R&D Systems (Minneapolis, MN). GM-6001 (25 μM), a broad-spectrum hydroxamic acid matrix metalloproteinase (MMP) inhibitor [3-(N-hydroxycarbamoyl)-2(R)-isobutylpropionyl-L-tryptophan methylamide], was obtained from Calbiochem (La Jolla, CA). Montelukast (10 μM), a specific cysLT₁ antagonist, was provided by Merck Frosst Canada.

Cells

BEAS-2B bronchial epithelial cell line (derived from adenovirus 12-SV40-transformed normal human bronchial epithelium) were obtained from the American Type Culture Collection and maintained in DMEM/F-12 with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C under 5% CO₂. BEAS-2B cells were cultured in 75-cm² tissue culture flasks and used between passages 15 and 30. Medium was changed every other day, and cells were passaged when they reached 80–90% confluence. Following passage, cells were dissociated using trypsin and cultured in an air-liquid interface system. BEAS-2B cells were seeded (20,000/cm²) onto Transwell clear culture inserts (0.4-μm pore size; Costar, Cambridge, MA). Cells were cultured submerged for the first 3–4 days, and
medium was changed every day. On the 5th day, an air-liquid interface was established by removing the medium from the apical surface, exposing only the basal surface of the cells to medium. Cells were cultured for a further 5–7 days, and the medium (RPMI medium containing 5% FBS) was changed daily. Cells were serum-starved 24 h before all experiments. Medium from the bottom chamber was collected for all experiments.

NHBE cells cultured in air-liquid interface were generously provided by MatTek (Ashland, MA) and were cultured on 12- and 24-well inserts in a humidified incubator at 37°C and 5% CO₂ using commercial primary epithelial cell medium (MatTek) that was changed daily. Medium from the bottom chamber was collected following experiments for analysis. Cells were harvested from lung tissue that was procured from disease-free, non-smoking donors.

**Real-time PCR**

To determine basal expression level of the cysLTr1 in BEAS-2B and NHBE cells, real-time quantitative PCR (RT-qPCR) was performed using a LightCycler (Roche). Cells were serum-starved overnight before isolation of RNA with an RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's protocol. Each cDNA sample was synthesized using 0.5 μl of random hexamer primers, 0.5 μl of oligo(dT) primers, 1 μl of dNTP mix, 8 μl of RNA (1 μg), 10 μl of RNA-free H₂O, 2 μl of 10× cDNA synthesis buffer, 2 μl of 0.1 M DTT, 1 μl of RNaseOUT, and 1 μl of SuperScript III (Invitrogen). RT-qPCR was run using RT²qPCR Primer Assay for Human
CysLTr1 (SABiosciences). Briefly, for each reaction, 2.5 μl of RT² SYBR Green qPCR master mix, 10.5 μl of distilled H₂O, 1.0 μl of RT²First-Strand cDNA, and 1.0 μl cysLTr1 10 μM PCR primer pair stock were used. S9 was used a normalizing control. Conditions for real-time PCR were as follows: 15-s melting at 95°C, 10-s annealing at 59°C, and 15-s amplification at 72°C. Forty-five cycles were performed. Analysis was done using commercial software (LightCycler software package version 3.5.3). To verify the size of the amplification product, PCR reactions were analyzed on an ethidium bromide-stained 2.5% agarose gel.

**Immunocytochemistry for the CysLTr1**

Cells grown on culture inserts were fixed with 4% paraformaldehyde for 10 min. The membranes on which the cells were grown were removed from the inserts and embedded in paraffin. Sections (5μm) of the membrane were cut by microtome to allow visualization of the cells adherent to the membrane. Sections were stained with hematoxylin and eosin for assessment of cellular morphology, and immunohistochemical staining for cysLTr1 was performed with the Vectastain avidin-biotin-peroxidase complex (ABC) kit (Vector Laboratories, Burlingame, CA). Following deparaffination of sections, the slides were immersed in antigen-unmasking solution (Vector Laboratories) for 8 min. Sections were washed for 5 min twice with Tris-buffered saline (TBS; 0.5 M Tris·HCl, 1.5 M NaCl, pH 7.6). Cells were permeabilized using 0.2% Triton X-100 for 20 min, and universal blocking solution (Vector Laboratories) was applied for 20 min. Primary antibodies against human cysLTr1 (Cayman
Chemical) and control normal goat serum (Vector Laboratories) used at a dilution of 1:250 were applied to tissue sections for incubation in a humidified chamber at room temperature for 1 h. The sections were then washed with TBS twice for 5 min. Biotinylated rabbit anti-goat IgG (Vector Laboratories) was applied to the tissue sections at a 1:50 dilution and incubated at room temperature for 45 min. Sections were washed twice with TBS. Then, the slides were incubated with avidin-biotin complex alkaline phosphatase (Vector Laboratories) for 45 min. Last, Vector Red alkaline phosphatase (Vector Laboratories) was used to develop the sections for 15 min. For NHBE sections, nuclear staining was performed with Hoechst dye (Sigma-Aldrich) at a dilution of 1:2,000 in H₂O for 5 min. Following Hoechst staining, sections were washed in H₂O for 5 min. Sections were mounted with Vectamount mounting medium (Vector Laboratories). Immunofluorescence microscopy was used to visualize the cysLTr1.

Measurement of EGFR ligands and CXCL8 by ELISA

We assessed protein levels for HB-EGF, amphiregulin, TGF-α, and CXCL8 following stimulation with LTD₄ or HB-EGF at indicated time points and concentrations. Four hours following stimulation, cell supernatants were collected and frozen at −80°C. In some experiments, AG-1478 (10 μM), PD-153035 (50 μM), GM-6001 (25 μM), neutralizing antibody against HB-EGF (2.5 μg/ml), or montelukast (10 μM) were added to the cell cultures 30 min before (AG-1478, PD-15035, GM-6001, and montelukast) or simultaneously with (neutralizing antibody against HB-EGF) the addition of LTD₄. The
concentrations of various EGFR ligands and CXCL8 in the cell culture medium were determined using sandwich ELISA assays (R&D Systems).

**EGFR knockdown using EGFR-specific siRNA transfection**

BEAS-2B cells were seeded into 6-well plates at 50% confluence (2 × 10^5 cells per well) in antibiotic-free DMEM/F-12 supplemented with 10% FBS overnight. EGFR-specific siRNA (SC-29301; sense strand A, 5′-CUCUGGAGGAAAAGAAAGU-3′; sense strand B, 5′-CGUAAAGGAAAUCACAGGG-3′; sense strand C, 5′-ACAGUGGAGCGAAUCCU-3′) and the negative control siRNA (SC-37007; silencer negative control) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A concentration of 80 pmol of siRNA targeting EGFR gene and nonspecific silencer region was transfected into cells according to manufacturer’s protocol. Cells were allowed to recover for 48 hours and were put in serum-free medium and stimulated with 1 μM LTD₄ or 250 pg/ml HB-EGF for 4 hours. Supernatant and cell lysates were collected for ELISA and Western blot analysis.

