The roles of cysteinyl leukotriene receptors in chlorine mediated irritant induced asthma

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Contents

Abstract

In allergic asthma, signalling through cysteinyl leukotriene receptors 1 and 2 (CysLTR1 and 2) mediates bronchoconstriction, increased vascular permeability, pulmonary edema, mucus secretion and clearance and airway smooth muscle hyperplasia. However, little is known about the role of these receptors in irritant asthma. In this project, we expanded on previous findings suggesting a protective role for CysLTR1 in a murine model of acute chlorine induced irritant asthma and explored potential roles of CysLTR2. Our first aim was to replicate previous findings that CysLTR1 knockout (CysLTR1 -/-) mice had increased inflammation and airway hyperresponsiveness (AHR) at 24h following chlorine exposure. As it was previously hypothesized that CysLTR1 -/- mice display increased AHR and inflammation due to the inability of nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor responsible for induction of many antioxidant mechanisms, to translocate to the nucleus, we also compared the phenotypes of Nrf2 -/- mice to their CysLTR1-/- counterparts at 48h. The results showed an increase in AHR but not inflammation in CysLTR1 -/- mice 24h post exposure and no difference 48h post exposure compared to their wild type counterparts. As Nrf2-/- mice have unresolved inflammation and AHR at 48h post chlorine, we concluded there was no direct mechanism linking signalling through CysLTR1 and Nrf2 translocation in the pathogenesis of irritant asthma. This was reinforced by *in vitro* experiments on a bronchiolar epithelial cell line, BEAS-2B.

Our second aim was to determine if the increase in AHR and inflammation seen in CysLTR1-/- mice could be explained by increased signalling through CysLTR2 in the absence of CysLTR1. We explored this by inhibiting CysLTR2 using HAMI 3379, a selective antagonist of CysLTR2,

in CysLTR1-/- mice and looking at response 24h post chlorine. Mice administered HAMI 3379 displayed a slightly more severe phenotype in terms of AHR than CysLTR1-/- mice administered vehicle alone, disproving our initial hypothesis. Since CysLTR2 is most commonly reported in the literature for its role in vascular leak, we evaluated the concentration of fibrinogen in the bronchoalveolar lavage (BAL) as a marker of vascular leak at 30 minutes and three hours post chlorine exposure in wild type and CysLTR1-/- mice. Fibrinogen concentration peaked at 30 min in both genotypes and returned to basal levels by three hours. Therefore, we evaluated fibrinogen levels in CysLTR1-/- mice administered HAMI 3379 at 30 min post chlorine exposure to determine the role of CysLTR2 in vascular leak. The data suggest that CysLTR2 mediates microvascular leak, although this event is not crucial in the overall pathogenesis of irritant asthma. Further study is needed to explore the mechanisms causing increased AHR and inflammation in CysLTR1-/- mice and explain the switch of CysLTR2 from a pathogenic role immediately following irritant exposure (30 min) to a protective one in later stages (24h). Irritant asthma remains a poorly understood asthma phenotype which requires further study to ensure appropriate treatment.

Résumé

Dans le contexte de l'asthme allergique, il est bien connu que la signalisation par les récepteurs cysteinyl leucotriènes 1 et 2 (CysLTR1 et 2) induit la bronchoconstriction, la perméabilité vasculaire, l'œdème pulmonaire, la sécrétion et élimination de mucus ainsi que l'hyperplasie des muscles lisses des voies aériennes. Cependant, il existe peu d'informations concernant les rôles du recepteur CysLTR1 et 2 dans l'asthme irritant. Dans ce projet, nous avons approfondi des résultats antérieurs suggérant que CysLTR1 a un rôle protecteur dans une modèle murin de l'asthme irritant provoqué par l'exposition aiguë au chlore.

Notre but premier était de confirmer les résultats antérieurs montrant que les souris ne disposant pas du gène pour CysLTR1 (CysLTR1-/-) présentent une augmentation de l'inflammation des cellules innées inflammatoire dans la lavage broncho-alvéolaire et de l'hyperréactivité bronchique (HRB) 24h avant l'exposition au chlore. Auparavant, l'hypothèse a été faite que l'augmentation de l'inflammation et de l'HRB des souris CysLTR1-/- est causée par l'incapacité du facteur érythroïde nucléaire 2-comme 2 (Nrf2), un facteur de transcription qui contrôle une grande partie des réponses antioxydantes, à se déplacer vers le noyau. Pour cette raison, nous avons comparé les réponses des souris Nrf2-/- avec les CysLTR1-/-. Nos résultats montrent une augmentation de l'HRB mais pas de l'inflammation chez les souris CysLTR1-/- 24h après l'exposition au chlore en comparaison avec les souris exprimant le gène CysLTR1. Il n'y avait pas de différences en terme d'inflammation et de l'HRB à 48h. L'inflammation et l'HRB n'étaient pas résolus chez les souris Nrf2-/- à 48h. Nous avons alors conclu qu'il n'existe pas de mécanisme direct entre la signalisation de CysLTR1 et la translocation de Nrf2 dans la pathogenèse de l'asthme irritant. Ce résultat a été confirmé par des expériences menées en

utilisant une lignée de cellulaires bronco-épithéliales, Beas-2B. Notre but secondaire consistait à déterminer si l'augmentation de l'inflammation et de l'HRB dans les CysLTR1-/- étaient causés par une augmentation de la signalisation de CysLTR2 en l'absence du récepteur principal. Nous avons inhibé CysLTR2 avec HAMI 3379, un antagoniste sélectif pour CysLTR2 dans les souris CysLTR1-/- et avons observé la réponse 24h avant le chlore. Il y avait une petite augmentation de HRB dans les souris ayant reçu HAMI 3379 par rapport aux contrôles mais pas de changement de en terme d'inflammation. Ces résultats réfutaient notre hypothèse. CysLTR2 étant plus connu pour son rôle de fuite vasculaire, nous avons évalué la concentration de fibrinogène dans les lavages broncho-alvéolaires (LBA), entre 30 min et 3h pour les souris normales et CysLTR1-/-. La fuite vasculaire s'avérait être la plus grande à 30 min et était résolue à 3h dans les deux cas. Nous avons donc évalué l'effet de l'administration de HAMI 3379 sur la fuite vasculaire à 30min. Nos résultats montraient que CysLTR2 induisait la fuite micro-vasculaire mais que la fuite vasculaire n'était pas importante dans la pathogenèse de l'asthme irritant. Nous planifions des expériences additionnelles pour déterminer la raison pour laquelle CysLTR2 est pathologique immédiatement après exposition au chlore (30min) mais protecteur à 24h. L'asthme irritant reste encore une énigme et il est impératif que nous fassions plus de recherches pour comprendre comment le traiter.

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Contribution of Authors

Tim Sun performed the Beas2B experiments and analysis of the corresponding data presented in this paper as part of his honours project.

Chapter 1: Introduction

Asthma

Definition of Asthma – Clinical Phenotypes and Endotypes

Asthma is a chronic respiratory disorder characterized by airway inflammation, airway hyperresponsiveness (AHR) and reversible airway obstruction. This triad of abnormalities leads to the common symptoms of cough, wheeze, dyspnea and chest tightness. In some individuals, frequent exacerbations can be associated with irreversible airway remodelling resulting in fixed obstructive airway disease.[1] The World Health Organization (WHO) estimates about 235 million individuals are affected by asthma worldwide.[2] The prevalence of asthma is increasing not only in low and middle income countries, but in many high income countries as well.[3] In the United States alone approximately 28 million people are afflicted with asthma, 7 million of which are pediatric cases.[3] Patients with asthma have a decreased quality of life as measured by disability adjusted life years (DALY) [4] and create a large socioeconomic burden due to both direct and indirect costs of prolonged management.[1, 2, 4] There is a high correlation between urbanization and asthma which has been postulated to result from increased exposure to indoor and outdoor pollutants. Prevalence is also especially high in rural areas of middle/ low income counties that burn solid fuel such as manure, wood and crops in open fireplaces coupled with extremely limited access to medical resources.[1]

Two distinct types of asthma have been identified based on causative agent; allergic asthma and non-allergic or irritant induced asthma. Allergic asthma is the most recognized type, and is characterized by a TH2 adaptive response resulting in production of Ig-E and eosinophilia

following subsequent allergen exposure. Based on observable clinical symptoms, asthmatics have traditionally been categorized as intermittent or persistent; the persistent cases are further subdivided into mild, moderate and severe. These groups are based mainly on spirometric measurements, specifically, the forced expiratory volume in 1 second (FEV_1) and the ratio of FEV_1 to forced vital capacity (FVC), frequency of use of short acting ß-agonists, daily symptoms, number of hospitalizations and necessity of interventions with oral, systemic corticosteroids (Table 1).[5]

As asthma is an extremely heterogeneous multifactorial disease, these diagnostic criteria have not been particularly effective in determining treatment course or prognosis. To try to address this problem, physicians have created many other phenotypic groupings to further classify asthma. These categories further subdivide patients based on age of onset, cause of disease onset, serum levels of IgE, blood eosinophilia, immune cell profile in patient sputum or bronchoalveolar lavage (BAL), corticosteroid resistance, extent of airway remodelling as well as spirometric values.[6] In recent years, there has been more enthusiasm for endotyping; grouping patients by the cellular pathways responsible for specific clinical symptoms. Often endotypes are also based on classifying patients by their response to treatment as this implies similar mechanisms are responsible for the symptoms seen among specific endotype groups.[7]

Table 1: Diagnostic Criteria for Asthma Severity

Asthma Classification	FEV_1	FEV_1/FVC	Use of short acting ß-agonists	Symptoms	Number of Hospitalizations
Mild/intermittent	$FEV_1 \geq 80\%$	Normal	More than twice weekly, no more than once daily	Minor limitation to normal activity	None in the last year*
Moderate	$60\% < FEV_1 < 80\%$	Reduced by <5%	Daily	Some limitation to normal activity	One hospitalization in the past year*
Severe	$FEV_1 \leq 60\%$	Reduced by >5%	More than once daily	Extreme Limitation to normal activity, daily symptoms	At least one hospitalization in the last year*

*hospitalization can result from lack of adherence to medication so this parameter is not specific for diagnosis of asthma severity.

Factors affecting asthma development

Asthma is a multifactorial disease associated with both genetic and environmental factors. In terms of childhood-onset asthma, there appears to be a strong genetic predisposition in individuals with a family history of allergy and/or asthma. There is a high risk of heritability in children with two asthmatic parents.[8] Early exposure to respiratory viruses seems to be associated with asthma incidence in children [9], as is bacterial colonisation of the hypopharyngeal region [10]. Asthma is most frequently allergy-induced and well controlled with inhaled corticosteroids. Other risk factors which affect childhood asthma include exposure to allergens, smoking both pre- and post-natally, environmental pollutants and obesity.

The mode of inheritance is complex, involving multiple genes that can contribute to disease development and subsequent severity. The development of disease is strongly influenced by "gene-environment interactions[11]". In adult-onset asthma, environmental factors are the primary determinants of whether a genetic predisposition leads to development of asthma. Other risk factors include chronic inflammation in the upper respiratory tract, such as rhinitis and sinusitis [12], as well as acute respiratory infections. As with childhood onset, exposure to first- and second-hand smoke, pollutants and obesity are all risk factors for asthma development in adulthood. Many adults develop asthma due to workplace exposure to sensitizing agents or irritants. While some of these exposures cause the typical TH-2-mediated eosinophilic asthma with Ig-E specific for the sensitizing epitope, other substances induce non-eosinophilic phenotypes that are neither TH-2 nor Ig-E mediated.[13, 14] These mechanisms appear to be distinct from those causing allergic asthma.

Irritant induced asthma (IIA), often interchangeable with non-allergic occupational asthma, is part of the non-TH2 endotype [6] involving the innate immune system, requiring no latency period from initial exposure to development of symptoms. It can develop from a single high dose exposure to irritants (commonly referred to as reactive airway distress syndrome, RADS) or chronic low dose exposures. Irritants take the form of gases, fumes or smoke including chlorine gas, formaldehyde, sulfur dioxide, ozone and inhaled diesel fumes.[15] Chlorine gas exposure has been an area of specific interest as many potential sources of exposure have been identified including swimming pools [16], water purification plants, military campaigns, combining household cleaning agents [17] and industrial processes that produce chlorine as a by-product. Once inhaled, chlorine gas dissolves in the fluid lining the respiratory tract forming hypochlorous acid and hydrochloric acid which interact with reactive nitrogen and oxygen species to form highly oxidative molecules.

