Redox Control Of Allergic Airway Disease: Impact Of Glutaredoxin-1 On Epithelial Driven Inflammation And Allergen-Induced Airway Remodeling

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REDOX CONTROL OF ALLERGIC AIRWAY DISEASE: IMPACT OF GLUTAREDOXIN-1 ON EPITHELIAL DRIVEN INFLAMMATION AND ALLERGEN-INDUCED AIRWAY REMODELING

A Dissertation Presented

by

James D. Nolin

to

The Faculty of the Graduate College

of

The University of Vermont

In partial fulfillment of the Requirements for the Degree of Doctor of Philosophy Specializing in Cellular, Molecular and Biomedical Sciences

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ABSTRACT

Asthma is a multi-faceted chronic inflammatory disease accompanied by loss of airway epithelial integrity leading to remodeling of the airways. Perturbations to the lung redox environment, including alterations in glutathione (GSH) content, have been reported in asthma. GSH can be conjugated to protein cysteines, controlling protein function in an oxidant-dependent process known as protein S-glutathionylation (PSSG). The thioltransferase, glutaredoxin-1 (Glrx1), de glutathionylates proteins under physiological conditions, restoring sulfhydryl groups of target proteins. Glrx1 is emerging as a critical player in settings of allergic airway disease, but its function in regulating epithelial cell responses to asthma-relevant cytokines has not been examined. Furthermore, the role of Glrx1 in controlling the extent of airway remodeling in response to house dust mite (HDM) in vivo is still not well understood.

Interleukin-17A (IL-17A) is a potent cytokine that stimulates epithelial cells to produce pro-inflammatory mediators, in part by activating the nuclear factor kappaB (NF-κB) pathway, a key regulator of inflammation. We demonstrate that interleukin-17A (IL-17A) induces rapid activation of both classical and alternative NF-κB, while simultaneously resulting in protein oxidation and PSSG. In particular, we show IL-17A induces S-glutathionylation of RelA (RelA-SSG) and IKKα (IKKα-SSG), which is enhanced following siRNA-mediated knockdown of Glrx1. We also demonstrate that absence of Glrx1 leads to increased nuclear content of RelA and RelB and enhanced production of NF-κB-driven pro-inflammatory genes, KC and CCL20 while decreasing IL-6 expression. Finally, we show that siRNA-mediated knockdown of IKKα attenuates nuclear RelA and RelB and dampens pro-inflammatory gene production. Together, these data indicate a crucial role for the Glrx1/PSSG axis in controlling RelA-SSG, IKKα-SSG and epithelial cell responsiveness to IL-17A.

Mice lacking Glrx1 were previously shown to display enhanced resolution of allergic airway disease induced by ovalbumin (Ova) challenge. In this study, we determined the role of Glrx1 in a HDM model of allergic airway disease. Wild type (WT) mice and Glrx1 deficient (Glrx1−/−) mice demonstrated similar total lung cell counts, but Glrx1−/− mice displayed fewer neutrophils than WT mice. Conversely, mice overexpressing Glrx1 specifically in CCSP positive cells in the lung (Epi-Glrx1) showed attenuated total lung cell counts and lung eosinophils compared to control mice. Immunohistological analysis of remodeling markers revealed that Glrx1−/− mice displayed increased HDM-induced mucus metaplasia, α-smooth muscle actin (αSMA) positivity and collagen staining compared to WT mice. Evaluation of total lung collagen showed that Glrx1−/− mice had significantly higher collagen content compared to WT mice. In Epi-Glrx1 mice, attenuation of mucus metaplasia, αSMA content and collagen staining was observed compared to control mice. Furthermore, Epi-Glrx1 mice also demonstrated significantly impaired collagen production compared to control mice. We also demonstrate that Glrx1 absence results in decreased expression of the epithelial cell marker, E-cadherin, and increased expression of αSMA, a mesenchymal marker. Together, these studies demonstrate a critical role for Glrx1 in controlling epithelial cell responses to IL-17A and in mediating in vivo collagen production in response to chronic allergen exposure.
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Asthma

Overview

Asthma is a chronic disease of the airways that affects approximately 25 million people in the U.S. and 300 million people worldwide. Typical characteristics of asthma include reversible airflow obstruction, chronic inflammation of the airways and airway hyperresponsiveness and are sometimes accompanied by development of irreversible airway remodeling. These features give rise to the clinical manifestations of asthma, consisting of tightening of the chest, wheezing and difficulty breathing, which can range from mild to severe. Adding to the complexity of the disease, several different subtypes of asthma, recently termed ‘endotypes’, exist and include such classifications as exercise- or cold air-induced asthma, fixed airflow limitation (associated with neutrophilic influx) and allergic asthma [1]. For the purposes of this review, the focus will be on allergic asthma and the characteristics involved with the development and exacerbation of the disease.

Allergic Asthma

Exposure to many allergens occurs on a daily basis (e.g. house dust mite), yet the exact mechanisms that lead to the development of allergic asthma remain largely unknown. This is due in large part to the heterogeneous nature of the disease. There is a general consensus that a genetic component exists, though validation of specific genetic
risk factors has proven difficult. One genome-wide association study (GWAS) concluded that risk of asthma development cannot be readily determined by genetic analysis, owing in part to differences observed in genes involved in development of childhood asthma versus late-onset asthma [2]. The same study, the largest GWAS done on asthma-related genes to date, also concluded that many genes implicated in disease pathogenesis appear to be involved in the communication of damaged epithelium with cells of the immune system, not necessarily polymorphisms associated with predisposition to the disease [2].

The airway epithelium, discussed in greater detail in the next section, acts as a protective barrier between the external environment and the submucosa and can aid in coordinating immune responses via production of mediators that attract and activate immune cells. It is generally thought that events in the airway epithelium, including production of immune regulators IL-25, IL-33 and thymic stromal lymphopoeitin (TSLP), are the first step leading to downstream signaling events that drive a type 2 immune response, characteristic of asthma. The type 2 response is distinguished by production of IL-4, IL-5 and IL-13, which correspond with high levels of airway eosinophils and production of IgE [3]. Although CD4+ T-helper 2 (Th2) cells were originally thought to be the major contributor of type 2 cytokines, there is strong evidence that the recently described type 2 innate lymphoid cells (ILC2s) may also be important in regulating asthma development [4]. ILC2s produce a cytokine profile similar to Th2 cells, but are unique in the cell surface receptors they express. For instance, ILCs do not express any T or B cell lineage markers; additionally, ILCs do not appear to be myeloid or erythroid in origin and are instead considered non-B non-T cell source of...
Th2-associated cytokines [5, 6]. The presence of IL-25, IL-33 and TSLP in the lung can lead directly to activation of ILC2s, whereas the Th2 population requires communication with antigen presenting cells and a substantial lag period during expansion. Therefore, innate cells such as ILC2s may allow for more acute responses to allergens while the Th2 population is undergoing clonal expansion.