**Western analysis of EGFR**

BEAS-2B cells were cultured and transfected with EGFR siRNA or the appropriate control (scrambled siRNA) as described above. Cultured cells were lysed with the following lysis buffer: 50 mM Tris·HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 2 mM DTT, and 1:100 dilution of protease inhibitor cocktail (Complete Mini Roche). Equal
amounts of cell lysate (20 μg) were separated by an 8% SDS-PAGE gel and then transferred onto a nitrocellulose membrane, which was further incubated for 1.5 h with 5% skim milk in TBS containing Tween 20. Membranes were cut to separate high and low molecular mass proteins and incubated overnight at 4°C with a rabbit polyclonal antibody for total EGFR (cat. no. SC-03; 1:500 dilution; Santa Cruz Biotechnology) and β-actin mouse monoclonal antibody (dilution 1:10,000; Sigma-Aldrich) as a loading control. Proteins were visualized using secondary antibodies conjugated to horseradish peroxidase (Amersham, Oakville, Ontario, Canada) and enhanced chemiluminescence (ECL; Amersham) on a FluorChem 8000 imaging system (Alpha Innotech, San Leandro, CA).

**Statistical analysis**

All data are expressed as means ± SE with at least n = 3 independent observations per experiment. In experiments that contained only two groups, statistical differences were determined by an unpaired Student's t-test. When comparing more than two means, two-way ANOVA was used, and post hoc analysis was performed using the Newman-Keuls test. GraphPad Prism (La Jolla, CA) was used to evaluate the data. Differences were considered significant for P values of <0.05.
6.5 Results

*CysLTr1 expression on epithelial cells*

To confirm the capacity of BEAS-2B and NHBE cells to express cysLTr1 mRNA, we performed semiquantitative RT-PCR. Following cell culture in an air-liquid interface system, both BEAS-2B and NHBE cells were tested. RT-qPCR confirmed that both cell types express cysLTr1. Similar crossing points were noted for both BEAS-2B and NHBE, indicating cysLTr1 mRNA expression to be comparable and supporting the relevance of findings on BEAS-2B for primary cells (NHBE) in our experiments. Amplicon size for cysLTr1 (85 bp) was verified on 2.5% agarose gels (Figure 6.1). We demonstrated the presence of the cysLTr1 at the protein level by immunocytochemistry. Using a polyclonal cysLTr1 antibody and immunofluorescent secondary antibody, we were able to detect expression of cysLTr1 protein (Figure 6.2, red) on the cell membrane in both BEAS-2B and NHBE cells.

*Effects of LTD₄ on release of EGFR ligands by epithelial cells*

Once we confirmed mRNA and protein expression of the cysLTr1 on BEAS-2B and NHBE cells, we examined the effects of LTD₄ on the chosen three EGFR ligands. We found significant increases in the release of HB-EGF by both BEAS-2B and NHBE cells (Figure 6.3, A-B). However, under identical conditions, we did not detect increases in TGF-α (Figure 4.3 C-D) or amphiregulin (Figure 6.3 E-F). Next, we constructed a concentration-response curve to LTD₄ for the release of HB-EGF from the cells (Figure 6.4A). A
significant release of HB-EGF was observed at 1 μM LTD₄. Using this concentration, we examined the time course of release of HB-EGF over a 24-h period (Figure 6.4B). We chose the 4-h time point for subsequent experiments in this study.

Role of the EGFR in LTD₄-induced release of CXCL8

We performed a time course over a 24-h period in BEAS-2B cells and found that LTD₄ caused a time-dependent increase in the release of CXCL8 (Figure 6.5A). The 4h incubation time with LTD₄ was chosen for the remainder of the experiments. Additionally, we confirmed that LTD₄-induced CXCL8 release was present in both cell types (Figure 6.5B-C). CXCL8 release has been demonstrated to occur downstream of the activated EGFR.[19] To confirm the involvement of EGFR in the release of CXCL8, we showed that treatment of the cells with 250 pg/ml HB-EGF caused a robust release of CXCL8 in both cell types (Figure 6.6). Additionally, a selective tyrosine kinase inhibitor, AG-1478, was successful at blocking CXCL8 release from exogenously administered HB-EGF in both cell types (Figure 6.6A-B). To further implicate specific involvement of the EGFR in the release of CXCL8 from epithelium by LTD₄, two selective tyrosine kinase inhibitors were used, AG-1478 (10 μM) and PD-153035 (50 μM). Both AG-1478 and PD-153035 potently inhibited the release of CXCL8 from LTD₄-stimulated BEAS-2B and NHBE cells (Figure 6.7A-B). Previous work has shown HB-EGF to be cleaved from its pro-HB-EGF membrane-bound form to its soluble free form by the action of MMPs.[13] Therefore, we tested the effects of a broad-spectrum MMP
inhibitor, GM-6001 (25 μM), on the release of CXCL8. GM-6001 blocked CXCL8 release in both BEAS-2B and NHBE cells (Figure 6.7A-B). To assess the contribution of HB-EGF to the release of CXCL8, we used a neutralizing antibody to HB-EGF (2.5 ng/ml). The neutralizing antibody treatment attenuated the release of CXCL8 following LTD$_4$ stimulation (Figure 6.7A-B). In each instance, the inhibitors reduced CXCL8 levels to values at or below basal levels (Figure 6.7A-B). The inhibitors on their own did not affect basal CXCL8 levels (Figure 6.7A-B).

*Effects of EGFR knockdown with siRNA on HB-EGF and LTD$_4$ induced CXCL8 release*

We used a specific siRNA targeting the EGFR to knockdown this pathway in BEAS-2B cells. Following a 24-h transfection period and a further delay of 48 h, we examined the level of EGFR by Western analysis. There was a substantial reduction in EGFR induced by siRNA compared with the scrambled siRNA treatment (Figure 6.8A). This result is representative of three experiments. The treatment of BEAS-2B cells with specific siRNA completely abrogated the release of CXCL8 by LTD$_4$ (Figure 6.8B). It also blocked the release of CXCL8 triggered by HB-EGF (Figure 6.8B).

**6.6 Discussion**

In the current study, we have shown that LTD$_4$ induces the release of CXCL8 by bronchial epithelial cells through an EGFR-dependent mechanism. We demonstrated the transcript and protein for the cysLTr1 on the epithelial cells, and using specific pharmacological inhibitors, a neutralizing antibody
against HB-EGF and EGFR-specific siRNA, we demonstrated that the cysLT1
mediated the effects of LTD₄ by a cascade of reactions involving MMPs,
release of HB-EGF, and downstream activation of the EGFR. Furthermore, we
confirmed the role of HB-EGF as the dominant ligand driving this mechanism.

CysLTs have long been recognized as contributors to the pathogenesis
of asthma. In our study, we used human bronchial epithelial cells that were
donor (NHBE) and cell line-derived (BEAS-2B) to evaluate the mechanisms
by which LTD₄ may cause remodeling through EGFR-dependent pathways
involving the airway epithelium. We first confirmed the presence of the
cysLT1 by RT-PCR. Interestingly, both cell types demonstrated similar levels
of mRNA expression for cysLT1. Diffuse immunoreactivity for the receptor
was present on BEAS-2B cells maintained in an air-liquid interface cell culture
system, whereas NHBE cells demonstrated denser staining on the apical
surface. The differences in staining between cell types may have been due to
the formation of tight junctions and cell polarization in NHBE cells, attributes
lacking in the BEAS-2B phenotype. CysLTs have been shown to be mitogenic
for primary human epithelial cells in culture with LTC₄ showing the highest
potency.[20] Montelukast inhibits LTC₄-induced synthesis and release of
TGF-β₁ by airway epithelium [21], again supporting the presence of a
functional cysLT1 on the epithelium.