Studies of acute chlorine exposure of human are limited; most of the data collected are based on hospital reports from individuals exposed to excessive amounts of chlorine. Information from emergency room charts of patients admitted in the South Carolina accident suggested that exposed individuals tended to wheeze rather than cough during the first 13 hours post exposure.[18] Follow up studies on victims of the train accident in Youngstown, Florida report an increase in morning cough and mucus secretion in ex/non-smokers between 3 weeks and 6 years post exposure.[19] Multiple studies report air trapping, airflow obstruction, increased lung residual volume which resolved over time and airway hyperreactivity in the first 24h post exposure.[20, 21] The impact of irritant exposure long term is still a contentious issue; while some groups report persistent decreases in FEV_1 and airway hyperreactivity,[20, 22] others report complete resolution of symptoms within months.[19] In murine models, irritants

(specifically chlorine) induce oxidative stress resulting in neutrophilia, induction of antioxidant genes, epithelial cell damage and AHR. [18] The specific mechanisms through which chlorine induces these responses have not been clearly elucidated, and the extent to which these mechanisms differ from those documented in allergic asthma remains unclear.

Pathophysiology

Inflammation

As previously stated, asthma is an extremely heterogeneous disease. The inflammatory profiles of asthma can differ extensively based on whether onset was associated with allergen sensitization and asthma severity. In typical allergic asthma, airway inflammation is dominated by eosinophilia during exacerbation; this is associated with a TH2 inflammatory signature including induction of interleukin (IL)-4, IL-5, IL-13 and IL-33.[23] However, many studies demonstrate that in severe asthma, there is a dysregulation of TH1 cytokines including interferon (IFN)-γ resulting in a more complex immune profile and increased severity.[24, 25] There are several types of identified patterns of airway inflammation based on an examination of leukocytes in sputum including eosinophilic, neutrophilic, mixed granulocytic (both eosinophilic and neutrophilic) and paucigranulocytic (few granulocytes). Neutrophilic inflammation is often observed is severe asthmatics and has been associated with TH17 activation resulting in increased levels of IL-17 and IL-8, a key neutrophil chemoattractant.[26] Irritant induced asthma is mainly neutrophilic with a phenotype that is very similar to that seen in severe asthmatics. As a result, there is a lot of interest in the pathobiology of IIA as it may allow for better understanding of severe asthma.

Airway Remodelling

One of the major characteristics of asthma as an obstructive airway disease is airway remodelling. Over time, there is an increase in airway smooth muscle (ASM) mass, epithelial cell hyperplasia, deposition of excessive matrix proteins in the reticular basement membrane and goblet cell metaplasia.[27] The increase in ASM mass is postulated to be a result of both ASM hyperplasia and hypertrophy.[28] There is often a concurrent increase in angiogenesis and myofibroblast recruitment to the sub-epithelium.[27] There is no current consensus as to whether airway remodelling occurs in IIA, partially because exposure cannot be anticipated so sample sizes in clinical studies are quite small. Takeda et. al found that between 4 and 20 years following acute accidental exposure to an oxidant caused increased thickening of the reticular basement membrane resulting in epithelial detachment. They also reported increased TGF-β, MMP-1, MMP-9, IL-8, VEGF and β-FGF in these subjects all of which favour a pro-fibrotic environment thus contributing to extracellular matrix deposition in the basement membrane.[29] Mo et al. described enduring changes to the structure of the airway epithelium, with abnormal distributions of club cells and ciliated cells following epithelial regeneration. They showed these changes led to pneumonitis independent of bacterial infection.[30] Other studies suggest that the long term effects of chlorine exposure depend on genetics, as evidenced by the differences in outcome among different mouse inbred strains.[31] Some of these finding were specific to the animal model used and have not been robustly described in human subjects. In addition, many have failed to find any evidence of long-term impact of chlorine exposure from patient data. There is currently no consensus as to the extent of airway remodelling in IIA and whether this is specifically dependent on the level of exposure and/or duration of exposure.

Epithelial damage

The airway epithelium is in direct contact with gases entering the lungs and is therefore highly affected by chlorine inhalation. In mouse models, chlorine inhalation resulted in mass epithelial cell damage particularly in the bronchi and subsequent detachment from the basement membrane. Up to 24h post exposure there was a corresponding increase in epithelial cell apoptosis; in some areas, enough dead cells were sloughed off to cause complete denudation of the airways.[32] Many groups have reported a period of epithelial proliferation over the next 24h allowing for regeneration of the airway epithelium. [32-34]

Airway Hyperresponsiveness (AHR)

Despite years of investigation, the exact pathophysiology resulting in AHR remains elusive. AHR is clinically defined as excessive airway narrowing in response to various stimuli, which can be identified by exposing patients to a series of progressive doses of a bronchoconstrictor such as histamine or methacholine.[35] Airway responsiveness is quantified by determining the provocative dose causing a decrease in FEV_1 of 20% from baseline; in patients with AHR the provocative dose is much lower than that of healthy subjects. Indeed, many healthy subjects have minimal if any fall in FEV_1 after methacholine. Therefore, AHR can be characterized by a shift to the left in the dose response curve as well as an increase in the slope of the curve.[36] In patients, increased inflammation often accompanies AHR during exacerbations; however, while anti-inflammatory compounds reduce inflammation in patients they do not necessarily decrease AHR. In addition, some patients manifest AHR in the absence of inflammation. Together this information suggests AHR can be mechanistically independent of inflammation.[35] Different components of airway remodelling in asthma have been implicated in AHR including changes in

smooth muscle cell phenotype and increased bronchial vascularization.[35] As the findings of many of these studies are based on animal models and cannot be studied in patients, the mechanisms of AHR are still an area of active investigation.

Cysteinyl Leukotrienes

Cysteinyl leukotrienes, LTC_4, LTD_4 and LTE_4, are proinflammatory derivatives of lipoxygenase mediated arachidonic acid metabolism. In this pathway, arachidonic acid in converted to LTA_4 through interaction with 5-lipoxygenase enzyme (5-LO) and FLAP. LTA_4 is subsequently hydrolyzed to LTB_4 through the action of LTA_4 hydrolase or converted to LTC_4 by LTC_4 synthase (LTC_4S). LTC_4 is rapidly converted by γ-glutamyl transpeptidase to LTD_4 and subsequently cleaved by a dipeptidase to LTE_4.[37, 38] Cysteinyl leukotrienes specifically LTC_4 and LTD_4, are potent bronchoconstrictors and play a role in inflammation, vascular permeability, pulmonary edema, mucus secretion and clearance and airway smooth muscle hyperplasia.[39, 40] There are a number of CysLT receptors that have been identified with specific affinity for the CysLTs including CysLT receptor 1 (CysLTR1), CysLT receptor 2 (CysLTR2) and GPR99. CysLTR1 is a transmembrane G-coupled protein receptor with highest affinity for LTD_4 and moderate affinity for LTC_4. It is expressed by bronchial and vascular smooth muscle cells, airway epithelial cells, macrophages, monocytes, eosinophils, basophils, mast cells and dendritic cells as well as lymphocytes.[41] Activation of CysLTR1 has been implicated in intracellular calcium signaling in bronchial smooth muscle [42], TH2 response [43], eosinophilic inflammation [44, 45] and vascular permeability (summarized in Figure 1). CysLTR1 selective antagonists such as montelukast are currently used to treat severe asthma.

There are also data which suggest that CysLTR1 signaling is involved is structural changes in cells. For example, exposure to LTD_4 of HCT116 cells (human colorectal adenocarcinoma cell line) resulted in an increase in β-catenin after 24h; while there was an overall reduction at the plasma membrane, there was increased nuclear accumulation. Nuclear translocation of β-catenin induced pro-proliferative gene expression. E-cadherin was also reduced at the plasma membrane resulting in increased migration. Interestingly, other cell lines with more E-cadherin and less CysLTR1 did not respond in the same way.[46] Studies have also implicated CysLTR1 in increases in airway smooth muscle (ASM) area following allergen exposure in rats. Administration of MK571, a forerunner of montelukast, decreased ASM compared to untreated controls in the repeatedly exposed rat allergen model.[47] Administration of LTD_4 to the rat mimicking the allergen exposures recapitulated the effects of allergen exposure.[48] Montelukast has also been shown to reverse allergen induced ASM remodeling in a murine model.[49]

In contrast, very little is known about the role of signaling through CysLTR2 and its role in asthma. CysLTR2 is also a G-protein coupled receptor with equal affinity for LTC_4 and LTD_4; however, the binding affinity is less than that of LTD_4 to CysLTR1. Neither receptor has a strong binding affinity for LTE_4; most LTE_4 signaling is mediated through GPR99.[50] CysLTR2 and CysLTR1 are co-expressed on macrophages, monocytes, eosinophils, basophils, mast cells and dendritic cells as well as lymphocytes and endothelial cells. In most cells, CysLTR1 expression dominates; endothelial cells are the only cells which express higher levels of CysLTR2.[51, 52] Although CysLTR2 is not able to induce calcium flux in ASM cells, it does influence changes in calcium in human umbilical vascular endothelial cells (HUVECs).[53] Mice lacking CysLTR2 are protected from bleomycin induced pulmonary fibrosis and cutaneous

IgE mediated microvascular leak, reinforcing the importance of CysLTR2 in the endothelium.[54] (summarized in Figure 1) Experiments conducted by Sekioka et al. (2017) studying the effect of LTC_4 inhalation in guinea pigs demonstrate that inhibition of CysLTR2 but not CysLTR1 prevents air trapping and vascular hyperpermeability.[55]

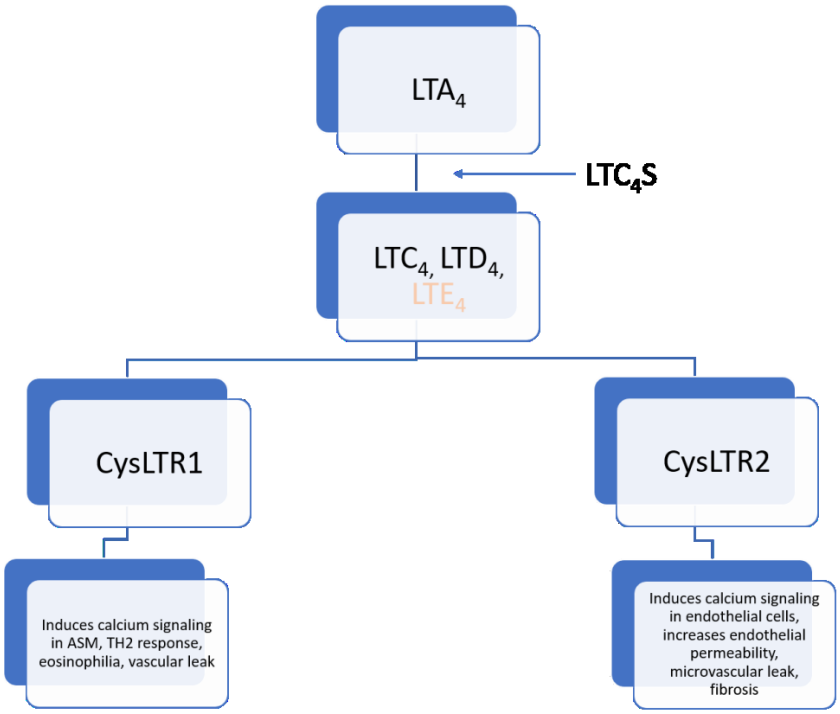

Figure 1: Role of CysLTR1 and 2 signalling in asthma and respiratory dysfunction

Antioxidant Response

Inhalation of chlorine results in the formation of free radicals which induce oxidative stress in the epithelial cells lining the airways.[18] The level of oxidative stress is increased by the infiltration of innate immune cells such as neutrophils which release both oxidants and proteolytic enzymes. In response, surrounding structural cells mount an antioxidant response to prevent oxidation of key molecules in the cell. One of the key molecules in regulating the antioxidant response is the nuclear factor erythroid 2-related factor (Nrf2); a transcription factor

for the antioxidant response element (ARE). Under normal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like EHC binding protein 1 (KEAP1); a protein which enables an E3 ubiquitin ligase to bind Nrf2, targeting it for lysosomal degradation. KEAP1 acts as a sensor for changes in oxidative state; oxidative stress causes a conformational change which frees Nrf2 from KEAP1 preventing Nrf2 degradation.[56] The KEAP1/Nrf2 complex forms a quaternary structure with β-catenin and E-cadherin which anchors this complex within the cytoplasm. Experiments conducted by Kim et al (2012) demonstrated that E-cadherin prevents nuclear accumulation of Nrf2 in a β-catenin dependent manner. Furthermore, β-catenin knockdown prevented the binding of both KEAP1 and E-cadherin to Nrf2 *in vitro*.[57] The introduction of oxidative stress alters the configuration of KEAP1, allowing Nrf2 to translocate to the nucleus and bind ARE. This enables the transcription of phase II detoxification enzymes such as NADPH, NAD(P)H quinone oxidoreductase1 (NQO1), glutathione peroxidase (GPx) and heme oxygenase 1 (HO-1). These enzymes have antioxidant properties and are involved in anti-inflammatory pathways (process summarized in Figure 2).[56] Interestingly, previous studies in our lab on Nrf2-/- mice show that there is no difference in AHR or inflammation 24h post chlorine exposure. However, Nrf2-/- mice show sustained inflammation and AHR at 48, suggesting a role for Nrf2 in resolution of oxidative stress and associated inflammation.[58]