More recently, there has been mounting evidence that Th2-associated asthma is just one aspect of a much more complex set of diverse phenotypes. This in part resulted from the discovery of the recently described CD4+ Th17 cell, characterized by the production of a unique panel of cytokines, including IL-17A and IL-22. In contrast to Th2 cytokines which are more involved in eosinophil recruitment and IgE production, IL-17A signaling in epithelial cells stimulates production of cytokines, such as KC (CXCL1; IL-8 in humans), G-CSF and others, involved in the recruitment of neutrophils [7]. The resulting phenotype is dominated by a more destructive neutrophilic influx, and the presence of Th1 cells are often observed in abundance along with Th17 cells [8]. Asthmatics presenting with excessive IL-17A levels, both protein and mRNA, are often classified as having severe asthma, and IL-17A content in the sputum and serum is often used as a potential marker for severe asthma [9, 10], although the characteristics are often much more diverse. Th17 cells are thought to be the primary source of IL-17A [11, 12], but since their initial description, several other cell types have been shown to contribute to the IL-17A pool. Among these cells are γδ T cells [13], cytotoxic CD8+ (Tc17) T cells [14], invariant natural killer (iNKT) T cells [15] and ILC3s [16], many of which are involved in IL-17A production in the lung. There is evidence to suggest that neutrophils
and mast cells may also contribute to IL-17A production in synovial inflammation [17], although this has not been demonstrated in the setting of inflammatory lung diseases.

A prominent role for various IL-17A-producing cells has been shown in lung disease, expanding beyond the Th17 paradigm [18]. ILC3s are similar to Th17 cells in that both rely on the transcription factor RORγt for proper functioning and both can express IL-17A, but like the aforementioned ILC2s, ILC3s lack any lineage markers [19, 20]. Interestingly, ILC3-derived IL-17A can directly mediate AHR in a mouse model of obesity-induced asthma, independent of adaptive immunity [16]. ILC3s have also been observed in the BAL fluid of individuals suffering from asthma, further indicating the clinical importance of innate immune cells residing in the lung and participating in disease pathogenesis [16]. Specifics involved in IL-17 signaling and its involvement in inflammation will be discussed further in the next section.

**The Airway Epithelium**

Epithelial cells line the airways spanning the nasal cavities, trachea, bronchi and terminal bronchioles and are crucial in maintaining proper lung homeostasis. Previously, the epithelium was thought to act primarily as a physical barrier to pathogens and toxins and only after the epithelial layer was compromised and subsequently breached would an immune response be elicited. Recently, however, it has become apparent that the airway epithelium is not an innocent bystander in this process, but rather plays an active role in initiating and propagating immune responses [21]. Proper barrier function is exceedingly important and is a complex network of highly organized epithelial cell subtypes in the
conducting airways and in submucosal glands that produce mucus, surfactant proteins and several other components that aid in protection, killing and clearance of bacteria and viruses. Once trapped in the mucus layer, foreign material can stimulate the release of many of these factors, including β-defensins, amplifying the antimicrobial action of the epithelium [22]. Any offending material is then swept up the lung lining via mucociliary action where it is finally expelled from the airways.

The conducting airways, including the trachea, bronchi and bronchioles, are largely pseudostratified and consist of three primary types of epithelial cells: ciliated, non-ciliated and basal cells. Of these subtypes, ciliated cells are the most abundant found in primates, making up over 50% of the epithelial cell population, compared to rodents in which secretory cells are the dominant epithelial cell type. Despite this inherent difference, both rodent and primate lungs contain basal cells that lie at the basement membrane and act as a foundation for other epithelial cell types. Basal cells are also important in that they can function as progenitor cells, aiding in repair and regeneration of damaged epithelium [23, 24]. Other prominent epithelial subtypes found on the airway surface include goblet cells, which are principally responsible for producing the large mucin glycoproteins that make up the complex mucus meshwork; serous cells, important in maintaining the proper hydration of mucus secreted by goblet cells; and club cells (formerly known as Clara cells), which secrete surfactant proteins [25], anti-proteases and CCSP (or CC10), which exhibits some immunosuppressive functions and acts as an indirect phospholipase A2 inhibitor, among other putative functions [26]. Collectively, all
of these processes aid in protection of epithelial cell integrity and thus homeostasis of the lung.

As the inhaled air is cleansed of pathogens and harmful compounds by the discriminating landscape created by the conducting epithelium, it travels further into the lung where it reaches the terminal bronchioles and alveoli. The cellular composition in the alveoli consists of type I and type II pneumocytes, each with very distinct functions. Interestingly, type I cells comprise roughly 40% of the alveolar cell population, but due to their large surface area, they cover approximately 95% of the alveolar space. The primary function of type I cells is to facilitate efficient gas exchange to and from the blood, an ability made possible by their unique morphology and close contact to endothelial cells. In contrast, type II cells primarily secrete surfactant proteins and lipid mediators, such as dipalmitoylphosphatidylcholine (DPPC), which act to reduce alveolar surface tension, maintain alveolar shape and aid in innate immunity [27].

Because of the emerging data supporting the role of the epithelium in disease pathogenesis beyond simple barrier function, epithelial cells have become a central focus in understanding how immune responses are coordinated in the lung [28-30]. Airway epithelial cells can directly respond to inhaled allergens and toxins in part via pattern recognition receptors (PRR), such as toll-like receptors (TLR), which can recognize bacterial or viral components that penetrate the protective mucosal layer [31]. Upon ligand binding, TLRs can activate various transcription factors, including NFκB, leading to the induction of numerous genes involved in host defense and innate immunity [32]. As previously noted, the airway epithelium can produce cytokines that directly influence
the activation of innate immune cells in the lung, namely IL-25, IL-33 and TSLP. The epithelium can also indirectly contribute to the immune response by producing chemokines like KC, CCL20, MCP-1 and eotaxin, that act as chemotactic signals for neutrophils, dendritic cells, T-cells and eosinophils, respectively [33-36].