Previous work has implicated the EGFR in cysLT-mediated airway
remodeling in allergen-sensitized and -challenged animals.[18, 22]
LTD₄ causes airway epithelial and smooth muscle remodeling in the rat, an
effect attenuated by the use of the selective EGFR tyrosine kinase inhibitor, AG-1478. Morphometric analysis of HB-EGF protein expression demonstrated an increase localized to the airway epithelium of LTD₄-treated rats, suggesting this ligand as the potential cause of the EGFR transactivation. Recently, studies have shown that cysLTs can induce transactivation of the EGFR in airway smooth muscle cells, but whether this was ligand-dependent was not established.[23] LTD₄ synergizes with EGF in promoting growth of fibroblasts, but the effect does not seem to involve transactivation of the EGFR and is not mediated by either cysLT₁ or -2.[24] The current study provides a potential link between the remodeling effects of LTD₄ and activation of the EGFR via the release of HB-EGF. LTD₄ induced the release of HB-EGF protein from both primary and bronchial epithelial cell lines in a time- and concentration-dependent manner. Several other G protein-coupled receptor agonists (endothelin, thrombin, and lysophosphatidic acid) have been also implicated in the activation of the EGFR, although again the release of EGFR ligands that might have mediated these effects was not examined.[25]

IL-8 (CXCL8) is a potent chemoattractant for neutrophils and a key mediator of neutrophilia in asthma.[26] LTD₄ is able to induce release of CXCL8 in a time-dependent manner, and this release is dependent on EGFR activation. Furthermore, we demonstrate that exogenously administered HB-EGF induces CXCL8 release, an effect that is also attenuated by AG-1478. Additionally, AG-1478, PD-153035, and a neutralizing antibody against HB-EGF prevent LTD₄-induced CXCL8 release. Taken together, these data
indicate that LTD₄-induced CXCL8 release is predominantly dependent on HB-EGF ligand binding leading to activation of EGFR.

Cleavage of EGFR ligands has been linked to various MMPs. MMPs cleave the proform of HB-EGF, solubilizing it and allowing it to bind to EGFR. The precise MMP responsible for this action in our current experiments has not been elucidated. However, it appears that more than one MMP may be potentially responsible for the mechanism of cleavage. We demonstrate that LTD₄-induced CXCL8 release acts via an MMP-dependent mechanism as using the broad-spectrum MMP inhibitor GM-6001 blocks CXCL8 release. This result is consistent in both BEAS-2B and NHBE cells. Whether the same MMP is responsible for the cleavage of HB-EGF in response to other stimuli such epithelial compression [29] or wounding [16] has not been elucidated. There is added significance to the current findings in relationship to mechanisms of airway remodeling mediated by cysLTs since CXCL8 itself is a mitogen for airway smooth muscle and may also cause migration of airway smooth muscle cells.[13]

CysLTs may not be the only agonists to lead to activation of the EGFR in the setting of airway disease. Previous studies have demonstrated that activation of the EGFR follows mechanical compression of the airway epithelium [14, 29, 30]. This also involves HB-EGF, as a neutralizing antibody prevents the phosphorylation of the EGFR and downstream activation of mitogen-activated kinases. TLR agonists have also been shown to activate a cascade involving ADAM17 and the release of TGF-α.[17] However, we were
not able to detect an increase in TGF-α following LTD₄ stimulation, suggesting mechanisms of actions distinct from those that follow TLR activation involving ADAM17. Finally, we used siRNA specific against EGFR to test the direct involvement of EGFR in LTD₄- or HB-EGF-induced CXCL8 release. The treatment of BEAS-2B cells with specific siRNA for EGFR successfully reduced protein expression, and the release of CXCL8 induced by LTD₄ and HB-EGF was abrogated entirely. We noted an increase in baseline levels of IL-8, an effect most likely attributable to activation of TLR3. Previous studies have shown TLR3 activation to be induced by siRNA in epithelial cells, and IL-8 levels have been shown to increase following TLR3 activation.[19, 31] Despite this, our data indicate that specific knockdown of EGFR inhibits LTD₄- and HB-EGF-induced IL-8 increase. Additionally, these findings support the conclusions derived from the use of the pharmacological antagonists of the EGFR.

We conclude that stimulation with LTD₄ sets off a cascade of signaling events that lead to the release of potent asthma mediators. These mediators have historically been implicated in the pathogenesis of asthma and remodeling. In this study, we show for the first time that the mechanism by which this process is taking place is hinged on EGFR activation through an HB-EGF-dependent system.
Figure 6.1

Expression of cysLT1 cysteiny1 leukotriene receptor (cysLTr1) mRNA in primary and epithelial cell line

RT-PCR was performed to determine presence of cysLTr1. RNA was isolated from confluent cells grown in an air-liquid interface. cysLTr1 was detected in both transformed bronchial human epithelial (BEAS-2B; A) and normal human bronchial epithelial (NHBE; B) cells. PCR products from both cell types were run on an agarose gel to confirm the expected amplicon size of 85 bp. +, PCR products that were run with cysLTr1 primers; −, negative control PCR products; Lad, ladder. \( n \geq 3 \).
Figure 6.2

Bronchial epithelial cells express cysLTr1 protein

Confluent BEAS-2B and NHBE cells grown in an air-liquid interface were formalin-fixed and embedded in paraffin. The membrane was cut into 5-μm sections and stained with hematoxylin and eosin to visualize cell morphology in BEAS-2B (A) and NHBE (B) cells. Sections were stained with cysLTr1 primary antibody and developed with a secondary red fluorescent antibody for BEAS-2B (C) and NHBE (D) cells. NHBE cells were also stained with Hoechst to visualize the nuclei (D). No positive staining was seen using the isotype control in either cell type (E and F). No nuclear staining was detected (B). n ≥ 3. Scale bar = 10 μm. Neg, negative.
Figure 6.3

Leukotriene D4 (LTD4) induces release of heparin-binding EGF-like factor (HB-EGF) but not amphiregulin or transforming growth factor-α (TGF-α)

BEAS-2B and NHBE cells grown in air-liquid interface were stimulated with LTD4 (1 μM) for 4 h. Following incubation, supernatant was collected and assayed by ELISA for presence of EGF receptor (EGFR) ligands HB-EGF, amphiregulin, and TGF-α. Both cell types released significant amounts of HB-EGF (A and B), but no changes were seen in the other ligands (C–F). \( n \geq 3; \quad *P < 0.05 \).
LTD₄ induces concentration- and time-dependent release of HB-EGF

Four hours after exposure to LTD₄, there is a concentration-dependent increase in the release of HB-EGF in BEAS-2B cells. A final concentration of 1 μM was chosen as it caused a significant increase in HB-EGF release (A). n ≥ 3; *P < 0.05. B: cells were exposed to 1 μM LTD₄, and supernatant was collected over a 24-h period. At 2, 4, 8, and 24 h, there was a significant increase in HB-EGF in the supernatant of LTD₄-exposed cells compared with controls. n ≥ 3; *P < 0.05, **P < 0.01.
A

HB-EGF (pg/mL)