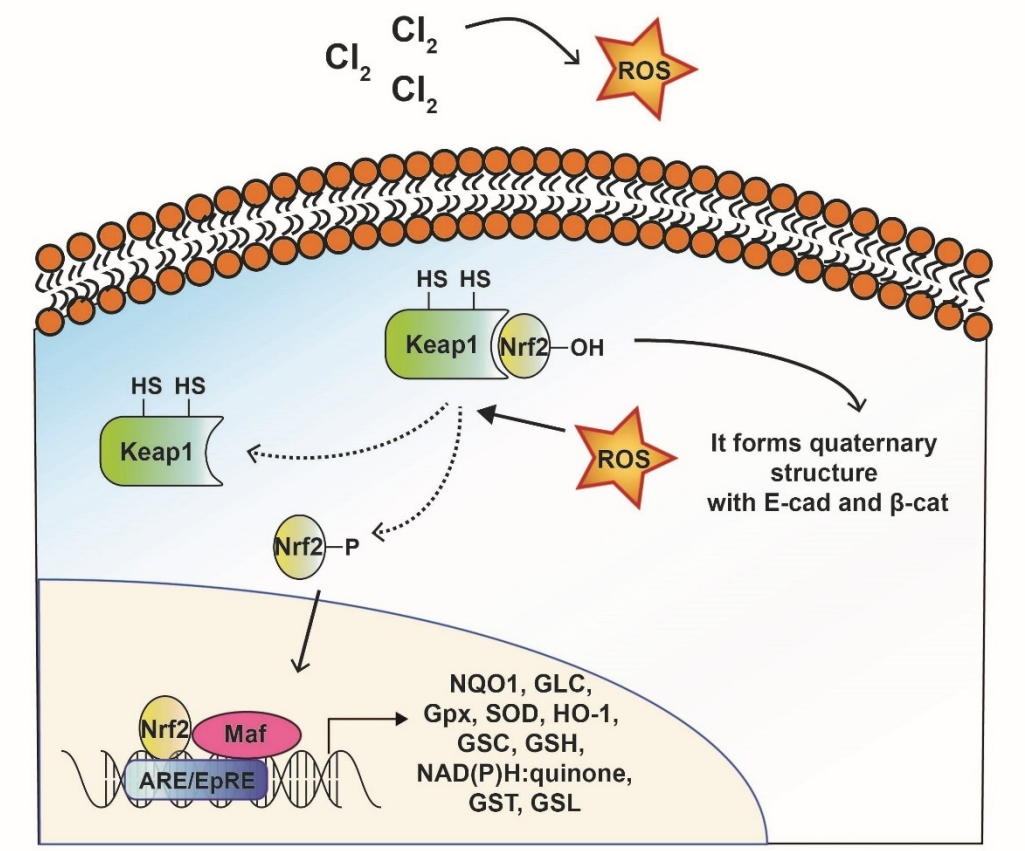

Figure 2: Schematic for the role of Nrf2 in response to chlorine exposure.

The Role of Cysteinyl Leukotrienes in IIA

The role of cysteinyl leukotrienes (CysLTs) have in irritant induced asthma is mostly speculative. Previous murine studies in our lab indicate chlorine exposure (100ppm for 5min) results in an increase in transcription of CysLT-biosynthetic enzymes in pulmonary tissue at 6h post exposure followed by increased levels of CysLTs in the bronchiolar lavage (BAL) 24h post exposure. CysLTR1 was also upregulated based on transcriptional levels at 24h.[59]

The Role of CysLT receptors in IIA

Previous studies completed in our lab demonstrate that mice lacking CysLTR1 have increased neutrophilia and AHR 24h following chlorine exposure. At baseline CysLTR1-/- mice have excessive amounts of E-cadherin in airway epithelial cells and display increased soluble E-cadherin in the BAL 24h post Cl_2 exposure. CysLTR1-/- mice also have an aberrant anti-oxidant response, and fail to upregulate transcription of many Nrf2 dependent (ex.HO-1, NQO1, Gpx2) and independent genes (ex. SOD1, CAT) as seen in wild type mice. The absence of CysLTR1 also had no impact on the increase in KC following chlorine exposure.[60]

In contrast, administration of montelukast at least partially prevented the increase in AHR due to Cl_2 exposure in mice and decreased both neutrophilia and eosinophilia compared to vehicle exposed animals. Unlike the CysLTR1-/- mice, treatment with montelukast did not alter transcription of antioxidant genes post Cl_2 exposure. Montelukast prevented increases in IL-6 and VEGF in response to chlorine but had no effect on KC.[61]

Aims of this study

In this project, we addressed the role of CysLTR1 in chlorine induced irritant asthma and the potential link between CysLTR1 signalling and the induction of an Nrf2-dependent antioxidant response. We also established a role for CysLTR2 in irritant asthma compared to that of CysLTR1.

Our first aim was to determine whether induction of CysLTR1 alone could induce Nrf2 translocation in bronchial epithelial cells *in vitro*, as measured by transcription of Nrf2-dependent enzymes. As CysLTR1-/- mice have excessive levels of E-cadherin in the bronchial epithelium, we postulated that signalling through CysLTR1 could be linked to cycling of

structural proteins such as E-cadherin and ß-catenin that sequester Nrf2 in the cytoplasm. To examine this link, we exposed BEAS-2B cells to LTD_4 *in vitro*, and determined the impact of CysLTR1 signalling and oxidative stress on Nrf2 activity based on transcription of Nrf2-dependent enzymes and transcription and changes in structural proteins. CysLTs are constitutively formed within the airways and therefore epithelial cells experience a background stimulation of the CysLTR1. Therefore, we also primed cells with LTD_4 before exposing the cells to hypochlorite (an oxidative stress) to determine whether activation of CysLTR1 before exposure to oxidative stress alters the cellular response.

Our second aim was to replicate previous findings demonstrating an increase in AHR and inflammation in CysLTR1-/-mice post chlorine exposure and compare the phenotype of CysLTR1-/- mice at 48h post Cl_2 to that previously observed in Nrf2-/- mice. Replication of the initial study of CysLTR1-/- mice was especially important as the animal facility currently housing mice differs from that pertaining to the initial study of cysLTR1-/- mice. Based on previous data from Nrf2-/- mice, Nrf2 is most heavily implicated in the resolution of AHR and inflammation following chlorine exposure we hypothesized that CysLTR1-/- mice should mimic the phenotype of Nrf2-/- in mice 48h post chlorine.

Since CysLTR1-/- mice displayed the greatest difference in inflammation 6h post chlorine exposure; therefore, we explored the role of CysLTR1-/- in inflammation at 3h post chlorine. If CysLTR1 is involved in induction of an early inflammatory response, we reasoned it might be possible to see differences in the inflammatory profile very early in the cell recruitment process.

Our final aim was to characterize the role of CysLTR2 in irritant asthma based on findings from the first two aims. We hypothesized that in the absence of CysLTR1, LTC_4 and LTD_4 would be

available to bind to CysLTR2, resulting in enhanced CysLTR2 signalling; this could explain increase in AHR and inflammation 6-24h post chlorine. As previous studies have implicated CysLTR2 in microvascular leak, we explored the time course of the appearance of fibrinogen in the BAL fluid to assess vascular leak in wild type and CysLTR1-/- mice. We used a CysLTR2 selective antagonist to evaluate the role of CysLTR2 in vascular leak post chlorine and determine whether the excessive AHR and inflammation in CysLTR1-/- 24 hours post Cl_2 could be attributed to excessive signalling through CysLTR2. In summary, the project provided increased insight into the mechanisms driving excessive AHR and inflammation in CysLTR1-/- mice, defined a role for CysLTR2 in irritant asthma and provided a comparison between the roles of CysLTR1 and 2.

Chapter 2: Materials and Methods

Epithelial Cell Culture

BEAS-2B cells were cultured in growth medium: DMEM (Thermo Fisher, Waltham, MA) with 5% FBS (Thermo Fisher) and 1% Antibiotic-Antimycotic (Life Technologies, Carlsbad, CA). All experiments were conducted in starvation medium consisting of DMEM with 0.01% FBS (and 1% Antibiotic-Antimycotic).

LTD_4 Preparation

LTD_4 dissolved in isopropanol at a concentration of 50 µg/100 nMol (Cayman Chemicals, Ann Arbor, MI) aliquots were stored at -80°C until the day of experimentation. Aliquots were thawed

as required and the isopropanol was evaporated with compressed nitrogen. The LTD_4 was re-dissolved in 10µL DMSO and diluted to the appropriate concentration in starvation medium.

Treatment of BEAS-2B cells with LTD_4

BEAS-2B cells were plated in 6 well plates at a density of 200 000 cells/well in growth medium. The following day, the medium was replaced with starvation medium (day 2). On day three, the cells were exposed to either 0, 10nM or 100nM LTD_4. Cells were harvested at each concentration at 6, 24 and 48h for cellular RNA, protein and culture supernatant.

Hypochlorite Preparation

Reagent grade sodium hypochlorite, NaOCl with 10-15% available chlorine (Sigma Aldrich, St. Louis. MO) was dissolved in starvation medium. Based on previously developed protocols in our lab, we diluted 7.5µL of NaOCl in 4mL of starvation medium. We further diluted this 10-fold in starvation medium to obtain a final working concentration of 0.3mM OCl^-.

Priming of BEAS-2B cells with LTD_4 before hypochlorite exposure

Cells were plated in 6 well plates at a density of 50 000 cells/well in complete medium. On day 2 the medium was replaced with starvation medium. On day 3 cells were exposed to LTD_4, 100nM or fresh starvation medium. On day 5 (48h post LTD_4 priming) these cells were exposed to hypochlorite solution for 4h and harvested for mRNA and protein and the supernatant stored for analysis. A separate group of cells was exposed to LTD_4 on day 4 instead of day 3, resulting in a 24h priming period before hypochlorite exposure. These cells and their controls were also harvested 4h post hypochlorite exposure for measurement of mRNA of Nrf2 dependent and

independent antioxidant enzymes, structural proteins sequestering Nrf2 in the cytoplasm and IL-8and protein level of the aforementioned structural proteins.

RNA extraction and qPCR

Supernatant was removed from plates followed by a single wash with PBS. Total RNA was extracted using RNeasy mini kit (74104, Qiagen, Venlo, Netherlands) according to manufacturer's instructions. Extracted RNA quantity was assessed using nanodrop technology. RNA was reverse transcribed to cDNA using AffinityScript cDNA synthesis Kit (Aligent, Santa Clara, CA). The amount of RNA per sample was normalized before cDNA synthesis to create a total reaction volume of 20µL. The samples were run using the following PCR program:

1. 5 min at 25 °C
2. 15 min at 42 °C
3. 5 min at 95 °C
4. 4°C for ∞

Real-Time (RT) PCR

cDNA libraries created from total cell RNA were probed for multiple genes (Table 2) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) according to manufacturer's instructions and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The program used consisted of three distinct stages; a holding stage at 90°C for 30s. a cycling stage with 40 cycles at 95 °C for 15s and 60 °C for 30s, and a melting curve stage with an incremental temperature increase of 0.5°C per cycle from 65 – 95 °C for 5s per cycle.