In Vivo Models of Allergic Airway Disease

Our understanding of the mechanisms involved in asthma pathogenesis has been formed through extensive research using animal models (Reviewed in [37]). To maintain focus, only mouse models of asthma will be considered in this review. In vivo models of allergic airways disease, including the conventional Ova protocol, have been broadly used to induce allergic airways disease in mice [38]; however, these models have also been subject to widespread criticism due to the method of antigen exposure and sensitization. In the Ova protocol, the antigen (Ova) is administered via intraperitoneal injection along with an adjuvant, Aluminum hydroxide, to elicit an immune response. The immune response observed in the Ova model is primarily T-helper 2 (Th2), with characteristic eosinophilic influx and little to no neutrophils [28]. By avoiding sensitization via the airways, the Ova protocol omits a crucial step in the immune cascade, namely recognition of inhaled substances by the airway epithelium. Other models are currently being employed to recreate a more representative asthmatic phenotype, in which a mixed eosinophilic and neutrophilic response is observed. Modified protocols involve direct sensitization of the airways, such as the LPS/ova protocol, in which a mixed Th2 and Th17 response associated with a neutrophilic influx
and steroid resistance has been noted [39]. Again, this model demonstrates promising results, but lacks in administration of an allergen in a physiologically relevant manner.

More important still is a model in which a ubiquitous allergen that elicits immune responses in humans can also be used to evoke similar immune responses in animal models. The aeroallergen house dust mite (HDM; *Dermatophagoides* sp.), to which an estimated 85% of asthmatics are allergic, is one of the most common aeroallergens in nature [40]. HDM contains various immunogenic epitopes, TLR agonists and other substances that enhance its allergenicity [40]. In a mouse model utilizing HDM, the allergic inflammation observed was largely dependent on recognition by epithelial cells, particularly through the pattern recognition receptor, TLR4, which recognizes bacterial lipopolysaccharide (LPS) [31]. Furthermore, it has also been demonstrated that epithelial expression of the C-type lectin, dectin-1, which recognizes fungal β-glucan found on HDM, is a major factor in driving innate immunity [34]. These data indicate that the epithelium plays a more critical role in driving allergic responses in the airway than was previously considered.

**Interleukin-17**

The Interleukin-17 (IL-17) family of cytokines has recently received much attention in the area of inflammation and host defense, due in part to the discovery of the recently described Th17 cell [11, 41]. The hallmark cytokine of the Th17 cell is the potent elicitor of inflammatory responses, IL-17A. The receptors for IL-17A, IL-17RA and IL-17RC, are expressed ubiquitously in both mouse and man, including in the airway
epithelium [42]. Stimulation of epithelial cells with IL-17A leads to the TRAF6-dependent activation of NF-κB [43], and induction of genes important in downstream inflammatory responses, including IL-6 [44], GM-CSF [45], KC [7] and CCL20 [46], among others. In response to IL-17A, epithelial cells also express genes important in host defense against live pathogens, such as S100 proteins [47] and defensins [48]. When activated, the IL-17R complex can also signal via a TRAF5-dependent, TRAF6-independent manner, which results in the prolonged half-life of certain chemokine mRNAs, such as KC, thus leading to a protracted inflammatory response [49]. There is now evidence that epithelial cells can produce another IL-17 family member, IL-17C, which signals in an autocrine manner to promote inflammation similar to that induced by IL-17A/F signaling [50]. The receptor for IL-17C, IL-17RE, was shown to be most highly expressed in mucosal organs, including the trachea and lungs, and more specifically was found on cells of epithelial origin [50, 51].

A crucial role for IL-17A has been demonstrated in a plethora of disease models, mainly those involved in host defense against foreign pathogens. In many instances, however, IL-17 signaling can go awry leading to the pathogenesis and exacerbation of several autoimmune and inflammatory diseases including rheumatoid arthritis, multiple sclerosis, psoriasis, systemic lupus erythematosus and asthma [52-56]. In asthma, aberrant IL-17A signaling has been shown to drive airway hyperresponsiveness in a mouse model by enhancing IL-13 driven disease [57]. Typically, IL-17A levels in asthmatics have been correlated to disease severity and often linked to steroid resistant asthma. This link with severe asthma is at least partially due to the ability of IL-17A to
stimulate the production of neutrophil chemoattractants, in particular KC and G-CSF, as many patients presenting with a severe type of asthma often have abundant airway neutrophils [39, 58]. Prognostic markers have included IL-17A levels in the serum of asthmatics, which appear to be an indicator of severe asthma [9, 10, 59].

IL-17 signaling has also been implicated in the development of fibrosis, as blockade of IL-17A signaling led to resolution of both inflammation and fibrosis in the lungs of mice [60]. In the setting of experimental hypersensitivity pneumonitis, a disease in which repeated exposure to inhaled organic antigens results in inflammation and fibrosis, IL-17A also plays a central role in driving fibrosis, which is dependent upon recruitment of neutrophils to the airways [61]. This indicates a potential link between IL-17A, severe asthma and neutrophil recruitment which may lead to constant tissue damage and regeneration, ultimately resulting in airway remodeling.

**Airway Remodeling**

Airway remodeling is described as the structural alterations of the airways that occur following chronic injury and repair. Characteristics of remodeling include (1) goblet cell hyperplasia and mucus metaplasia; (2) increased smooth muscle mass, indicated by elevated αSMA; (3) release of angiogenic factors and vascular expansion; and (4) increased subepithelial collagen deposition (Fig. 1) [62]. Although the processes involved in airway remodeling in asthmatics remain poorly understood, the resultant changes ultimately contribute to the clinical symptoms observed in lung dysfunction. Disruption of the epithelial layer, including death of ciliated cells, goblet cell hyperplasia
and increased release of growth factors, such as TGFβ, are thought to create an environment that promotes irreversible structural changes to the airways [63-65]. This injurious phenotype is due in part to chronic inflammation, although the extent of the contribution of inflammation remains unclear [66]. Others have demonstrated that allergen- or methacholine-induced bronchoconstriction alone can lead to airway remodeling in asthmatic patients, specifically by increasing collagen production and mucus metaplasia [67], further illustrating the complex and heterogeneous nature of the disease.