Control  Vehicle  LTD₄ 1nM  LTD₄ 10nM  LTD₄ 100nM  LTD₄ 1μM

B

HB-EGF (pg/mL)

Time (in hours)

Control  LTD₄
Figure 6.5

LTD₄ induces the release of CXCL8 protein from bronchial epithelial cells

BEAS-2B cells were stimulated with 1 μM LTD₄ and allowed to incubate for given times over a 24-h period. Medium was collected at the indicated time points and assayed by ELISA for CXCL8 protein content. LTD₄ induced a significant release of CXCL8 at 4, 8, and 24 h (A). Both BEAS-2B (B) and NHBE (C) cells secreted a significant amount of CXCL8 following stimulation at 4 h. n ≥ 3; *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 6.6

**CXCL8 release induced by exogenous HB-EGF is inhibited by AG-1478**

A concentration curve using various concentrations of HB-EGF was done to establish comparable levels of CXCL8 at 4 h as to those following LTD₄ stimulation (data not shown). A concentration of 250 pg/ml HB-EGF was chosen as it induced comparable amounts of CXCL8 release from both BEAS-2B and NHBE cells 4 h following stimulation. Following incubation with HB-EGF, supernatant was collected, and protein concentration was determined by ELISA. Exogenous HB-EGF (250 pg/ml) induced significantly high levels of CXCL8 in both cell types (A, \( P < 0.01 \); B, \( P < 0.05 \)). Pretreatment with AG-1478 (10 μM) 30 min before addition of HB-EGF attenuated this effect, limiting CXCL8 levels to that of non-HB-EGF-stimulated cells (A and B). AG-1478 alone had no effect on CXCL8 release on either cell type. \( n \geq 3 \); \( P < 0.05, P < 0.01 \)
LTD₄-induced CXCL8 release is inhibited by EGFR inhibitors, metalloproteinase inhibitors, a cysLT₁ antagonist, and a neutralizing antibody

BEAS-2B and NHBE cells were stimulated with 1 μM LTD₄ for 4 h. Thirty minutes before stimulation, various inhibitors were added. AG-1478 (10 μM), PD-153035 (PD; 3 μM), GM-6001 (25 μM), and montelukast (MK; 10 μM) all attenuated the increase in CXCL8 release compared with cells that received LTD₄ only (A and B). There was no difference among any other groups. In addition, a neutralizing antibody against HB-EGF (nAB; 2.5 μg/ml) was effective at limiting the increases in CXCL8 in both cell types (A and B). There were no differences among any of the groups that received LTD₄ (1 μM) plus inhibitors and baseline CXCL8 levels. Neither the inhibitors nor the neutralizing antibody on its own caused any changes in basal release of CXCL8. n ≥ 3; *P < 0.05.
Figure 6.8

Effects of EGFR knockdown with siRNA on HB-EGF and LTD₄ induced CXCL8 release.

Small interfering (si) RNA knockdown of EGFR results in diminished release of CXCL8 following LTD₄ and HB-EGF stimulation. siRNA targeted against EGFR was transfected into BEAS-2B cells, and cells were harvested for analysis by Western blotting 72 h later. Cells treated with siEGFR had less EGFR expression than those treated with scrambled siRNA (A). B: following transfection, transfected cells were stimulated with LTD₄ (1 μM) or HB-EGF (250 pg/ml) for 4 h. Supernatant was assayed for CXCL8 by ELISA and showed that knocking down EGFR limits the release CXCL8 following stimulation. n ≥ 3; *P < 0.05, **P < 0.01.
6.7 References


Chapter 7

Discussion and Conclusions
7.1 Effects of Cl\textsubscript{2} exposure in mice

The aim of the research presented was to assess the role of oxidative stress in the manifestations of this murine model of irritant induced asthma and its link to pulmonary neutrophilia. Additionally, we wished to explore the role of cysLTs and the cysLTr1 and their links to airway dysfunction. Oxidative stress-induced asthma and the complexities associated with understanding how hallmarks such as neutrophilia affect outcomes like AHR proved a daunting but exhilarating pursuit. What began as a relatively straightforward, step-wise approach describing the murine response to Cl\textsubscript{2} inhalation evolved into a complicated and at times, counter-intuitive mechanism involving the well-established pro-asthma receptor, cysLTr1. This discussion will address my work in the same manner that led to the conclusions regarding the importance of cysLTr1 in Cl\textsubscript{2} induced asthma; a step-wise process of addressing an observation followed by an attempt to test and answer relevant mechanistic questions that arose from that observation.

In Chapters 2 and 3, we established that Cl\textsubscript{2} inhalation in mice was a reproducible stimulus for the elicitation of IIA and confirmed the presence of several hallmarks noted in IIA in humans including airway inflammation, epithelial cell denudation and subsequent proliferation, and AHR. To address the oxidative stress response we used two antioxidants, given either prior (DMTU) or following (DMTU or AEOL10150) Cl\textsubscript{2} inhalation to confirm that oxidative stress was a driving mechanism responsible for the changes in airway function and airway damage observed in mice. We found that Cl\textsubscript{2}
inhalation resulted in concentration-dependent, severe AHR in response to the bronchoconstrictor, MCh, expressed as respiratory system resistance and respiratory system elastance. We observed a high degree of neutrophilia in the lungs of mice following Cl₂ inhalation, which peaked at 6 hours and remained elevated until 24 hours, returning to baseline by 48 hours. These findings, AHR and neutrophil-dominated inflammation, are consistent with the human response to acute Cl₂ inhalation as are the changes we observed in epithelial cells that are readily shed from airways and shed cells correlate with the concentration of Cl₂ administered.\[1, 2\] Previous work has shown that airway remodeling is present in mice up to 10 days following Cl₂ administration, in the form of increased smooth muscle mass, collagen deposition, and epithelial cell proliferation.\[3\] While long-term effects of Cl₂ and subsequent remodeling processes are highly relevant, we chose to focus on acute (<24 hours) effects in the hopes of understanding the mechanism of damage, rather than repair processes. We observed increases in oxidative stress following Cl₂, with large increases in 4-HNE in lung tissue and changes in GSH and GSSG confirming that Cl₂ kick-started processes involved in the endogenous antioxidant response.

The most important outcomes observed in Chapters 2 and 3 were not the effect of Cl₂ on the lungs or that these effects could be prevented using antioxidants, but rather the timing of antioxidant administration. Given that Cl₂ is scrubbed from the airways fairly rapidly, we hypothesized that administering antioxidants one hour following Cl₂ would allow observation
of direct versus indirect effects of Cl₂ in the context of oxidative stress. While it has been established that the underlying mechanism by which Cl₂ exerts is effects are indeed oxidant driven, whether these effects were caused directly by Cl₂ exposure or some other indirect, Cl₂-induced mechanism remained ambiguous. Despite the fact that by-products of Cl₂ include potent oxidants like HOCl and HCl, we observed that antioxidant administration given at least one hour following Cl₂ administration was able to prevent AHR, neutrophilia, and 4-HNE increases, our first evidence of Cl₂’s indirect action. It appeared that Cl₂ itself, and despite its oxidant by-products, was not necessarily sufficient to provoke mechanisms leading to neutrophil recruitment, a key finding considering activated neutrophils provide an oxidant burden through the production of HOCl. One might consider that pulmonary neutrophilia and likely the damage from HOCl release from these cells may explain the observed increases in 4-HNE in lung tissue as high levels of neutrophils have been previously shown to be associated with increased 4-HNE levels in the lungs of COPD patients. [4] Importantly, AEOL administration post-Cl₂ exposure prevented epithelial cell proliferation 72 hours following Cl₂ exposure, further supporting the hypothesis that Cl₂ damage is indirect. Cl₂ exposure results in epithelial cell damage and turn-over only if the endogenous antioxidant response is not bolstered by exogenous antioxidants administered at time points after Cl₂ has likely been scrubbed.