Table 2: Primers used for RT-PCR in cell experiments

Protein Name	Gene name	Forward primer (5’ – 3’)	Reverse primer (5’ – 3’)
Interleukin-8	IL8	GAGCACTCCATAAGGCAC	ATGGTTCCTTCCGGTGGT
Vimentin	VIM	GAGAGGAAGCCGAAAACA	CAACCAGAGGGAGTGAAT
Catalase	CAT	TCATCAGGGATCCCATAT	CCTTCAGATGTGTCTGAG
Superoxide dismutase 1	SOD1	TCATCAATTTCGAGCAGA	GCAGGCCTTCAGTCAGTC
Thioredoxin	TXN	TTACAGCCGCTCGTCAGA	AAGGCTTCCTGAAAAGCA
NAD(P)H Dehydrogenase [quinone] 1A	NQO1A	CAGCTCACCGAGAGCCTA	GAGTGAGCCAGTACGATC
Heme oxygenase 1	HO-1	GGCAGAGGGTGATAGAAG	AGCTCCTGCAACTCCTCA
E-cadherin	CDH1	AGAGAAGCCATTGCCAAGTAC	AACGAATCCCTCAAAGACCG
B-catenin	CTNNB1	TGACCTGAGACTGGATGTAGAAAA	CCCCATCACTCAGCTCATAAA
Ribosomal Protein S9	RPS9	CTGCTGACGCTTGATGAGAA	CAGCTTCATCTTGCCCTCA

Protein Extraction and Western Blot

At harvest, medium was removed from cells and cells were washed once with PBS. Protein lysis buffer consisting of NP40 cell lysis buffer (FNN002, Thermo Fisher, Waltham, MA) with 1% 100X Halt™ Protease and Phosphatase Inhibitor Cocktail (78426, Thermo Fisher,Waltham, MA) was added to each well and plates were incubated on ice for 15min. Cells were completely detached from the plates using a cell scraper and the entire volume from each well was transferred into separate 1.5mL tubes. Lysates were centrifuged at 13,000 RPM for 3 minutes at 4°C to pellet lipids; supernatants containing protein were collected in a separate tube.

Western Blot

We combined 5µg sample protein with 4 µL 4X Laemmli Sample Buffer (Bio-Rad), 0.32 µL TCEP solution (Sigma-Aldrich) and added ddH_2O to a final volume of 14µL. Samples were denatured for 6min at 95°C and loaded into a precast 4 – 15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) with 10µL of Precision Plus Protein™ Kaleidoscope™ Standards Protein Ladder (Bio-Rad). Gels were run at 200V at room temperature until the loading buffer ran to the bottom of the gel (approximately 30 min). The gel was then transferred onto Immune-Blot® PVDF Membrane with 0.2 µm pore size (Bio-Rad) and left in the transfer chamber under 250mA for 2 hours at 4 °C. The membrane was transferred to blocking solution consisting of 5% bovine serum albumin (BSA) and tris-buffered saline with Tween®20 (TBST), and incubated overnight at 4 °C. The next day, the membrane was exposed to primary antibody diluted in 5% BSA TBST and incubated at 4°C overnight (see concentrations in Table 3). The membrane was washed and incubated with secondary antibody diluted in TBST for 1h at room temperature. The membrane was washed, Clarity™ Western ECL substrate (Bio-Rad) was added and imaged

with ChemiDoc MP system (Bio-Rad).

Table 3: Concentrations of Antibodies Used for Western Blot

Company	Antibody	Clone	Dilution
Abcam	Rabbit monoclonal anti-beta Catenin IgG	E247	1:8000
Abcam	Mouse monoclonal anti-E Cadherin IgG	GT477	1:2000
EMD Millipore	GAPDH	6C5	1:3000
Cell Signaling	HRP-linked anti-rabbit IgG	7074	1:5000
Cell Signaling	HRP-linked anti-mouse IgG	7076	1:2000

Animal Models

Genotyping and Husbandry

Heterozygous female mice for the CysLTR1 knockout were crossed with homozygous Balb/C males. Tail samples of progeny were taken when weaned to determine genotype. DNA was extracted by vortexing tail samples in 120µL of 50mM NaOH. Samples were heated to 95°C for 1h, vortexing intermittently. Once removed from the heat, 10µL of 1M Tris-HCL (pH 7.0) was added to each sample. Samples were immediately vortexed followed by centrifugation at room temperature for 10min at 7200XG. Supernatant was removed and stored at -20°C. Three primers were used for PCR at 100nM concentrations: CysLTR1 sense (5'- AAA ACA ATG ACG TGC ACT ATA AAG-3'); CysLTR1 anti- sense (5'- AAT CAT GTA TAC TTG GAA

GGC TGA -3'); CysLTR1 neo anti-sense 5'- ATC TTG TTC AAT GGC CGA TCC CAT -3'). DNA was amplified using Platinum® Taq DNA Polymerase Kit (10966-026, Life Technologies/ Thermo Fisher, Waltham, MA, USA). For each sample, added 1.0 µL mouse DNA to a mix of 5.0µl 10×PCR Buffer; 1.5 µl 50mM MgCl2 solution; 1.0µl 10mM dNTP mix, 1. 0µl CysLTR1 neo antisense primer (10 µM);1.0µl CysLTR1 sense primer (10µM); 1.0µl CysLTR1 antisense primer (10µM); 0.2µl Platinum Taq Polymerase and 38.3µl DNAse free water to a total reaction volume of 50µl. Samples were amplified using the following PCR template: Step 1: 95℃ for 30 sec, Step 2: 55℃ for 30 sec, Step 3: 72℃ for 60 sec for 35 cycles. Samples were run on a 3% agarose gel with ethidium bromide at a voltage of 100V for 1h. Bands were visualized using UV light. Since the CysLTR1 knockout gene was created by inserting DNA into the CysLTR1 wild type gene, mice with the wild type allele had a band of 284bp and the mice with the knockout gene had a band of 333bp. Heterozygotes had both bands (Figure 3). Since CysLTR1 is X-linked only females were heteroz ygotes. Homozygous breeding pairs were established to create a knockout colony. (Figure 3)

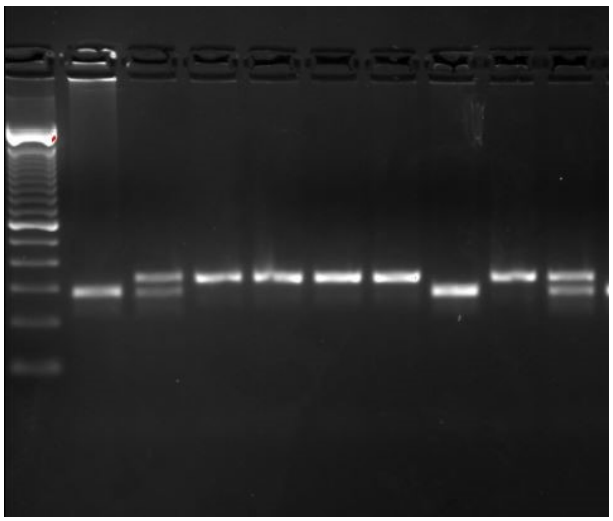

Figure 3: Gel electrophoresis for determining CysLTR1 genotype.

The CysLTR1 null allele was created by knocking in small non-coding segment of DNA into the coding region for the gene. Therefore, wild type mice have one band at 284bp, CysLTR1-/- mice have one band at 333bp and heterozygous mice have both

Chlorine Exposure

Wild type and CysLTR1 -/- mice were exposed to 100ppm Cl_2 for 5min in a nose only exposure chamber using Dynacalibrator (model 230-28A, VICI Metronics, WA, USA) as previously described by our group.[62]

Measurement of airway responsiveness to methacholine

At either 24 or 48h post chlorine exposure, mice were sedated with xylazine hydrochloride (10mg/kg i.p.) and anesthetized with sodium pentobarbital (50mg/kg i.p.). Mice were tracheotomized and the trachea was cannulated with an 18-gauge cannula which was connected to the small animal ventilator (Flexivent FX1,Scireq, Montreal, Canada) to enable ventilation of mice. Mice were administered 0.2mg/kg i.p. rocuronium bromide to induce muscle paralysis thereby inhibiting spontaneous breathing efforts and challenged with progressively increasing concentrations of methacholine (MCh); 0, 6.25, 12.5, 25 and 50mg/mL in PBS. The software took 6 measurements per dose each of which yielded values for resistance (Rrs), elastance (Ers), Newtonian resistance (Rn), tissue damping (G) and tissue elastance (H). The mechanics parameters were determined using both a single compartment model and the constant phase model. [63] Rrs estimates the magnitude of airway constriction in the lungs and Ers is a dynamic measurement of the rigidity of the lungs to mechanical inflation. Rn is an estimate of the narrowing affecting the large conducting airways, G is similar to tissue resistance and measures the consequences of narrowing of the distal airways and H is a measurement of the dynamic elastic properties of the lungs. Changes in G are related to airway narrowing whereas changes in H are associated with airway closure and hyperinflation.[64] The maximum value for each

measurement at each dose with a coefficient of determination greater than 0.75 was recorded for compilation and analysis.

Bronchoalveolar lavage (BAL)

BAL fluid was collected in PBS 0.01% BSA; 1ml was slowly injected into the lung through the tracheal cannula, slowly withdrawn and immediately placed on ice. BAL samples were spun down for 6 minutes at 3000Xg at 4°C. Supernatant was removed and placed in fresh tubes and stored at -80°C. For mice euthanized at 3h, the cell pellet was resuspended in 500µL PBS. Cells were counted with Trypan Blue to determine total cell count and mounted on slides using a cytospin. Slides were stained with Diff-Quik (Medical Diagnostics, Düdingen, Germany) and differential cell count was determined based on the morphology of at least 300 cells.

Harvesting of lung tissues

Lungs were extracted from mice and flash frozen in liquid nitrogen. Lungs were stored at -80°C.

Collection of BAL for vascular leak assessment/cell counts at 3h

Wild type and CysLTR1-/- mice were exposed to chlorine as described in 2.1.3. Mice were euthanized with 100mg/kg pentobarbital i.p. at 30min, 1h or 3h post exposure and BAL, lungs and blood were collected for further investigation. BAL was spun down as described in 2.1.4; cell counts and differentials were only conducted on 3h samples. Vascular leak was assessed using a mouse fibrinogen ELISA kit (ab213478, Abcam, Cambridge, UK) following manufacturer's instructions except the volume of substrate added was doubled from 50µL to 100µL and the initial incubation was changed from 1h at room temperature to overnight at 4°C.

HAMI 3379 administration/optimization

Since HAMI 3379 (*10580*, Cayman Chemical, Michigan, USA) has never been used in mouse models, initial dosage and delivery method was based on a study conducted by Shi et al (2015). In this study, the effective dosage for rats in a model of focal cerebral ischemia brain was 0.1-0.4mg/kg IP.[65] Therefore, the original protocol assessed inflammation at 6h after chlorine exposure (the time point where inflammation is highest) using a single injection of 0.2mg/kg or 0.4mg/kg drug or vehicle. Although there was a trend towards less inflammation at 6h post chlorine exposure in drug vs vehicle treated mice, the difference was not significant (Supplementary Figure 1). Based on previous studies conducted in our lab with montelukast which has a higher affinity constant for its receptor, the protocol was modified to two doses of 10mg/kg HAMI 3379 given 18h before and 1h before chlorine exposure by gavage. To compare results to others previously collected in the lab, the 6h time point was discarded and 24h was chosen as the time point of interest for AHR and inflammation.

Assessment of Airway Hyperresponsiveness

Flexivent based assessment of methacholine responsiveness was performed 24h post chlorine exposure using the same methods outlined in 2.1.3.

BAL Extraction and Processing

BAL was extracted and analyzed as described in 2.1.4.

Data analysis/statistical analysis

Both cells in the BAL and Flexivent data were analyzed using Graphpad Prism 5. The cell data were analyzed using a non-parametric Kruskal-Wallis test and a Dunn's multiple analysis post

test. Flexivent data were analyzed using a 2-way repeated measures ANOVA. Statistical significance was defined as a p value less than 0.05. (N=6 per group for air exposed mice, 8 per group for Cl_2 exposed mice).

Time point optimization in wild type mice and data for vascular leak at 30 min in wild type vs CysLTR1-/- at baseline and post chlorine were analyzed using a non-parametric Kruskal-Wallis test and a Dunn's multiple comparison post test. Differences in vascular leak at 3h were measured by t-test between chlorine exposed CysLTR1-/- mice and wild type mice as controls are the same for this time point and the 30min time point. (N=6-8 per group).

Results

LTD_4 treatment does not alter transcription of anti-oxidant enzymes or structural proteins in BEAS-2B Cells

Stimulation of BEAS-2B cells with LTD_4 (100nM) had no effect on the transcription of either Nrf2 dependent or independent genes (mRNA) at 6h, 24h or 48h post treatment. There was also no change in the transcriptional or protein level of E-cadherin or β-catenin at any time point (Figure 4).