Mucus metaplasia is the overproduction of mucus in the airways that can lead to airway obstruction and symptoms common of asthma. The type 2 cytokine, IL-13, has been shown to drive mucus production from the epithelium, promote fibrosis, induce AHR and is critical in the pathogenesis of asthma [68, 69]. In vitro culture of airway epithelial cells with IL-13 results in increased positivity of mucus staining and increased Muc5AC message, but following removal of IL-13 from culture, goblet cell metaplasia is reversed and ciliated epithelial cells repopulate the culture [70]. In mice administered the antigen Ova, distal airways were less susceptible to IL-13-induced mucus metaplasia, likely owing to lower expression of IL-13Rα1 [71]. IL-13 induces expression of Muc5AC and Gob5 from epithelial cells via JAK/STAT6-dependent signaling and direct administration of IL-13 into the airways of mice results in robust induction of both Muc5AC and Gob5 [72]. This IL-13-dependent induction was not observed in mice systemically lacking STAT6, indicating a specific mechanism for IL-13-induced mucus production [72]. Therapeutic inhibition of STAT6 signaling is therefore being touted as a
viable treatment option for certain subsets of asthmatics [73]. Interestingly, this therapy may not be useful in patients expressing high levels of IL-17A, which can induce mucus metaplasia independent of STAT6 signaling, bypassing any benefit potentially seen by STAT6 or IL-13 blockade [74]. These data illustrate the difficulties associated with creating a single therapeutic to encompass all asthmatics and point to the need for development of more targeted individual therapies.

A layer of smooth muscle resides around the airway epithelium and aids in contraction and relaxation of the airways. In asthmatics, the thickness of the airway smooth muscle (ASM) layer is increased, reportedly due to both ASM hyperplasia and hypertrophy [75-78]. This is of clinical relevance, as an enlarged ASM layer can contribute to symptoms of asthma. In a mouse model, hypertrophic ASM was shown to positively correlate with AHR, particularly central airway resistance, following chronic exposure to the antigen Ova [79]. Furthermore, acute exposure of mice to Ova did not lead to a correlation between increases ASM mass and AHR, but did result in active proliferation of ASM [79]. This indicates that prolonged exposure to an allergen/antigen evokes an immune response that can thus amplify ASM proliferation. Recent evidence demonstrates that human ASM cells can recognize and subsequently proliferate in response to IgE, increasing ASM mass [80]. This amplification loop may be dysregulated in certain subsets of asthma, including severe asthma that is often refractory to treatment with corticosteroids. Adding to the complexity, certain chemokines such as eotaxin, RANTES and IL-8, typically involved in the recruitment of immune and inflammatory cells, appear to induce ASM cell proliferation and survival [81]. Commonly associated
with fibrosis, transforming growth factor beta (TGFβ) has also been implicated in ASM hyperplasia, although the role of TGFβ in ASM hyperplasia still remains controversial and context-dependent [82].

TGFβ is front and center in many fibrotic diseases and plays an active role in structural alterations observed in airway remodeling. In fact, TGFβ expression tends to correlate with collagen levels and is further enhanced with increasing asthma severity [83]. Therefore, in settings of lung remodeling and fibrosis, targeting TGFβ signaling or upstream mediators of TGFβ production has been a central avenue for therapeutic development [84]. Therapies directed at inhibition of IL-13 signaling (described above), may have impacts beyond ASM mass. In addition to increasing ASM mass, IL-13 can also increase levels of active TGFβ and matrix metalloproteinase 2 (MMP2), driving expression of type 1 collagen and contributing to subepithelial fibrosis in asthma [85].

Another recently discovered player in fibrotic remodeling is periostin, originally thought to be an adhesion molecule with the ability to bind matrix proteins and contribute to subepithelial fibrosis [86]. Periostin was initially considered to be limited to mesenchymal cells, but further investigation revealed that bronchial epithelial cells produce periostin in response to IL-13 and stimulate TGFβ signaling and collagen production from fibroblasts [87]. Interestingly, incubation of recombinant periostin with type 1 collagen resulted in increased elastic modulus, expanding its role beyond simply binding to matrix proteins, but also actively modifying the matrix itself [87]. Clinically, a correlation has been noted between levels of periostin and AHR in asthmatic children, leading to a potential new therapeutic target other than TGFβ for fibrotic remodeling.
[88]. Novel therapeutics directed at targets upstream of TGFβ or IL-13 signaling may offer new avenues for therapeutic intervention and treatment of severe forms of asthma accompanied by abundant remodeling.

Another common manifestation of remodeling is production of angiogenic factors leading to increased number of blood vessels surrounding the airways. In fact, elevated vascular endothelial growth factor (VEGF), a potent mediator of angiogenesis produced by cells via hypoxia inducible factor (HIF) transcription factors [89], has been observed in asthmatic airways [90] and has been associated with exacerbation of asthma [91]. More importantly, epithelial-derived VEGF has been shown to enhance antigen sensitization in the lungs and drive Th2-associated inflammation, partially through down-regulation of micro-RNA (miR)-1 expression in the lung [92, 93]. Despite advancements in our understanding of mechanisms involved in various aspects of airway remodeling, the clinical impact of vascular remodeling is still largely unknown [94]. It is possible that infiltrating immune and inflammatory cells trafficked into the airways via newly formed blood vessels may contribute to vascular remodeling by production of damaging cytokines, but there is a paucity of evidence to support this. Crosstalk between structural cells and immune/inflammatory cells appears to be a major contributing factor [94], but large gaps in our understanding of the clinical implications of vascular remodeling prevent any firm conclusions as to its direct impact on disease. Regardless, it is clear that factors involved in vascular remodeling are a valuable consideration in the development of targeted therapeutics.
Severe Asthma

According to both the European Respiratory Society and the American Thoracic Society, severe asthma is loosely defined as asthma that requires high doses of inhaled corticosteroids (ICS) in conjunction with other treatments, including systemic corticosteroids, to control potential exacerbations [95]. Severe asthmatics represent only a fraction of those afflicted with asthma, but account for nearly 80% of annual healthcare costs [96]. Therefore, development of novel therapeutics for targeting severe asthma has been a large focus in the field of asthma research. In certain subsets of severe asthmatics, patients are unresponsive to corticosteroid treatment, offering even fewer avenues available for symptom management. Although the precise mechanisms leading to severe asthma remain unidentified, severity of the disease tends to correlate with some key markers, including increased levels of IL-17A and presence of IL-17A-producing cells [97]. It should be noted, however, that the pathogenic role of IL-17A in asthma appears to vary greatly depending on the patient subpopulation [98]. Thus, while IL-17A is often a useful predictor of asthma severity, several other factors can contribute to drive a more severe phenotype. Options for disease management continue to evolve, as is evidenced by many newly developed therapeutics that show some promise in asthmatics (Reviewed in [99]).

Typical treatment regimens include a combination of ICS and a β2-agonist, which works well for managing mild or moderate asthma [100]. Additionally, leukotriene receptor antagonists (LTRA) have also been used in conjunction with ICS, but no further benefit has been observed by this addition beyond what would be seen simply by
increasing the ICS dose [101]. These typical treatment regimens often fail to completely resolve asthmatic episodes in severe asthmatics; thus, clinicians and researchers alike continue to search for an effective therapy. Certain invasive methods are also being employed in severe asthmatics with some promise. One such method is bronchial thermoplasty, which utilizes radio frequencies to briefly heat the surrounding airway to roughly 65°C, destroying some of the underlying smooth muscle [102]. This therapy was first approved by the FDA in 2010, and has been used with some success. Unlike therapy using biologics, there is no need for sustained treatment and results are often seen within weeks [102]. This offers a promising therapeutic avenue for severe asthmatics that do not respond effectively to ICS or other treatment regimens.