With regard to AHR, the observation that Cl₂-induced increases in resistance and elastance could be prevented by AEOL or DMTU
administration is noteworthy as these results were consistent with the aforementioned results. At the time of the study, AEOL was the first drug to provide therapeutic value as a potential rescue treatment in the context of Cl₂ exposure and was slated to begin clinical trial testing. And, while the results of the AHR data were encouraging, they also raised many questions regarding a mechanism by which Cl₂ could induce AHR in the first place, and how an antioxidant given post-exposure could prevent this.

It should also be stated that we chose to perform experiments using the inbred mouse strain Balb/C. Although this strain of mice has been frequently used to model both allergic [5-8] and non-allergic asthma [9-12], due to the inbred nature of these mice, consideration must be given to genetic predispositions towards phenotypic responses. For example, in response to allergen-induced asthma, Balb/C mice have been shown to have greater bronchial hyperresponsiveness, increased numbers of mast cells and increased production of cytokines IL-5, and IL-13 compared to another commonly used strain, C57Bl6. C57Bl6 mice showed greater peribronchial eosinophilia as well as pulmonary neutrophilia compared to Balb/C.[13] Care must be taken when drawing conclusions from data collected using murine models of disease, as these responses may differ greatly from humans. However, with regard to irritant-induced asthma, evaluation of several strains of mice have demonstrated that Balb/C mice best replicate pathologies that manifest in humans.[14]
7.2 Depletion of neutrophils as a potential mechanism for AHR in Cl₂ induced asthma

Given the “hallmarks” established in our modeling with Cl₂ which included neutrophilia, oxidative stress and AHR, we felt that exploration of the role of neutrophils would be a relevant task as the sheer numbers of neutrophils infiltrating in the lungs of mice following Cl₂ would likely have an effect on the other outcomes such as oxidative stress and potentially AHR. When comparing AHR responses, it appeared that mice with lower neutrophil numbers i.e. Cl₂ exposed mice given AEOL or DMTU, had drastically reduced AHR, despite exposure to Cl₂. No other inflammatory cell type we observed was so affected by antioxidant administration. Furthermore, outside of our observations, it has been well-established that neutrophils contribute to oxidant burden in several disease and injury models.[15-18]

To address the role of neutrophils, we used an antibody directed against Gr-1, a receptor known to be highly expressed on neutrophils. [19] While the mechanism has not been fully clarified, it is thought that anti-Gr1 exerts its action in two ways; by halting neutrophil production in the bone marrow and temporarily sequestering neutrophils at their current location thus preventing migration.[15, 20] Anti-Gr1, administered six hours prior to Cl₂ exposure, proved to be a highly effective agent, preventing neutrophil influx into the lungs such that BAL neutrophil counts were equal to those of air exposed mice. Almost no neutrophils were observed in the lung tissue of
mice exposed to Cl$_2$ following immunostaining with the neutrophil specific antibody NIMP r-14 and by H & E staining.

Once convinced that anti-GR1 was effective, airway function was assessed in mice exposed to Cl$_2$ and pre-treated with or without anti-GR1. Results of these experiments were remarkable and somewhat perplexing. In response to MCh, anti-Gr1 was highly effective at preventing increases in respiratory system resistance and Newtonian resistance, suggesting neutrophils affect large, central airway response. Despite Cl$_2$ exposure, mice treated with anti-Gr1 maintained resistance levels equal to those exposed to air. With regard to respiratory system elastance, and tissue damping, anti-Gr1 had no effect, and mice treated showed AHR equal to that of their Cl$_2$-only treated counterparts. It appears that neutrophils play an important role in determining the reactions of the large airways, as evidenced by the Newtonian resistance, but have little effect on damage induced by Cl$_2$ to the parenchyma, or peripheral airways measured by elastance and tissue damping, in our model. A more detailed set of experiments utilizing a strain of knock-out mice carrying a specific deletion of myeloperoxidase (MPO), an enzyme produced by neutrophils and required for HOCl production, would have been beneficial to evaluate more direct effects of neutrophil-derived HOCl with regard to AHR. Recent work has shown that a potent murine neutrophil recruitment chemokine, KC, affects sinonasal ciliary action, an important biophysical process related to efficiency of airway clearance, and perhaps an effector of AHR. [21] Evaluation of KC, a murine ortholog to
human IL-8, would be worthwhile and serve to better establish the time-line of neutrophil recruitment and infiltration into the lungs. With this information, we could administer exogenous KC to mice through an intratracheal instillation followed by evaluation of AHR in the absence of Cl2 exposure and thus further elucidate the direct action of neutrophil presence in the airways. In addition antibody neutralization of KC or inhibition of its receptor would provide information concerning the role of neutrophils in AHR.

Obviously, experiments detailing the direct role of neutrophils would give greater strength to our hypothesis that neutrophils play a direct role in AHR observed in mice following Cl2, however; as these experiments were not available at the time, we returned to our histological data in an attempt to characterize and identify the significance of neutrophil presence. This examination revealed that in many of the airways, especially large airways of mice that had been exposed to Cl2, neutrophils were occupying space in the airway lumen. In many cases, the space that these “clumps” of neutrophils occupied accounted for nearly 1/3 of the airway lumen. In mice exposed to Cl2, but treated with anti-Gr1, no airways exhibited this phenomenon. Given this observation, we can perhaps speculate that neutrophils, when recruited in large numbers and begin their emigration out of the lungs, may be causing increased resistance through a mechanical mechanism. Is it possible that in an acute model of asthma, before remodeling has time to take effect, this decrease of air flow reflected by increased resistance may serve as a viable
explanation for our observation. This explanation may also be supported by the fact that regardless of Cl₂ exposure or anti-Gr1 treatment all mice have the same baseline measurements of lung mechanical properties. It is not until MCh concentrations increase that we observe differences in mice whose lungs are packed with neutrophils. Scaled for lung volume, the large airways of the mouse are enormous by comparison to other species, like humans. The result of this is that air has ample space to move, until such time as the airways are constricted, induced by substances such as MCh. It is when this forced constriction occurs that differences in resistance are most apparent, and given that our data are collected at 24 hours, well before remodeling takes place, it seems a reasonable rationale that these airways would exhibit increased turbulence as the clumps of neutrophils become compressed and limit luminal space. This hypothesis has not been formally tested; however, would likely be a worthwhile endeavor. One method of testing this hypothesis would including utilizing synthetically constructed bioairways in an in vitro system. These airways are being developed in Canada through the BRONCH program with the goal of creating “a multi-cellular, tubular construct from human airway cells that functions to all intents and purposes, exactly like the airways in our own lungs.” These engineered airways include differentiated epithelium and smooth muscle cells grow within a tubular scaffold seeded with fibroblasts. We could construct a culture system that included the addition of varying numbers of neutrophils within these bioairways and test
what, if any, flow limitations were present with increased numbers of neutrophils and at different levels of constriction.