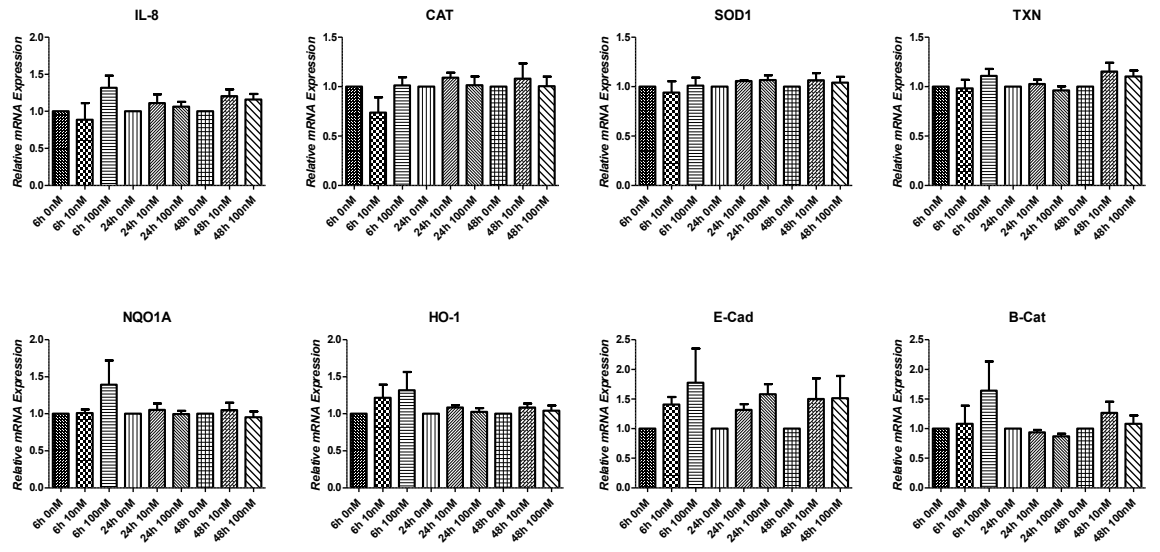

Figure 4: The effect of LTD_4 on Beas2B cell mRNA expression. There were no significant differences in mRNA expression of any protein measured. However, there seems to be a dose dependent incremental increase towards significance in E-cad and β-cat at 6h.

Hypochlorite exposure, LTD_4 pre-treatment and changes in antioxidant enzyme transcription and structural proteins

Hypochlorite treatment of Beas-2B cells did not result in significant changes in the transcription of antioxidant enzymes, E-cadherin, β-catenin or IL-8 4h post exposure. However, there is a trend towards an increase in HO-1; more experiments are needed to confirm the effect of hypochlorite exposure on cellular anti-oxidant response. Priming cells with LTD_4 either 24 or 48 hours prior to hypochlorite exposure did not result in any significant differences in antioxidant enzyme transcription, IL-8, E-cad or β-cat. (Figure 5). Western blot also showed no significant increase in protein levels of E-cad or β-cat between any experimental groups (data not shown).

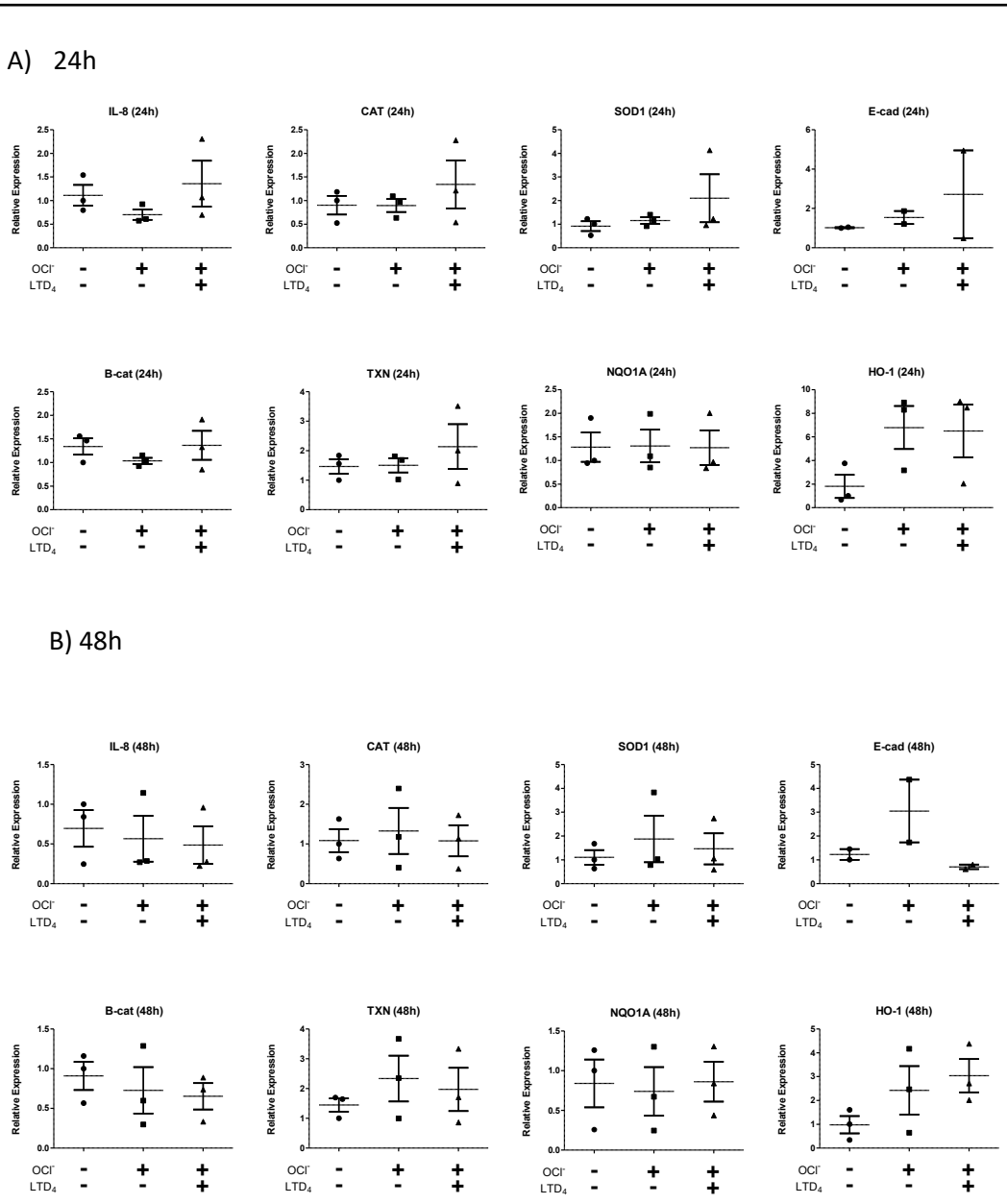

Figure 5: The effects LTD_4 treatment before hypochlorite exposure in Beas-2B cells on transcription of anti-oxidant enzymes, structural proteins and IL-8. A) LTD_4 was administered 24h prior to chlorine exposure. There were no significant differences in transcription levels of enzymes/ proteins probed in any group.

Chlorine exposure increases MCh Responsiveness and Inflammation in CysLTR1-/- mice at 24h

We measured AHR and inflammation in wild type and CysLTR1-/- mice at 24 hours post Cl_2 exposure. At 24h there was a significant increase in AHR in both wild type and CysLTR1-/- mice exposed to chlorine in comparison to their controls. At the highest dose of methacholine there were significant increases in resistance, tissue elastance and tissue damping in chlorine exposed CysLTR1-/- mice vs wild type exposed (Figure 6).

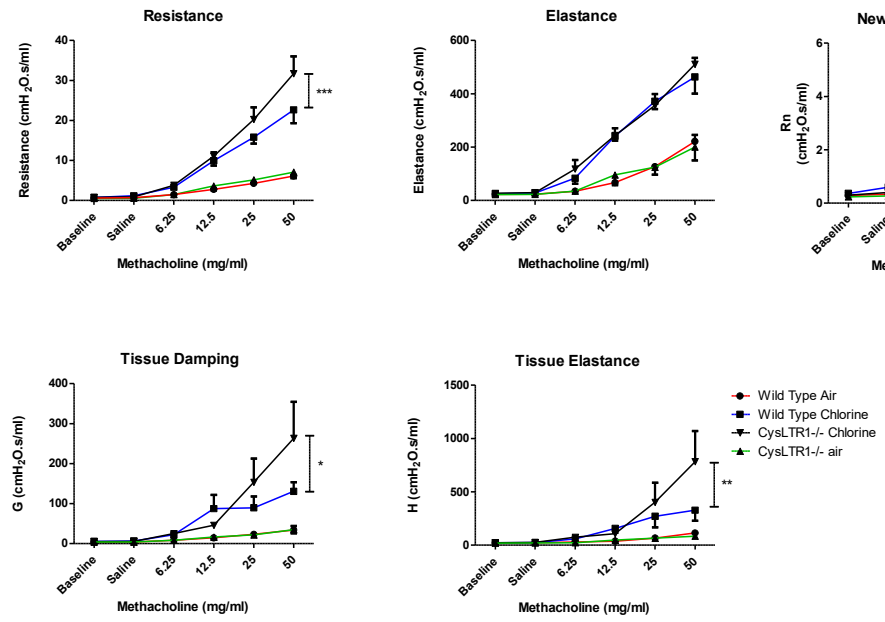

Figure 6: AHR in wild type vs. CysLTR1 -/- mice 24h after chlorine exposure. There were significant increases in resistance, tissue elastance (H) and tissue damping (G) in chlorine exposed CysLTR1-/- mice. There were no differences in elastance and Newtonian resistance in the two chlorine exposed groups. There were significant differences in air vs chlorine exposed mice, these differences are not depicted in this figure. *p<0.05,**p<0.01, *** p<.001; n = 6-8 per group.

There were no significant differences in inflammation between the chlorine exposed groups (Figure 7).

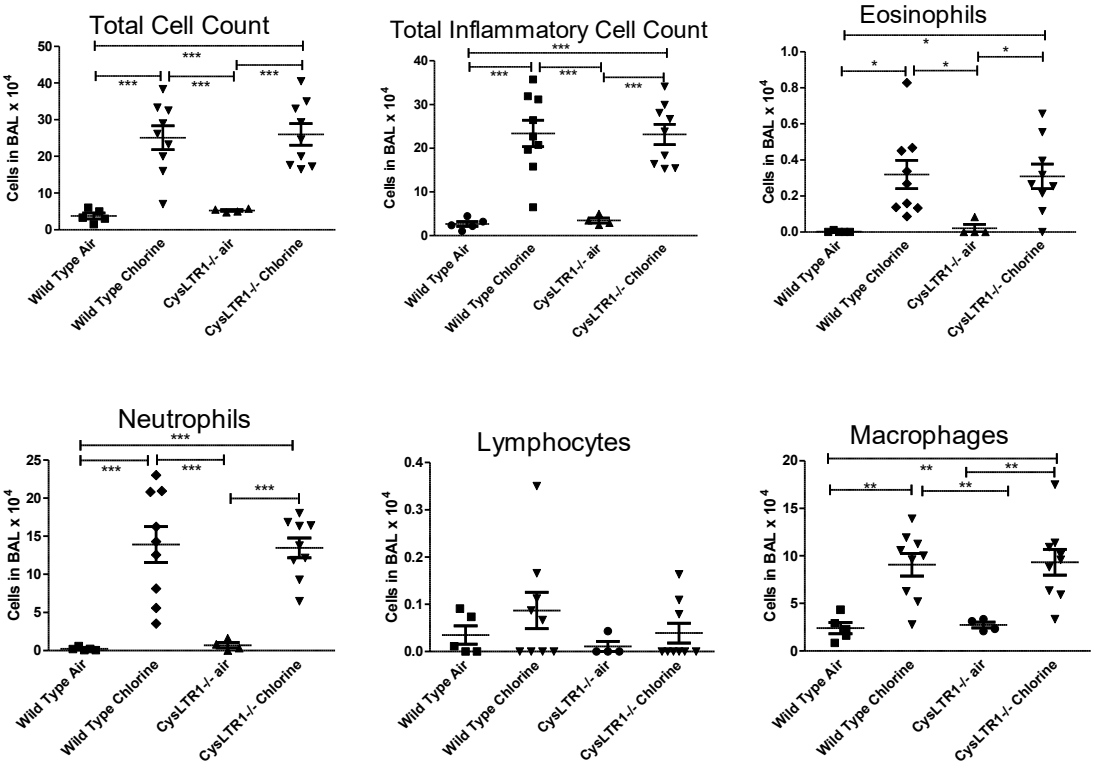

Figure 7: Inflammation in the BAL 24h post chlorine exposure. Exposure to chlorine induced significant increases in total cell count, total inflammatory cell count and number of neutrophils, eosinophils and macrophages in each genotype with respect to their air exposed controls. There were no significant differences between chlorine exposed CysLTR1-/- and wild type mice. *p<0.05, **p<0.01, *** p<.001.