**Nuclear Factor-kappa B**

*NF-κB Signaling: Classical and Alternative Pathways*

Nuclear factor-kappa B (NF-κB) is a transcription factor that is a central regulator of immune and inflammatory responses and also plays an important role in promoting cell survival and proliferation. The NF-κB pathway is present in almost every animal cell and is involved in transducing signals from numerous ligands [103, 104]. Because of its central role in modulating immune responses, aberrant NF-κB signaling is frequently implicated in the pathogenesis of several diseases, including asthma. Thus far, two arms of the NF-κB pathway have been described, aptly named classical and alternative, each consisting of unique players involved in signal transduction. The classical pathway consists primarily of the transcription factors RelA (p65) and p50, which are held inactive
in the cytosol by inhibitory IκB proteins under resting conditions (Fig. 2). Stimulation with a ligand, such as LPS or IL-17A, leads to assembly of adaptor proteins, such as TRAF6, and phosphorylation of the effector kinase TAK1. Critical for proper NF-κB signal transduction, TAK1 can then associate with the heterotrimeric IKK signalsome consisting of IKKβ, IKKα and NEMO (IKKγ), phosphorylating IKKβ [105]. This in turn leads to the phosphorylation and subsequent ubiquitination of IκBα, which is then degraded by the 26S proteasome, allowing nuclear entry of RelA/p50 and downstream gene induction. Genes induced by the classical NF-κB pathway have been associated with inflammation and immunity, and deregulation of this pathway can lead to a variety of diseases, including cancer and inflammatory/immune disorders.

The alternative pathway relies primarily on the transcription factors RelB and p52 and is associated with discrete cellular functions unique from classical NF-κB signaling. Activation of the alternative pathway is dependent on the activity of NF-κB-inducing kinase (NIK), which is constantly ubiquitinated by TRAF3 and signaled for degradation under resting conditions. Following stimulation with a unique set of ligands, TRAF3 itself is ubiquitinated and degraded, allowing for NIK to phosphorylate a homodimeric IKKα complex, independent of IKKβ and NEMO, which can then phosphorylate p100. P100 is typically bound to RelB, sequestering it to the cytosol and preventing nuclear entry. Phosphorylation of p100 at several serines in both the C and N termini by IKKα leads to p100 processing by the 26S proteasome to form p52, allowing RelB/p52 dimers to enter the nucleus [106, 107]. The alternative pathway has generally been studied in cells of the immune system and in the context of lymphoid organogenesis. Until recently,
it was widely accepted that these two pathways existed independently with little
crosstalk, leading to unique transcriptional profiles. However, a recent publication by our
group demonstrated that in fact both pathways cooperate extensively to control NF-κB
activation and downstream gene induction in lung epithelial cells in response to a variety
of stimuli [108]. This aids in expanding the role of alternative NF-κB to structural cells
and demonstrating a more complex relationship between the classical and alternative
NF-κB pathways than was previously known.

NF-κB Regulation

Being such a pivotal regulator of immune responses, NF-κB family members are
subject to extensive regulation through various post-translational modifications (PTMs).
These include phosphorylation, ubiquitination, acetylation as well as oxidant dependent
PTMs, including S-nitrosylation and S-glutathionylation. Depending on the target
protein, these modifications can either activate or inhibit NF-κB signaling, thus affecting
subsequent downstream signaling events. Important to this body of work are the oxidant-
dependent PTMs, in particular S-glutathionylation, the conjugation of glutathione with
reactive cysteines, which will be discussed in greater detail later. Several targets of the
NF-κB pathway have been shown to be targets for S-glutathionylation, including RelA,
IKKβ, p50, IκBα and IKKα [109-113]. S-glutathionylation of these proteins confers
inhibition of the NF-κB pathway, suppressing downstream proinflammatory gene
induction. Our group has also demonstrated that the glutathionylation of IKKβ and IKKα
are dependent on the activity of the enzyme glutaredoxin 1 (Glrx1) [110, 113], which,
under physiological conditions, can de-glutathionylate target proteins and restore their function. In settings of disease where there is increased oxidative stress, it may also follow that there are high levels of oxidized cysteines on proteins that may be targeted for S-glutathionylation, a topic covered in greater detail in the following section.

**Redox Biology and Cysteine Oxidation**

*Endogenous Sources of Oxidants*

Oxidants, such as hydrogen peroxide, are widely accepted to be involved in the pathogenesis of various chronic inflammatory diseases, including asthma. Despite recognition of this fact, the exact source of the oxidants involved in disease pathogenesis and mechanisms of their pathogenic action remain largely enigmatic. Both endogenous (e.g. mitochondrial respiration) as well as exogenous (e.g. environmental allergens) sources of oxidants exist, and the differential or synergistic impact of oxidants on disease pathogenesis based on their source is unknown. At the cellular level, several key sources of oxidants exist: namely enzymes that produce reactive oxygen species (ROS) like \( \text{H}_2\text{O}_2 \) and those that produce reactive nitrogen species (RNS) like \( \text{NO} \). Endogenous \( \text{H}_2\text{O}_2 \) is produced in large part by members of the NADPH oxidase (NOX) family of enzymes. Members include NOX1, NOX2, NOX3, NOX4 & NOX5 and dual oxidase 1 (DUOX1) and DUOX2. These enzymes exist as membrane-bound multi-subunit complexes that transfer electrons from NADPH to molecular oxygen to produce the ROS superoxide (\( \text{O}_2^- \)), which can be further reduced to form \( \text{H}_2\text{O}_2 \) [114]. NOX4 is unique from other NOXs or DUOXs in that it is constitutively active, leading to speculation that its activity
is regulated primarily at the level of transcription [115]. Another interesting characteristic of NOX4 is its apparent ability to produce H$_2$O$_2$ directly [116], though this still remains controversial, as others report the major ROS produced by NOX4 to be O$_2^-$ [117, 118]. DUOX1 and DUOX2 are the only other members of this family known to directly produce H$_2$O$_2$, a function demonstrated to have great importance in airway mucosal host defense [119-121]. In either case, NOX members have been implicated in a variety of lung diseases, including asthma and cancer, providing strong evidence linking aberrant oxidant production with disease pathogenesis [122-124].