Because elastance of Cl2-exposed anti-Gr1 treated mice rose in concert with those given Cl2-only, we attempted to identify perhaps another cell type that may influence this result. While anti-Gr1 is highly expressed on neutrophils, it is also expressed macrophages and eosinophils and its administration slightly affected BAL counts of these cells types, of note as these two cell types are often cited in development of asthma and AHR. Therefore, we chose to perform two further depletion studies for macrophages using clodronate loaded liposomes and anti-IL-5 for eosinophils. Despite both the clodronate liposomes and anti-IL-5 effectively depleting their respective targets, we saw no change in any parameter of AHR following Cl2 exposure. This result reassured us that neutrophils were likely the main cell type responsible for the observed increases in resistance; however, left unanswered questions with regard to elastance, an area that remains to be explored. As discussed in Chapter 5, it is possible that there are different neutrophil-dependent mechanisms affecting airway mechanics in the central and peripheral airways. It is difficult to estimate how neutrophils may influence airway mechanics in the peripheral airways but studies have supported a somewhat beneficial role for neutrophils as inducers of VEGF, a necessary protein for surfactant production in type II alveolar cells. [22, 23] Furthermore, VEGF promotes repair of the alveolar-capillary
membrane following acute lung injury in humans as well as animal models.[24]

Due to the likelihood that neutrophils play a role in the increases in oxidative stress observed in \( \text{Cl}_2 \)-exposed mice, we performed experiments to determine if lack of neutrophils would affect the endogenous antioxidant response. We found that indeed, presence of neutrophils has a strong influence on NRF2, a master regulator of the antioxidant response. This influence came in the form of NRF2 nuclear translocation, a necessary step for cells with antioxidant production capabilities to undergo in order to begin transcription of dozens of antioxidants. We observed the NRF2 translocation levels in bronchial epithelial cells using immunofluorescent labeling of NRF2. The data were quantified by measuring the intensity of NRF2 present in the nucleus as compared to the cytoplasm, where it remains during basal conditions. We found that NRF2 readily translocates to the nucleus following \( \text{Cl}_2 \) exposure and this is prevented by neutrophil depletion prior to \( \text{Cl}_2 \), suggesting a robust antioxidant response induced by the presence of neutrophils. To support these data, we measured mRNA levels of NRF2 and SOD-1 (an antioxidant dependent on NRF2) which increased following \( \text{Cl}_2 \) exposure. Both NRF2 and SOD-1 remained at basal levels following anti-Gr1 treatment, despite \( \text{Cl}_2 \) exposure. Of note, GPX-2, an antioxidant produced in bronchial epithelial cells of mice, and whose production is independent of NRF2 and was decreased in mice exposed to \( \text{Cl}_2 \), but not in mice given anti-Gr1, where it remained at basal level. Upon \( \text{Cl}_2 \) exposure,
bronchial epithelial cells undergo severe damage, as evidenced by Chapter 3’s Ki-67 proliferation data, and therefore these cells ability to function normally and produce certain antioxidants may be compromised by Cl₂. Chapter 4’s data serves to further implicate neutrophils as major players in the oxidative stress associated with Cl₂ exposure. Testing these hypotheses would involve relatively simple experiments in which Cl₂, HOCl or neutrophils could be applied to cultured bronchial epithelial cells. Evaluation of NRF2 cytosolic and nuclear translocation by Western blotting, immunofluorescent labeling of NRF2 and mRNA evaluation of NRF2, GPX2 and SOD-1 would further our understanding of the effects of the NRF2 mediated endogenous antioxidant response specifically in bronchial epithelial cells.