MCh responsiveness and inflammation 48h post Cl_2 exposure

We found in previous studies that the increase in AHR and inflammation in CysLTR1-/- mice resulted from an inability of Nrf2 to translocate to the nucleus.[59] Our studies of Nrf2-/- mice

exposed to chlorine displayed no significant differences in inflammation or AHR 24h post chlorine.[58] At 48h, Nrf2-/- mice maintained elevated inflammation and AHR; therefore we expected the same phenotype if Nrf2 activity was CysLTR1 dependent (Supplemental Figure 1). At 48h there were no significant differences in AHR in chlorine exposed wild type vs. CysLTR1-/- mice (Figure 8). There were no differences in inflammation in chlorine exposed wild type mice compared to CysLTR1-/- mice at 48h (Figure 9).

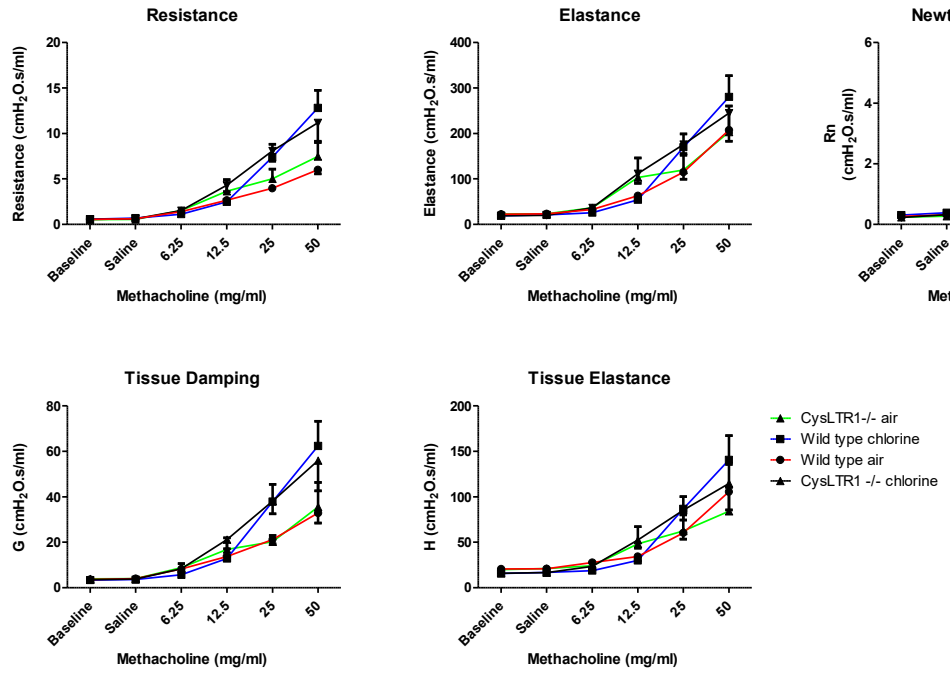

Figure 8: AHR in CysLTR1-/- and wild type mice 48h post chlorine exposure. There were no significant differences in any parameter of AHR 48h following chlorine exposure in the chlorine exposed groups. N=6-9 per group

Differences in BAL composition 3h post Cl_2 Exposure

Although there were no significant differences in cell counts in the BAL between wild type and CysLTR1-/- mice 3h post chlorine exposure, there were significant differences compared to their

respective air exposed controls. (Figure 10). In CysLTR1 mice there was a significant increase in neutrophils compared to their unexposed controls that was not seen in wild type mice.

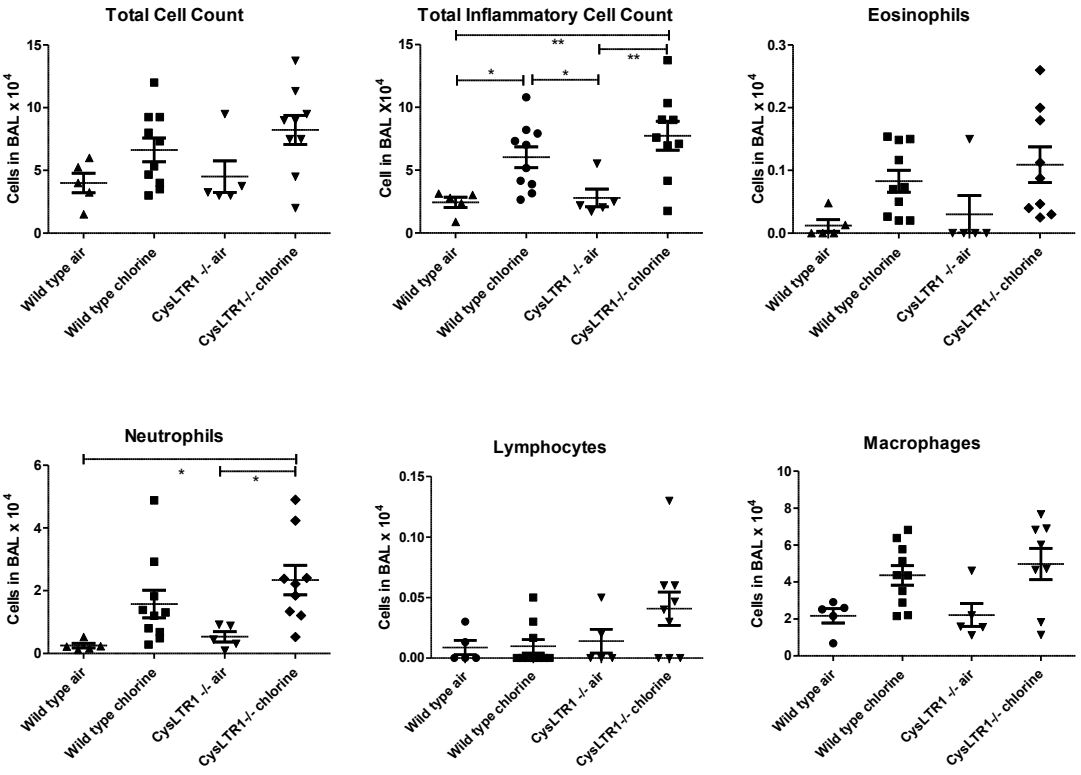

Figure 9: Inflammation in CysLTR1-/- and wild type mice 48h post chlorine exposure. There were no significant differences in inflammation between chlorine exposed groups. Both wild type and CysLTR1-/- mice still have residual inflammation as seen in the elevated level of total inflammatory cells compared to baseline. CysLTR1-/- mice also have significantly higher neutrophilia compared to baseline where as there is only a trend in wild type mice. *$p<0.05$, **$p<0.01$, *** $p<.001$

Conversely there was a significant increase in eosinophils and epithelial cells in wild type mice exposed to chlorine vs their air exposed controls; however, there was no significant difference in

CysLTR1-/- mice. There were no significant differences in number of macrophages between any of the groups.

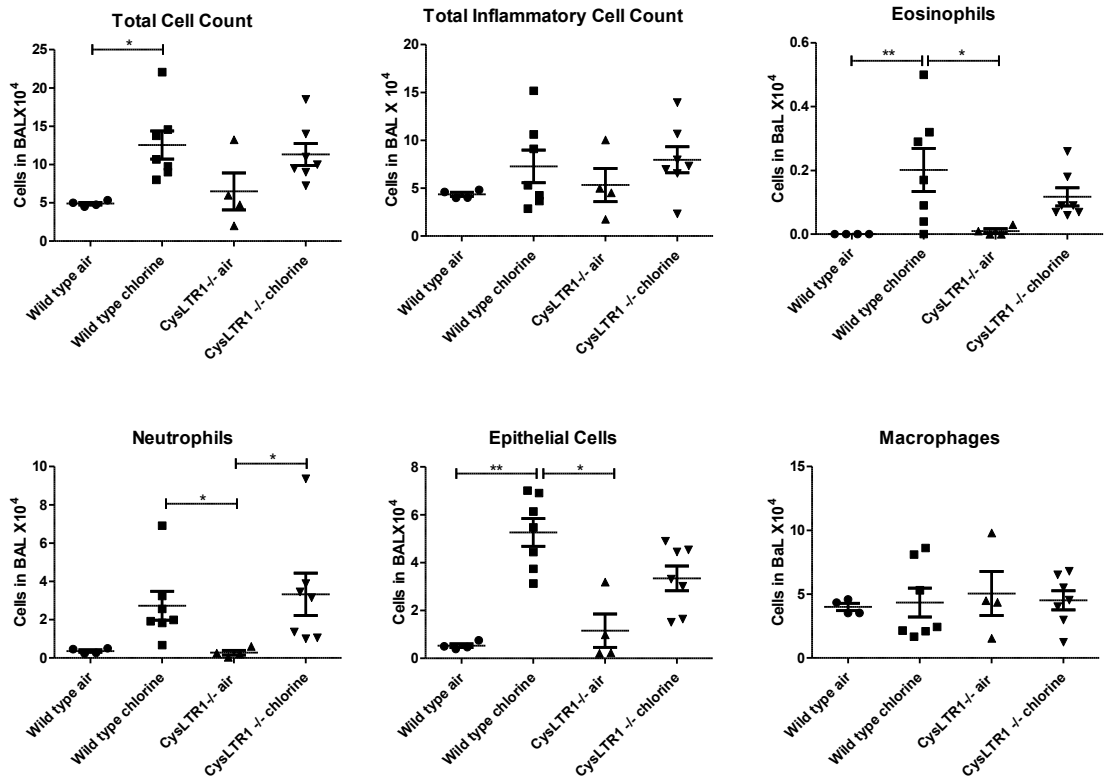

Figure 10: Inflammation CysLTR1-/- and wild type mice 3h post chlorine exposure. Although there are no significant differences in the two chlorine exposed groups, the composition of cells in the BAL in each genotype compared to their respective controls is different. Wild type mice exhibit a significant increase in total cell count, epithelial cell shedding and eosinophilia where as CysLTR1 -/- mice exhibit increased neutrophilia compared to their air exposed controls. *p<0.05, **p<0.01, *** p<.001

Cl_2 exposure and induction of pulmonary microvascular leak in mice

To assess microvascular leak, we measured levels of fibrinogen in the BAL at 30 min, 1h and 3h in wild type and CysLTR1 -/- mice. In wild type mice, there was a significant increase in fibrinogen in the BAL at 30 min but not at 1h or 3h. CysLTR1-/- mice displayed the same trend; however, baseline levels of fibrinogen were significantly higher than that of wild type mice. (Figure 11)

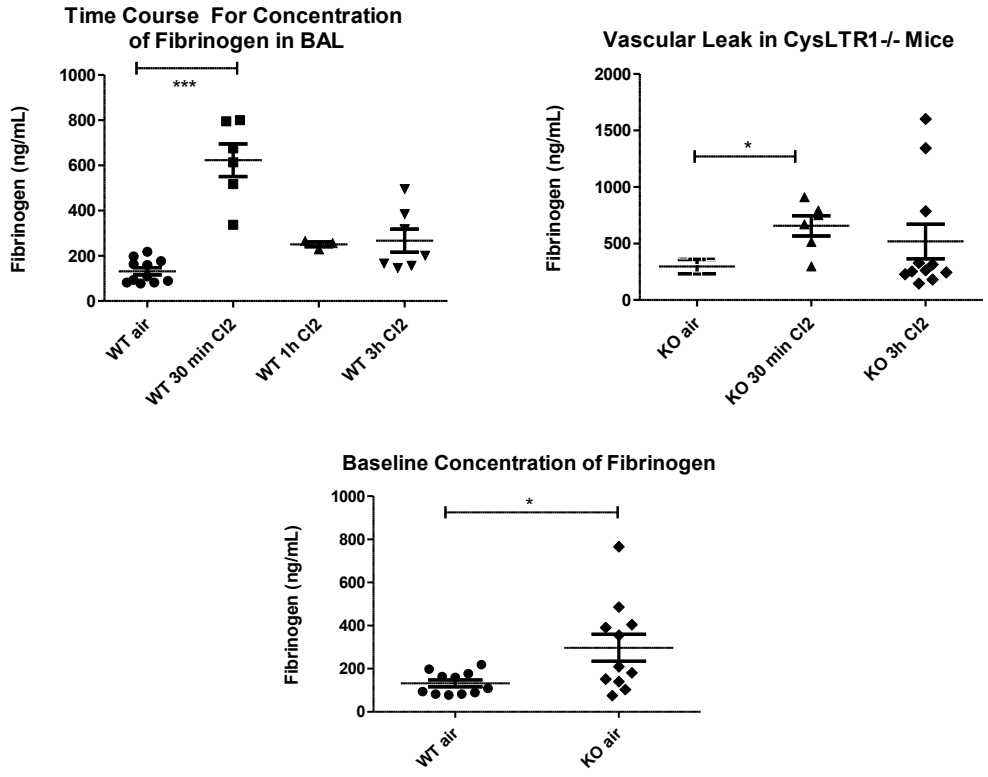

Figure 11: Induction of microvascular leak in wild type and CysLTR1-/- mice resulting from chlorine exposure. In both genotypes, there was a significant increase in vascular leak as measured by concentration of fibrinogen in the BAL. Baseline levels of fibrinogen were also significantly higher in CysLTR1-/- mice than in wild type. *p<0.05, **p<0.01, *** p<0.001

Effect of HAMI 3379 on MCh responsiveness and inflammation 24h post Cl2 in CysLTR1-/- mice

To determine the role of CysLTR2 in IIA we used a CysLTR2 selective antagonist, HAMI 3379, to block receptor activity in CysLTR1-/- mice. Administration of HAMI 3379 resulted in a significant increase in Rn at the maximum MCh dose (50mg/mL) 24h post chlorine. Although there was a trend towards an increase in total resistance in HAMI 3379 treated mice, the difference was not significant (Figure 12).