NO is mainly produced by nitric oxide synthase (NOS) enzymes, of which there are three known forms in mammals: neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2) and endothelial NOS (eNOS; NOS3). The amino acid arginine is used as the substrate by NOS to produce one molecule of NO and the byproduct citrulline. The NO produced in this reaction acts as a vasodilator and a bronchodilator, in part by the activation of guanylate cyclase and resultant production of cGMP, and is thus important for maintaining proper airway function [125]. Alternatively, NOS-derived NO can readily react with O$_2^-$ to form the RNS peroxynitrite (ONOO$^-$) and with the antioxidant tripeptide glutathione (GSH), to form the S-nitrosothiol, S-nitrosoglutathione (GSNO). Because NO readily reacts with other species, NO signaling is also believed to function via S-nitrosylation of proteins which can have drastic impacts on protein function [126]. It is known that GSNO is a potent bronchodilator, the concentrations of which are higher in the airways than NO itself [127]. GSNO was also shown to be depleted in the airways of asthmatics, and a study using an experimental asthma model determined that increased
GSNO reductase (GSNOR) is primarily responsible for the removal of GSNO and protein SNO, leading to more hyperresponsiveness following allergen challenge [128], implicating a protective role for SNO.

Both in vitro and in vivo studies demonstrated that the addition of ONOO\(^-\) is sufficient to drive hyperresponsiveness and damaging inflammation in guinea pigs [129] and abundant NO production has been observed in asthmatic patients, potentially leading to enhanced production of ONOO\(^-\) and subsequent damage [130, 131]. GSNO reductase (GSNOR) polymorphisms have been associated with the pathogenesis of asthma [132] and restoration of the GSNO pool via pharmacological inhibition of GSNOR was recently shown to be protective in a mouse model of allergic airways disease [133]. Elevated NOS activity is also observed in inflammatory lung diseases, and iNOS was shown to be critical in driving allergen-induced acute inflammation, as well as fibrotic remodeling, but not chronic inflammation [134], further illustrating the complexity of mechanisms in which oxidants contribute to disease.

It is now becoming recognized that oxidants control pathobiological functions following their compartmentalized production, including the mitochondria and endoplasmic reticulum (ER). As a byproduct of cellular metabolism, the mitochondrial electron transport chain leaks electrons which can then react with molecular oxygen to produce superoxide and eventually \( \text{H}_2\text{O}_2 \) [135]. Superoxide dismutase 2 (SOD2 or MnSOD), located in the mitochondrial matrix, is mainly responsible for converting harmful \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 \) and oxygen. From here, \( \text{H}_2\text{O}_2 \) can cross the mitochondrial membrane into the cytosol, where it can act locally as a second messenger in part via
oxidation of protein cysteines. The ER is also an important source of oxidants, particularly when undergoing the unfolded-protein response (UPR), in which abundant H₂O₂ is produced due to the refolding of proteins [136]. Much of the H₂O₂ produced is a consequence of disulfide bond isomerization, a process in which reduced protein disulfide isomerases (PDI) are oxidized and subsequently regenerated by ER oxidoreductase 1 (Ero1), generating H₂O₂ in the process. Excess H₂O₂ is often removed by the ER resident peroxiredoxin IV (Prx4), preventing rampant oxidative damage to properly folded proteins [137]. Partly derived from the ER, peroxisomes are responsible for carrying out important redox reactions and are therefore a major site of oxidant production. Long chain fatty acid catabolism can produce H₂O₂ as a byproduct, and this can be removed via peroxisomal catalase. Because of the ability of peroxisomes to make excess H₂O₂, their contribution to oxidative stress is becoming more widely appreciated [138].

Inflammatory cells are important in defense against pathogens, but in some cases, excess production of oxidants by these cells is thought to be pathogenic. Certain specialized cell types, neutrophils and eosinophils in particular, produce copious amounts of oxidants in response to pathogens or inflammatory signals. Neutrophil myeloperoxidase (MPO) can utilize H₂O₂ and chloride anion (Cl⁻) to produce hypochlorous acid (HOCl, bleach) and can also oxidize tyrosine residues to tyrosyl radicals using H₂O₂ [139]. Both HOCl and tyrosyl radicals are highly cytotoxic and are therefore used for killing bacteria or other invading pathogens during the respiratory burst. Eosinophils have a similar mechanism, utilizing eosinophil peroxidase (EPO),
which preferentially uses bromide anions (Br\(^-\)) over chloride anions to form hypobromous acid (HOBr) [140, 141], also used to kill invading pathogens. Proteins themselves can also be halogenated by either chloride or bromide anions via diverse mechanisms, including halogenases, further affecting protein function [142, 143]. When production of oxidants becomes dysregulated, the resultant oxidative stress and subsequent reactive products are believed to contribute to the pathogenesis of various lung diseases, including asthma [144, 145].

**Exogenous Sources of Oxidants**

Exposure to exogenous pollutants or allergens can lead to an inflammatory response in the lung, accompanied by the production of endogenous oxidants. Some exogenous toxins, such as cigarette smoke, which contains numerous oxidants, and ozone, a strong oxidant itself, can robustly induce oxidant production in the lung [146-148]. Increases in oxidative stress are also seen upon experimental exposure to other pollutants, including diesel exhaust and even man-made materials, such as carbon nanotubes [149, 150]. Although the lung is well-equipped to deal with oxidative flux with robust antioxidant systems in place, excess oxidant production is thought to contribute to tissue damage and chronic inflammation [151]. The ability of allergens to directly produce oxidants remains an active area of research, and has revealed some intriguing findings. As described earlier, among the most common allergens is house-dust mite (HDM), to which roughly 80% of asthmatics are allergic and which contains numerous antigenic proteins and pro-inflammatory compounds [40]. Although it has not been
determined if HDM contains any ROS-producing enzymes that affect their allergenicity, it is well-known that HDM can induce the production of oxidants in the airways and lead to inflammation [152]. Redox-related enzymes, such as Trx, are also important in controlling cellular responses to the oxidants induced by HDM [153]. In contrast, pollen from several plant species, including ragweed, possess intrinsic NOX-like activity, directly contributing to the imbalanced redox environment and subsequent inflammation in the airways via the production of H$_2$O$_2$ [154]. It is thought that the oxidant signal produced by pollen NOX enhances the subsequent inflammatory response to the pollen allergens [155]. Other allergens, such as the fungus Alternaria alternata (A. alternata), also have NOX-like enzymatic activity [156, 157]. This NOX-like-mediated production of H$_2$O$_2$ has been primarily studied in the plant pathology field and has not been investigated in the setting of human lung diseases caused by A. alternata. If NOX activity in A. alternata is comparable to that seen in ragweed pollen, similar downstream impacts including enhanced airway inflammation may also occur in response to A. alternata-produced H$_2$O$_2$.