7.3 The role of cysLTs and cysLTr1

CysLTs and their receptors have long been associated with asthma. While much research has examined the role of cysLTs and their receptors within the context of allergic asthma, little information is available regarding a mechanistic role in non-allergic asthma. Furthermore, despite strong correlative evidence demonstrating a link between oxidative stress and cysLTs, little work has been done on examining mechanisms of action in the lung. In chapter 5, began examination of the role of cysLTs and cysLTr1 in the lung following Cl₂ exposure. Exploration of the role of cysLTr1 in particular was possible as we were fortunate to have access to cysLTr1
deficient mice developed by Yoshi Kanaoka and Frank Austen at Harvard Medical School. Given the pro-inflammatory, bronchoconstrictive role of cysLTs and cysLTr1, we initially hypothesized that presence of cysLTs and upregulation of cysLTr1 would relate to the increased inflammation and, potentially, AHR that we observed. Indeed, we found that cysLTs in BAL increase in a time dependent fashion following Cl\textsubscript{2} exposure and that cysLTr1 mRNA as well as other biosynthetic genes related to cysLT production were increased. To relate these increases to our previously reported increases in oxidative stress, we measured the gene expression cysLT related biosynthetic genes following pre-treatment with the antioxidant DMTU. Antioxidant treatment effectively prevented increases in 5-LO and LTC\textsubscript{4} synthase as well as prevented cysLT increase in BAL following Cl\textsubscript{2}. These initial experiments supported our hypothesis that cysLTs increase in response to Cl\textsubscript{2}. However, as we delved deeper into the role of cysLTr1, major differences suggesting that lack of cysLTr1 had deleterious effects became apparent. While baseline measurements (air exposure) detected no discernible differences between strains, Cl\textsubscript{2} exposure provoked much more severe airway inflammation in cysLTr1\textsuperscript{-/-} compared to WT and this inflammation was predominated by neutrophils. Differences between strains were most apparent at 6 hours, with more than twice as many neutrophils found in BAL of cysLTr1\textsuperscript{-/-} compared to WT mice. Evaluation of AHR in response to MCh brought even greater surprises. CysLTr1\textsuperscript{-/-} mice showed drastic increases in AHR compared to WT mice following Cl\textsubscript{2}. These
differences manifested mostly as respiratory system resistance. Because Cl$_2$ exposure results in oxidative stress, either directly or potentially through the recruitment of neutrophils at later time points, we evaluated the antioxidant capacity of cysLTr1 mice hoping to shed some light on why these mice appeared to have such exaggerated AHR. Our previous work had shown that depletion of neutrophils could ameliorate AHR following Cl$_2$, and perhaps the exorbitant numbers of neutrophils we observed in cysLTr1-/- mice was responsible the increased AHR. We evaluated NRF2 translocation and found that WT mice translocate NRF2 to the nucleus following Cl$_2$. In contrast, cysLTr1-/- mice do not. Immunofluorescent evaluation of NRF2 in the airways indicated that this translocation occurred mostly in bronchial epithelial cells, with clear signals in wild-type and little, if any, translocation in the knock-out mice. It is indeed peculiar that with such high levels of neutrophils, that NRF2 would remain in the cytosol in cysLTr1-/- mice given the effect of neutrophil depletion on NRF2 in WT mice, especially considering that NRF2 translocation in epithelial cells can be directly induced by HOCl, the primary oxidant product produced by neutrophils.\[25\] Before tackling that conundrum, we first asked if NRF2 nuclear translocation in the cysLTr1-/- mice would result in measurable consequences within the airways. Thusly, we evaluated apoptotic signals in bronchial epithelial cells using TUNEL staining. CysLTr1-/- and wild-type mice had very similar epithelial cell apoptosis signals 6 hours following Cl$_2$; however, we observed that by 24 hours, while TUNEL signals had dissipated to baseline levels in WT mice,
cysLTr1-/ mice had ongoing, rising apoptosis signals. At this point, we understood that lack of cysLTr1 resulted in faulty NRF2 translocation, increased apoptosis, severe neutrophilia and enhanced AHR. Taking into consideration all these observations, we choose to examine e-cadherin levels in cysLTr1-/ and WT mice. The rationale behind involving e-cadherin came from several lines of evidence that seemed to relate to many of our findings. First, e-cadherin levels can interfere with NRF2’s ability to translocate to the nucleus.[26] This appears to be due to the fact that e-cadherin acts as a docking station for the c-terminus of the NRF2-KEAP1 complex.[26] Second, furin, a protein responsible for breakdown of e-cadherin through metalloproteainase production of MT1-MMP, are controlled in large part by cysLTr1 signaling by LTD4.[27] Lastly, upregulation of e-cadherin exacerbates apoptosis in carcinoma cells.[28] Taken together, it seemed reasonable to hypothesize that e-cadherin levels were pertinent to explore and we hypothesized that e-cadherin may be over expressed in cysLTr1-/ mice. Immunofluorescent staining using an e-cadherin specific antibody showed that cysLTr1-/ mice have an abundance of e-cadherin protein localized to the bronchial epithelial cells in their airways. This overexpression is innate. When examining e-cadherin following Cl2 exposure, we observed strong e-cadherin signals in what epithelial cells remained in the airways, although many were damaged (and perhaps apoptotic) and evaluation was difficult. Cl2 exposure did not appear to increase e-cadherin levels in WT mice. Nevertheless, these observations at baseline may be sufficient to being to
explain the effects of Cl₂ exposure in cysLTr1/- mice. It has been observed that activation of cysLTr1 by LTD₄ increases furin production through upregulation of the furin promoter, fur P1-Kpn1.[27] Upregulation of furin results in increased production of the TGF-β and the metalloproteinase MT1-MMP, known for its ability to degrade extracellular matrix and e-cadherin, in particular.[29] E-cadherin interacts with NRF2 and acts as a docking site for NRF2/Keap1. E-cadherin binds to the c-terminus of NRF2, of importance as it is the c-terminus of NRF-2 that contains the nuclear localization signal.[26] We speculate that mice lacking cysLTr1/- may not regulate furin production properly, and therefore likely will not produce MT1-MMP in quantities sufficient to effectively degrade e-cadherin. These mice have excessive e-cadherin, likely due to the lack of cysLTr1 causing furin-mediated MT1-MMP levels to be low and thus e-cadherin to accumulate. If e-cadherin levels are high, NRF2 translocation to the nucleus is impaired, as it remains bound in the cytosol to e-cadherin which retains its hold on the NRF2/KEAP1 complex. Without NRF2 nuclear translocation, many critical endogenous antioxidants are not produced, likely leading to airway damage. Finally, faulty NRF2 signaling results in excessive neutrophilia. Previous studies have observed an increased neutrophil burden, myeloperoxidase activity, and neutrophil elastase activity in the lungs of mice with NRF2 deficiency following oxidative stress injury [30-32] Taking into consideration the inability of cysLTr1/- mice to translocate NRF2, and the high likelihood that neutrophils play an important role in Cl₂ induced injury, we depleted
neutrophils in cysLTr1-/- mice with the hopes of connecting the increased neutrophilia to the observed AHR. We found that depletion of neutrophils successfully prevented increases in respiratory system resistance in cysLTr1-/- mice, but not in elastance, a result consistent with neutrophil depletion in wild-type mice. Figure 7.1 summarizes these hypotheses, beginning with lack of cysLT1r1 resulting in less furin production, less MT1-MMP activity and thus more e-cadherin, the consequence of which includes the inability for NRF2 to effectively translocate to the nucleus as it remains bound to e-cadherin on the cell membrane.

It is fascinating that deletion of cysLTr1-/- in mice has such deleterious effects on airway injury in an oxidative stress induced model given the traditional role of cysLTr1 in allergen induced asthma. There are many more questions regarding the mechanism of action of cysLTr1 in our model that remain to be explored. Elucidation of the role of cysLTr1, its effect on inflammatory cell recruitment and AHR following injury as well as the innate effects of its deletion may be important in understanding the role of this receptor in the context of oxidative stress. Given the frequent prescribing of cysLTr1 antagonists, it may be useful to explore if long term use of these antagonists has an effect on e-cadherin levels which may potentially affect a patient's endogenous antioxidant response. As a follow-up to our current experimental results, we will attempt to answer some of the questions regarding what effect a specific cysLTr1 antagonist, montelukast may have in humans by examining tissue biopsies from asthmatics that have
taken this drug long-term. We will start by evaluating e-cadherin levels, cysLTr1 levels and neutrophil numbers in lung tissue. In our mice, examining furin levels, MT1-MMP and treating mice with cysLTr1 antagonists will serve to further elucidate these mechanisms and hopefully confirm if our current proposed hypothesis can be validated.

7.4 A potential role for EGFR

Lastly, in elaborating on our current results, it will important to keep in mind the results of experiments presented in Chapter 6. These in vitro experiments showed that exogenous LTD₄ induces the release of IL-8 from human bronchial epithelial cells. Given that IL-8 is a strong neutrophil chemoattractant, it seems difficult to explain these experiments when considering the enormous neutrophil burden present in mice with cysLTr1 deleted. Firstly, the synthesis and release of IL-8 is not exclusively a result of cysTLr1 signaling. For example, activated neutrophils release substantial amounts of IL-8 potentially through a calcineurin-dependent pathway, [33] and mast cells are capable of releasing IL-8, through adenosine A2B receptors.[34] While LTD₄ is capable of inducing IL-8 release from bronchial epithelial cells, the crux of these experiments was to show a relationship between cysLTr1 signaling and EGFR. In Chapter 6, using siRNA, we show that exogenous LTD₄-induced IL-8 release from bronchial epithelial cells is dependent on EGFR. EGFR is an important receptor and its activation is often associated with repair damaged epithelium.[35, 36] EGFR expression is relatively low
under normal conditions in rodents, however, stimuli including oxidative damage induced by cigarette smoke, inhalation of noxious chemicals like naphthalene and bleomycin injury will induce its upregulation.[37] We have observed, increases in EGFR mRNA in WT mice at 6 hours following Cl₂, but not in cysLTr1-/-, where mRNA levels do not rise above baseline. It is possible that lack of cysLTr1-/-, has a negative impact on recovery from Cl₂ exposure should upregulation of EGFR prove beneficial in our model as a pathway related to airway repair. All of the experiments done in Chapter 6 were done using cell culture modeling, and this is likely the next logical step to explore the role of EGFR in cysLTr1-/- mice. Utilizing the same in vitro system of culturing epithelial cells on an air-liquid interface, we will culture primary tracheal cells from cysLTr1-/- mice and WT mice and evaluate EGFR levels and activity in response to sodium hypochlorite, a more stable form of HOCl. It is clear from our current in vivo studies that the epithelium is heavily involved in the damage and repair processes induced by Cl₂ exposure. Elucidating mechanisms specific to the epithelium and directly related to receptors known to be involved in cellular repair may prove useful. Furthermore, in accordance with our proposed mechanism highlighted in Figure 7.1, EGFR would likely serve to help complete this picture as furin expression [38], and furin-mediated functions including the release of TGF-β and matrix metalloproteases can be stimulated via EGFR signaling pathways.[39]
7.5 Summary and Conclusions