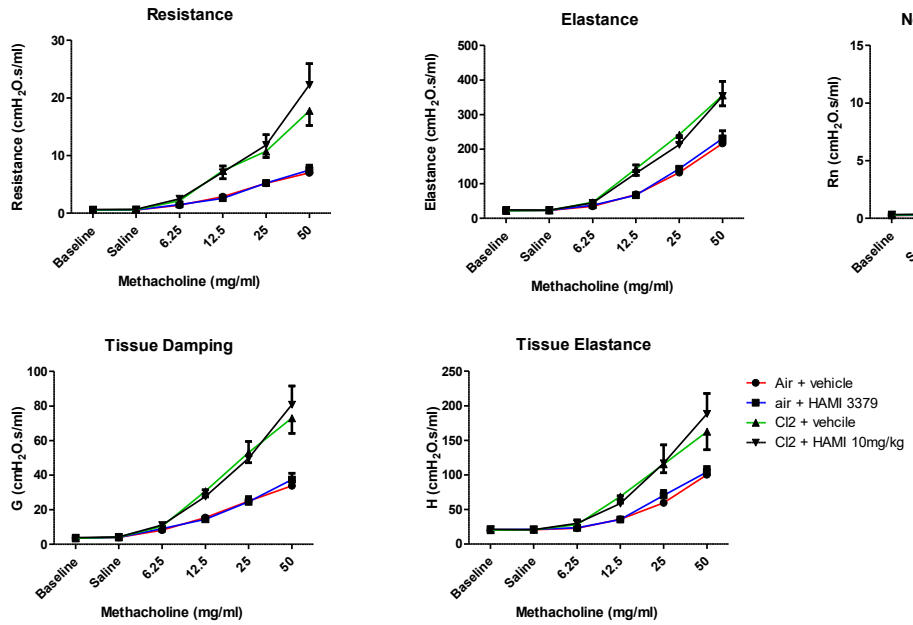

Figure 12: The effect of HAMI 3379 administration on AHR 24h post chlorine in CysLTR1-/- mice. Administration of HAMI 3379 resulted in a significant increase in Newtonian resistance compared to chlorine exposed vehicle controls. There is a trend towards a significant increase in respiratory system resistance, however, the difference is not significant. There are no other significant differences in AHR between exposed HAMI administered mice and their vehicle control exposed counterparts. HAMI 3379 has no effect on air exposed controls. *p<0.05, **p<0.01, *** p<.001

There were no differences in respiratory system elastance, tissue elastance or tissue damping, nor were there significant changes in inflammation or epithelial cell shedding between chlorine exposed groups (Figure 13).

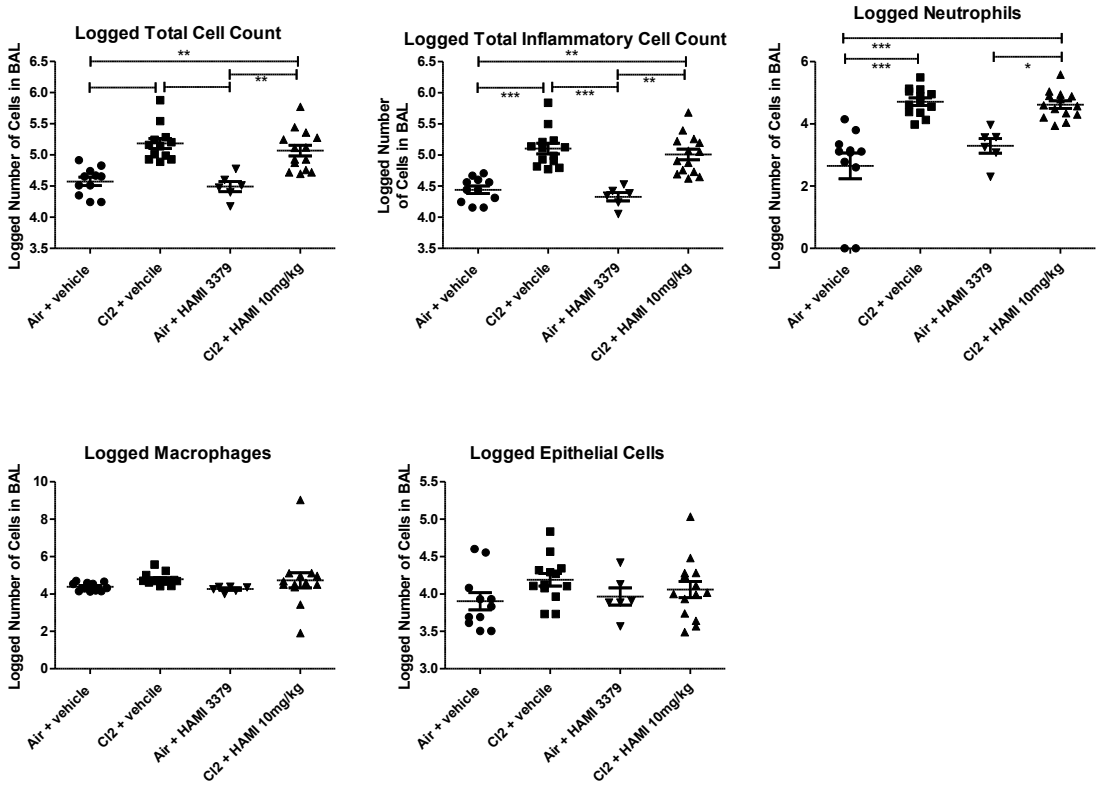

Figure 13: The effect of HAMI 3379 administration on inflammation 24h post chlorine exposure in CysLTR1-/- mice. Due to logarithmic transformation of cell counts, all graphs represent the logged value of cells in the BAL. As shown before, chlorine exposure resulted in an increase in total cell count, total inflammatory cell count, number of neutrophils. However, there were no significant differences in chlorine exposed groups.*p<0.05, **p<0.01, *** p<.001

Effect of HAMI 3379 on vascular leak

As 30min was the time at which vascular leak peaked, we used this time point for further experiments. We inhibited CysLTR2 in CysLTR1-/- mice using the same protocol as above and looked at the concentration of fibrinogen in the BAL at 30min post chlorine. Although not

significant, there is certainly a strong trend towards decreased fibrinogen in the BAL in mice administered HAMI 3379 in comparison to vehicle controls. (Figure 14)

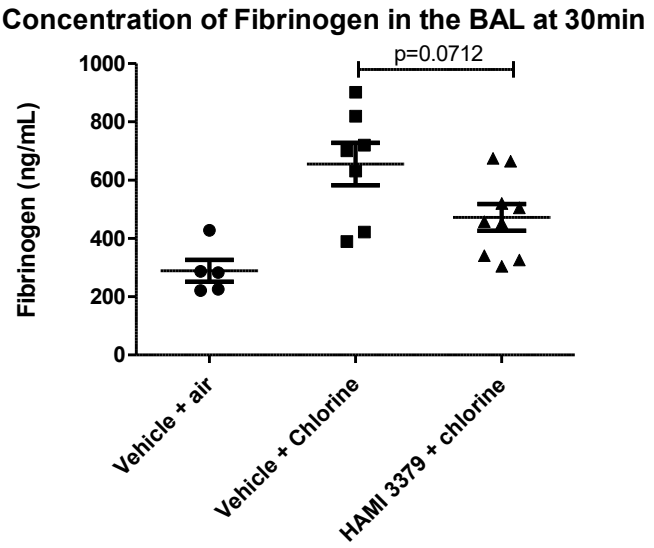

Figure 14: Effect of HAMI 3379 on microvascular leak 30 min post chlorine exposure. There is a trend towards a decrease in fibrinogen concentration in the BAL of HAMI 3379 administered mice. As fibrinogen concentration in a proxy measurement for microvascular leak, HAMI 3379 administration likely reduces vascular leak. *$p<0.05$

Chapter 4: Discussion

There is a consensus in the literature that CysLTR1 plays a pathogenic role in asthma through induction of airway inflammation, bronchoconstriction, TH2 cell activity and vascular leak.[38] It has been widely assumed that this role is conserved in all forms of asthma, independent of disease endotype. However, previous studies in our lab have demonstrated a potentially protective role for CysLTR1 in a chlorine model of acute irritant induced asthma.[59] In this project, we explored the concept of a protective role for the CysLTR1 in irritant asthma and the possible mechanisms which could explain the more severe phenotype seen in CysLTR1-/- mice at 24h post chlorine exposure.

First, we sought to confirm that deletion of CysLTR1 in mice results in increased airway resistance, elastance and neutrophilia 24h post chlorine exposure. Although we did find a significant increase in resistance, tissue damping and tissue elastance, we could not replicate the increase in elastance reported in previous data. We were also unable to reproduce previously identified increases in neutrophilia at 24h. All the data collected suggests that at 24h, CysLTR1 plays a protective role by controlling AHR specifically in the peripheral airways.

The differences mentioned above are likely explained by moving to a new animal housing facility in the period between the original study and my project as well as the use wild types littermates as controls instead of vendor purchased mice of the same age and strain. There are multiple studies in the literature that clearly demonstrate that changing facilities could significantly alter the outcome of experiments. Chang et al. (2012) clearly demonstrated that airway hyper-responsiveness in an asthma model is significantly different between sub-strains from different vendors, even after a five day acclimation period. This difference disappears if the

progeny from the sub-strains are reared in the same facility.[66] The cause of this has been attributed to stress from transport as well as different cage/bedding types, use of different food, frequency of cage cleaning and other environmental factors which could vary among vendor facilities. All of these factors play a role in development of the gut microbiome which has significant effects on the outcome of quantitative studies.[67] The new facility is also much cleaner than the previous one; therefore, mice bred in house may have been exposed to pathogens in the previous study which could contribute to the observed increases in immune response and AHR following chlorine exposure.

As our previous work indicates, the largest increase in inflammation was seen at 6h in the CysLTR1-/- mice, we wished to examine the role of CysLTR1 in earlier stages of inflammation. Since we have never reported the difference between CysLTR1-/- mice and wild type mice earlier than 6h and there were no significant differences in inflammation in wild type chlorine and air exposed at 2h[32], we chose 3h as our time point for exploration. Although there were no significant differences in the two chlorine exposed groups, there was a significant increase in neutrophils in CysLTR1-/- compared to air exposed controls that was not seen in wild type mice. Taken together these data suggest that CysLTr1-/- mice begin recruitment of neutrophils at an earlier time point, explaining the high neutrophil count at 6h. It seems CysLTR1 plays the largest role in IIA immune pathogenesis during initiation of inflammation and immune cell infiltration.

In previous studies, mRNA level of Nrf2 dependent and independent antioxidant enzymes was measured in the BAL of CysLtR1-/- 24h post chlorine. Nrf2 translocation was also quantified by staining paraffin-embedded lung sections with fluorescent-tagged anti-Nrf2 antibody and DAPI nuclear stain to determine cellular location. Results showed a decrease in mRNA for Nrf2 dependent enzymes 24h post chlorine in CysLTR1-/- mice and inability to translocate Nrf2 to the

nucleus by immunohistochemistry. It was hypothesized that if there was a direct mechanistic connection between Nrf2 and CysLTR1, NRF2-/- mice would exhibit the same phenotype on exposure to Cl_2; however, Nrf2 -/- mice did not exhibit increased inflammation or hyperresponsiveness at 24h after Cl_2 exposure.