**Cysteine Modifications**

The field of redox biology has advanced rapidly over the past several decades and has enhanced our understanding of the complex cellular mechanisms involved in managing oxidative stress. Although large amounts of oxidants can be damaging to cells (oxidative stress), it is now appreciated that low levels of oxidants such as H$_2$O$_2$ and NO are important in homeostatic functions (redox biology), but also potentially in signaling
cascades that contribute to disease (redox pathobiology). It is clear that a highly complex network of oxidant-dependent signaling exists within the lungs. Arguably one of the most important mechanisms of transducing oxidant dependent signals is through the amino acid cysteine (Cys, C) due to its sulfhydryl side chain (SH). Only certain cysteine residues have been denoted as ‘reactive’, meaning they can be readily oxidized, reduced and otherwise modified.

Reactive cysteines are normally characterized as having a lower pKa than that typical of cysteine (≤7 for reactive cysteine; 8-8.5 for normal cysteine). This microenvironment favors the loss of a proton from the SH group of cysteine, forming a thiolate anion (S⁻) which can then be oxidized to sulfenic acid (SOH), the initial oxidation product of cysteine [158]. Large-scale efforts to determine reactive cysteine residues have been undertaken, including the use of proteomics and computer modeling programs. Providing us with a useful tool for prediction, computer algorithms have had some success in determining reactivity of cysteines and thus potential redox targets [159, 160]. Proteomics have also been used to mine for reactive cysteine residues in proteins. Methods now use isotope-coded affinity tag (ICAT) reagents combined with mass spectrometry to directly compare two samples simultaneously, allowing determination of reactive cysteine residue [161-163]. These collective efforts have helped develop methods for the determination of reactive cysteine residues, but the next even more challenging step is to resolve the oxidation end-product that exerts its influence on the protein of interest.
Cysteine modifications vary, but all generally rely on the ability of the SH group of cysteine to be oxidized to SOH (Fig. 3). From here, SOH can be further irreversibly oxidized to sulfinic acid (SO$_2$H) and eventually sulfonic acid (SO$_3$H) in the presence of an oxidant, such as H$_2$O$_2$. Interestingly, the SO$_2$H form of cysteine is largely irreversible, leading to degradation, but there is one known case where the hyperoxidized (SO$_2$H) forms of peroxiredoxin I-IV (PrxI-IV) can be enzymatically reduced by sulfiredoxin (Srx) [164]. There is no known mechanism by which the SO$_3$H form of cysteine can be reduced, and this oxidation typically leads to degradation. Under certain circumstances, reactive cysteine can be conjugated to other molecules, such as GSH or NO, to form S-glutathionylated (SGG) or S-nitrosylated (SNO) proteins, respectively. These represent two well-studied redox-dependent post-translational modifications of cysteine and have been shown to play critical roles in redox regulation of protein function, launching a unique area of research [126, 165]. These modifications are thought in part to protect proteins from further irreversible oxidation, but also regulate protein function and signaling. SNO is the reversible, covalent conjugation of NO to a reactive cysteine in a protein that occurs without aid of enzymatic catalysis, whereas the reverse reaction can be catalyzed enzymatically via GSNOR, as well as Trx [166, 167]. Surfactant protein D is also a target for SNO in the lung, which enhances its pro-inflammatory function by allowing its binding to receptors, activating downstream NF-$\kappa$B [168]. These examples demonstrate the multifaceted role of SNO in lung inflammation.
**S-glutathionylation and Glutaredoxin**

S-glutathionylation (SSG) represents the covalent and reversible addition of GSH to reactive cysteine in proteins. SSG can occur spontaneously, in which oxidized GSH (GSSG) reacts with cysteine residues, leading to a glutathionylated protein. Unlike SNO, there is evidence that SSG can be catalyzed enzymatically, specifically by glutathione S transferase Pi (GSTP) [169] and to some extent GSTM, though the latter has not been shown to induce SSG in a biological setting [170]. While an exhaustive list of GSTP substrates for SSG has not been established, it is likely that several targets exist for this enzyme and others in the GST family. Interestingly, GSTO1, another member of the GST family, has been implicated in catalyzing the reverse reaction, acting as a deglutathionylating enzyme [171]. To date, numerous targets of SSG have been determined and studied in detail, including transcription factors, kinases, structural proteins and enzymes involved in metabolism [172, 173]. The reversible conjugation of GSH to protein cysteine can have profound impacts on signaling, as it alters the shape, charge and size of the target protein in question. Depending on the protein, SSG can either activate or inhibit its function. Specifically, our group has shown that SSG of NF-κB proteins leads to dampened NF-κB activity and decreased production of downstream inflammatory mediators, and this modification appears to be dependent upon the enzyme glutaredoxin 1 (Glrx1) [110, 113]. Glrxs are the main deglutathionylating enzymes in mammalian systems [174, 175], although other enzymes can also perform this reaction under certain conditions, including sulfiredoxin [176].
Glrxs are part of the larger thioredoxin fold family of enzymes which includes oxidoreductases, as well as other enzymes with diverse functions [177], as will be further discussed in the following sections. All oxidoreductases in this family share a highly conserved active site consisting of the motif CXXC or CXXS, involved in reducing disulfide bonds (S-S) or other oxidized cysteine and in some cases, reversing glutathionylated proteins [178]. In mammalian cells, there are at least four known Glrxs: Glrx1 (cytosolic), Glrx2 (mitochondrial/nuclear), Glrx3/PICOT (cytosolic) and Glrx5 (mitochondrial). The most well-studied of these are Glrx1 and Glrx2. Mitochondrial Glrx5 is primarily involved in [2Fe-2S] cluster maturation and transfer from scaffold proteins to their targets [179], a key mechanism in cellular iron regulation. PKC-interacting cousin of thioredoxin (PICOT), also known as Glrx3, was first discovered as a negative regulator of c-Jun N-terminal kinase (JNK) and NF-κB pathways via its Trx-like domain interaction with protein kinase C [180]. PICOT is unique in that it contains two CGFS motifs in its active site, highly conserved between plants and animals. Glrx1 and Glrx2 are very similar in function in that they are both very selective for deglutathionylation reactions, but Glrx2 displays a lower efficiency for catalyzing this reaction due to its increased active site cysteine pKa [181]. Glrx2 is primarily localized in the mitochondrial matrix and is involved in regulating the redox environment by controlling SSG of protein thiols within the mitochondria. Specifically, Glrx2 can deglutathionylate various targets in the mitochondrial inner membrane, including complex I of the electron transport chain [182, 183]. In mouse lens epithelial cells isolated from Glrx2-deficient mice, elevated oxidative stress and increased mitochondrial
glutathionylated proteins were observed, further demonstrating a specific, localized role of Glrx2 in maintaining mitochondrial redox balance [184].