CysLTs and their receptors have historically been viewed as pro-inflammatory and deleterious. In keeping with tradition, we initially conducted experiments that, at first glance, appeared to support much of the previous work involving cysLTs and cysLTr1. And, none of the studies presented in this thesis are inconsistent with work demonstrating that cysLTs are not in fact deleterious. Following Cl₂ exposure, we have found that cysLTr1-/- mice and WT mice produce equal amounts of cysLTs as measured in BAL fluid. CysLTr1 and its role, however, may be more complex. It was a fortunate series of events that allowed us to study cysLTr1 in the context of oxidative stress, as initially the effects of Cl₂ exposure in mice and the role of cysLTs in bronchial epithelial cells were uncoupled projects. Nevertheless, in the interest of completing a comprehensive thesis, and addressing previously unknown questions, evaluation of cysLTs in the context of oxidative stress was logical. Acquiring the cysLTr1-/- mice was somewhat of a requisite in being able to answer questions pertaining to the role of cysLTs and cysLTr1 following Cl₂, and we were quite fortunate to have been gifted these mice from Dr. Yoshihide Kanaoka. Our results with the cysLTr1-/- mice however, quickly and definitively diverged from the historical route, admittedly inducing a somewhat guttural fear response at the start. Two and half years later and after countless experiments, it has become clear that cysLTr1-/- mice have exaggerated AHR in response to MCh following Cl₂ exposure. The fact that cysLTr1 is required for these mice to mount an appropriate antioxidant response, effectively repair
their epithelium in the hours immediately following exposure and whose neutrophilia is quite apparent all result in the conclusion that presence of cysLTr1 is not deleterious, but in fact protective in the context of oxidative injury. Why it is protective is, of course, is much more interesting that the simple fact that it is. The role of neutrophils seemed a keystone in uncovering the consequence of cysLTr1 deletion. We have shown that absence of these cells can render exposure to Cl$_2$ nearly innocuous with regard to oxidative burden and large airway function. CysLTr1-/ mice have far greater numbers of neutrophils following Cl$_2$ and therefore it is not surprising that they report higher AHR. Why they are recruiting so many neutrophils in the first place poses a more difficult question. Here we observe a near lack of NRF2 activity, both at the transcriptional level as well as in regard to nuclear translocation. Again, it is not surprising that mice experiencing diluted endogenous antioxidant activity may experience greater airway damage and even AHR. In the end, it seems that the differences in WT and cysLTr1-/ mice may be predetermined at birth. Deletion of cysLTr1 results in an overexpression of e-cadherin, even in mice never exposed to Cl$_2$ and a fact that appeared harmless initially. Upon further examination it now appears that this overabundance of e-cadherin may be one of the most, if not the most, important findings. While much experimental work must be done in order to confirm and validate our preliminary findings, if it is fact, and e-cadherin overexpression is affecting mechanisms of endogenous antioxidant production, we may finally be able to directly implicate cysLTr1 as being critically important and protective in the
context of oxidative injury. Although many experiments have yet to be performed in order to elucidate the mechanisms driving the protective role cysLTr1 has, the work presented in this thesis has broken ground on the issue and solidified a robust model of oxidative injury in mice, shown neutrophils to be key players in our model, demonstrated the importance of epithelial cells both in vitro and in vivo with regard to cysLTr1, and shown that, regardless of mechanism, cysLTr1 is critical for normal response to oxidative stress.
Proposed Mechanism for e-cadherin overexpression in cysLTR1-/- mice.
7.6 References


Statement of Originality
Chapters 2 and 3 present original data demonstrating the efficacy of antioxidants administered post-Cl$_2$ exposure. We show for the first time that Cl$_2$-induced airway dysfunction, oxidative stress, airway inflammation and bronchial epithelial cell regeneration can be prevented by the post-exposure administration of two different antioxidants. These studies contributed to the understanding of how Cl$_2$ induces airway dysfunction by demonstrating that there are important indirect effects of Cl$_2$, and that it is primarily these effects that result in airway hyperresponsiveness (AHR), airway inflammation, increased oxidant stress and bronchial epithelial cell proliferation.

Chapter 4 provides original data demonstrating the role of neutrophils following Cl$_2$ exposure. Experiments in Chapter 4 revolve around depletion of neutrophils using a neutrophil depletion antibody, anti-Gr1. We successfully depleted neutrophils and found absence of this cell type completely prevented Cl$_2$ induced increases in airway resistance. Furthermore, depletion of neutrophils prevented both nuclear translocation of NRF2 and increases in antioxidant mRNA following Cl$_2$. We also depleted macrophages and eosinophils and found that depletion of these cell types was not able to attenuate Cl$_2$-induced AHR. These experiments advanced the knowledge of mechanisms, and specific cell types, that mediate a large portion of the effects of Cl$_2$ inhalation in mice.

Chapter 5 established, for the first time, that Cl$_2$ inhalation induces an increase in cysteinyl-leukotrienes and cysLT related biosynthetic enzymes. We also established that treatment with an antioxidant, DMTU, prevented Cl$_2$-
induced increases in cysteinyl-leukotrienes. We show, using cysLTr1/-deleted mice, that absence of cysLTr1 results in increased AHR, compared to wild-type mice following Cl2 exposure. We show that cysLTr1/- mice have increased neutrophilia and bronchial epithelial cell apoptosis. CysLTr1/- mice do not readily translocate NRF2 to the nucleus following Cl2, unlike their wild-type counterparts. Finally, in Chapter 5, we show that cysLTr1/- mice have higher than normal levels of e-cadherin on their bronchial epithelial cells. We propose a mechanism by lack of cysLTr1/- leads to increased e-cadherin levels impact NRF2’s ability to translocate to the nucleus, thus resulting in increased oxidative stress and neutrophilia, thus rendering cysLTr1/- mice more susceptible to Cl2-induced airway dysfunction. We show original data implicating the cysLTr1 as a protective receptor in a model of oxidative stress in the mouse.

Chapter 6 uses an established model of culturing bronchial epithelial cells in an air-liquid interface. Experiments presented in this chapter show that epithelial cells stimulated with leukoriene D4 (LTD4) release interleukin 8 (IL-8). We use several inhibitors to demonstrate that LTD4-induced IL-8 release is dependent on the epidermal growth factor receptor (EGFR) in both a bronchial epithelial cell line (BEAS2b) and primary bronchial epithelial cells (NHBE). We establish that the EGFR ligand HB-EGF, and not other ligands including TGF-β and amphiregulin is released following LTD4 stimulation of bronchial epithelial cells. Lastly, through the use of siRNA directed against EGFR, we further solidify our proposed mechanism that LTD4 induced IL-8 release is
dependent on EGFR activation. These data contribute to the understanding of the role of cysLTr1 and the effects of LTD$_4$ on bronchial epithelial cells.