As Nrf2 seemed to play a larger role in the resolution of inflammation and AHR, we hypothesized that if Nrf2 translocation is inhibited at 24h in CysLTR1-/- mice, the phenotype of the two models should converge at 48h. However, there were no significant differences in AHR and inflammation in CysLTR1-/- at 48 hours suggesting that their relative lack of Nrf2 activity and dysregulation of Nrf2 dependent enzymes are not solely responsible for the increases in inflammation and AHR in CysLTR1-/- mice 24h post Cl_2.

There are a few possible reasons that could explain the differences in phenotype between CysLTR1-/- mice, which were previously shown to have impaired Nrf2 activity, and Nrf2-/- mice following chlorine exposure. One possibility is the phenotype seen in CysLTR1-/- mice is not directly caused by impaired Nrf2 translocation in airway epithelial cells. Although it was postulated that the excess E-cadherin in CysLTR1-/- airway epithelial cells at baseline could inhibit Nrf2 translocation [59], we have not performed experiments to verify this pathway. At 24h post chlorine the level of E-cadherin in CysLTR1-/- epithelial cells is reduced to that of wild type mice. Based on this theory, Nrf2 would no longer be sequestered in the cytoplasm. CysLTR1 is also expressed on all the recruited immune cells and we have not investigated how the loss of the receptor impacts upon their function. It is also possible that if Nrf2 translocation is only impaired at for a short period by E-cadherin, subsequent translocation would enable resolution by 48h.

Our *in vitro* data supported the in vivo data, suggesting no direct mechanistic link between CysLTR1 signalling and Nrf2 activity in epithelial cells. The use of LTD_4 alone as a stimulus resulted in no changes at all in anti-oxidant enzyme transcription or E-cadherin and β-catenin transcription or protein level. We do not have enough replicates of the LTD_4 pre-treatment experiments to draw any conclusions concerning the effect of LTD_4 pre-treatment on anti-oxidant enzymes, E-cadherin or β-catenin following chlorine exposure. More experiments will be added to give sufficient power to draw concrete conclusions. We also plan to look at shed E-cadherin in the supernatant to see if the increase in soluble E-cad seen in mice [59] is replicated *in vitro*. In initial experiments, we also centrifuged the protein and removed the pellet containing membrane bound protein. Since β-cat and E-cad are usually associated with adherens junctions in structural cells,[68] they could be anchored in the plasma membrane and thus excluded from analysis by Western blot.

Based on these results we explored the possibility that an increase in CysLTR2 signalling could explain the increase in inflammation and AHR up to 24h in CysLTR1-/- mice and prolonged microvascular leak. Although previous papers do not show an increase in CysLTR2 transcription in CysLTR1-/- mice compared to wild type controls nor an increase in CysLTR2 transcription in response to chlorine exposure at 24h [59], we reasoned there could still be an increase in CysLTR2 signalling due to an increase in available substrate in the absence of the primary receptor (CysLTR1). To determine whether signalling through CysLTR2 could explain the phenotype seen in CysLTR1-/-mice we inhibited CysLTR2 with HAMI 3379, a selective CysLTR2 antagonist.[69]

At 24h post Cl_2, inhibition of CysLTR2 in CysLTR1-/- mice resulted in an increase in Newtonian resistance but no significant increase in total resistance or any other measure of AHR.

This suggests that CysLTR2 plays a minor role in Cl_2 induced AHR and its effect is localized to the proximal airways. However, there were no significant increases in inflammation, suggesting the increase in Newtonian resistance is not a result of excessive immune cell infiltration in the large airways. This finding clearly demonstrates that AHR and inflammation are not necessarily co-regulated; this phenotype requires further exploration to understand the specific mechanisms which drive AHR. In these experimental conditions, CysLTR2 also appears to play a protective role at 24h. It is possible that in the absence of CysLT receptors, cells are unable to mount an appropriate response to oxidative stress thus leading to increased airway hyperresponsiveness independent of immune cell location. In allergen models, CysLTR1 and 2 are synergistic; most of the reduction in AHR and inflammation can be attributed to CysLTR1 although CysLTR2 also plays a minor role. In our model of IIA, the two receptors also affect AHR. However while inhibition of the CysLTR2 has the same effect as the absence of the CysLTR1 it has the opposite effect as acute pharmacological inhibition of the CysLTR1 by montelukast.

Other groups have demonstrated that both CysLTR1 and CysLTR2 are protective in a model of cutaneous vascular leak.[54, 70] Therefore, we sought to determine the role of both receptors in vascular leak following Cl_2 exposure. Fibrinogen is a high molecular weight protein which is normally confined to the vasculature; therefore, we used the concentration of fibrinogen in the BAL as a measure of vascular leak. As this has not been previously reported in the literature, we looked at a 3 different time points 30min, 1h and 3h. In wild type mice, there was an increase in fibrinogen at 30 min but not at 1h or 3h post Cl_2. This suggests pulmonary microvascular leak is directly linked to oxidative damage from Cl_2 exposure and is very quickly resolved.

The CysLTR1-/- mice displayed a similar trend, with a significant increase in fibrinogen at 30 min post Cl_2 exposure. Since there was significantly more fibrinogen present in CysLTR1-/-

mice it, is likely that these mice are more prone to fibrosis due to increase in plasminogen activator inhibitor (PAI-1). This has been previously reported in the literature; CysLTR1-/- mice develop a much more severe phenotype in a bleomycin model of pulmonary fibrosis.[71] Based on this model it is clear that CysLTR1 does not mediate microvascular leak. However, it is possible that CysLTR1-/- mice have a pro-coagulative environment in the BAL which could lead to increased AHR as seen at 24h.

As 30 minutes post Cl_2 seemed to be the optimal time point to assess vascular leak, we administered HAMI 3379 or vehicle controls to CysLTR1-/- mice and assessed the concentration of fibrinogen in the BAL. There was a trend towards a decrease in fibrinogen in mice given HAMI 3379; further experiments are needed to determine if this difference is significant. Ideally, it would be better to try the experiment with a solvent which does not have anti-oxidant properties. In summary, CysLTR2 seems to mediate vascular leak; however, the effect is only relevant at very early time points. At later time points (24h) the role of this receptor switches from pathogenic to protective. Further study is needed to understand the mechanisms which lead to this functional change in role in pathogenesis.

The findings of this study may be applicable to other irritants such as diesel fumes, ozone and sulphur dioxide. Most irritants induce a similar response immune response to chlorine, including activation of antioxidant pathways, increases in IL-6, IL-8, neutrophilia, AHR.[72, 73] However, most have not been thoroughly studied in murine models, and existing data suggest that the specific pathway resulting in oxidative damage varies depending on the irritant. For example, when chlorine gas is inhaled, it dissolves in the mucous membrane and forms hypochlorous acid, hydrochloric acid and other chlorine metabolites. Chlorine can also bind to macromolecules altering signalling transduction and function.[18] Ozone, in contrast, induces

epithelial permeability through Toll Like Receptor 4 (TLR4). It has also been demonstrated that TLR4-/- mice are protected from AHR and inflammation in sub-chronic models of ozone exposure.[74] It is hard to speculate whether these mechanistic differences would alter the role of CysLTs in response to other irritants.

Chapter 5: Conclusions

Cysteinyl leukotrienes and their receptors play a large role in pulmonary inflammation and AHR; thus, it is important to understand their contribution to the pathogenesis of different pulmonary pathologies. In this study, we focused on the role of CysLTR1 and 2 in chlorine mediated irritant induced asthma. Our data strongly demonstrate a protective role for CysLTR1 and 2 specifically around 24h post Cl_2 exposure. Contrary to earlier studies, we did not find a direct link between Nrf2 translocation and CysLTR1 activation, nor does CysLTR1 seem to be involved in resolution of AHR and inflammation at 48h post Cl_2. Our data further demonstrate that the increase in inflammation and AHR in CysLTR1-/- mice cannot be explained by an increase in signalling through CysLTR2. Our data also demonstrate that CysLTR2 but not CysLTR1 could play a role in vascular permeability immediately following Cl_2 exposure. The findings of this project clearly indicate that our understanding of the mechanisms of IIA are extremely limited. It is critical that we expand on this knowledge to effectively identify and treat patients with IIA as it clearly differs significantly from allergic asthma.

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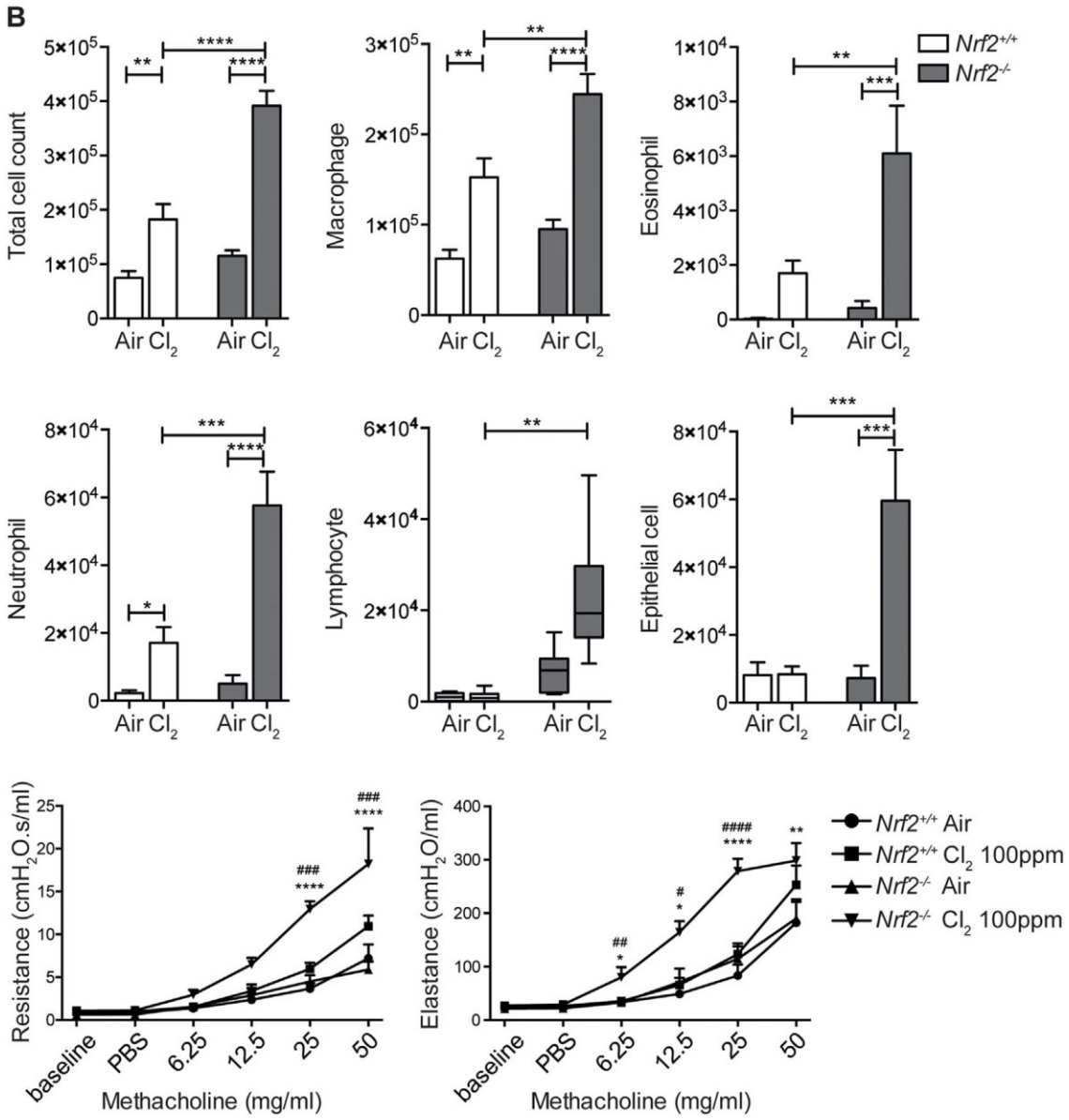
Supplementary Figures



Supplementary Figure 1: AHR and inflammation in Nrf2-/- mice 48h post Cl_2. Nrf2-/ mice have significantly higher levels of AHR and inflammation compared to their wild type controls 24h post Cl_2 exposure.

Adapted from Ano, S. et al (2017)[58]