The most extensively characterized isoform of the Glrx family is Glrx1. A primary role for Glrx1 is deglutathionylation of target proteins, several of which have been reported to date [109-111, 113, 185, 186]. Interestingly, Glrx1 is much more efficient at deglutathionylating proteins compared to other related enzymes [175]. Although primarily considered a cytosolic enzyme, studies have reported Glrx1 in the nucleus [187] and in the mitochondrial intermembrane space [188]. Its localization to the intermembrane space of the mitochondria is distinct from that of Glrx2, likely leading to control of diverse mitochondrial functions, including redox regulation of apoptosis [188]. Interestingly, Glrx1 has been detected in serum from healthy human donors, providing evidence that Glrx1 can be secreted, although the specific cell-type or types responsible remain unknown [189]. Further reports demonstrate secretion of thioredoxins (Trxs), peroxiredoxins (Prxs) and other Glrxs from myoblasts, which may be one cell type responsible for detectable serum levels in vivo [190]. Glrx1, like other Trx-fold members, lacks a typical cellular export signal, and when one group investigated the potential role of exosomal export of these enzymes, the results showed no detectable levels in the exosome fraction, but rather in the supernatant, leading to further questions about the mechanism of export and what the extracellular functions may be [190].

Membrane-bound Trx has been shown to act at the epithelial cell surface to reduce disulfide bonds in extracellular β-defensin 1, a cytotoxic peptide produced by neutrophils, enhancing its bactericidal potency [191, 192]. Another redox enzyme,
quiescin sulphydryl oxidase 1 (QSOX1), was demonstrated to possess extracellular activity, being necessary for the proper incorporation of laminin into the extracellular matrix deposited by fibroblasts [193]. Interestingly, this study also showed that matrix deposited in the absence of QSOX1 was defective in promoting cell adhesion, pointing to the importance of cysteine redox status in this process. No such activities have been demonstrated thus far for Glrx1, but detection of Glrx1 protein in the sputum of asthmatics [194] may indicate a potential role for modifying extracellular proteins in the airway lumen or in the extracellular matrix, similar to functions described above for QSOX1.

**Glutaredoxin in Asthma**

Abundant evidence has established that an altered redox environment is a contributing force to the development of asthma. More specifically, data from our laboratory suggests a potential role for Glrx1 in maintaining the redox balance in the lung in both mouse models [195, 196] and in asthmatic patients [194]. In human subjects, elevated sputum Glrx1 and decreased SSG were associated with impaired lung function, indicating a possible negative role for Glrx1 [194]. Interpretation of the limited clinical data is at best speculative, as it is unknown if alterations in Glrx1 and SSG content are causative or simply a consequence of the increased oxidative environment in these lungs. In response to allergen challenge, Glrx1 protein in the lung has been shown to increase and Glrx1-/- mice in the Balb/C background displayed enhanced resolution of airway hyperresponsiveness (AHR) following exposure to the antigen ovalbumin [196], further
suggesting a negative correlation between Glrx1 and lung function. Despite these efforts, the precise function of Glrx1 in disease pathogenesis is still not well understood and may include both intracellular and extracellular functions, some of which remain to be discovered.

**Statement of Hypothesis and Scope of Thesis**

As outlined in the literature review, perturbation of the cellular redox balance is becoming widely appreciated as a contributing factor driving the pathogenesis of various diseases, including asthma. S-glutathionylation of reactive cysteines has emerged as a major modification that affects a variety of signaling pathways, including NF-κB, due in large part to the existence of glutaredoxins (Glrx), namely Glrx1, which specifically regulate this process. There exists a paucity of data supporting a role for Glrx1 in animal models of allergic airway disease which utilize asthma-relevant allergens and even less data showing a role for Glrx1 in asthmatic populations. For that reason, we hypothesized that exposure of lung epithelial cells to IL-17A results in an altered cellular redox balance contributing to enhanced S-glutathionylation of NF-κB proteins and Glrx1 controls the extent of IL-17A-induced S-glutathionylation. Additionally, we hypothesized that Glrx1 status controls the extent of allergen-induced airway remodeling in a house dust mite model of allergic airway disease.

The goal of Chapter 2 was to determine whether IL-17A stimulation of lung epithelial cells leads to enhanced oxidative stress and a resultant environment conducive to protein S-glutathionylation. Furthermore, we sought to establish a role for the
Glrx1/S-glutathionylation axis in controlling epithelial activation of NF-κB and downstream gene induction. We also investigated the importance of IKKα status in lung epithelial cell responses to IL-17A via analysis of downstream pro-inflammatory gene expression.

In Chapter 3 of this thesis, we sought to explore the in vivo role of Glrx1 in settings of house dust mite (HDM)-induced allergic airway disease. To do so, we utilized two complementary approaches: a knockout mouse that globally lacks the Glrx1 gene and a transgenic approach in which Glrx1 is inducibly overexpressed in CCSP-positive lung epithelial cells. This allowed us to evaluate the impact of Glrx1 absence or increased expression on parameters of airway hyperresponsiveness, inflammation and airway remodeling following HDM exposure. We also utilized Glrx1-targeted siRNA or epithelial cells isolated from Glrx1−/− mice to assess the impact of Glrx1 on epithelial and mesenchymal markers in response to HDM and TGFβ.

Lastly, in Chapter 4 we discuss the impact of the findings obtained from these studies and offer insight into the role that Glrx1 plays in the pathogenesis of allergic airway disease. Furthermore, we discuss the possible role of Glrx1 in settings of human lung diseases, suggesting possible new targets that could be exploited in clinical settings of fibrotic lung disease.
Figure Legends:

Figure 1: Airway Epithelial Cells in Remodeling. Exposure of the lung epithelium to various stimuli, such as HDM, can damage the epithelial layer. This is normally resolved by mechanisms discussed in this thesis, resulting in a restored and functional epithelial layer. In cases of chronic injury, the injury/repair mechanism can become imbalanced, resulting in airway remodeling which includes mucus metaplasia, smooth muscle cell proliferation, excessive collagen production and secretion of VEGF, leading to vascular remodeling. This process can result in irreversible damage to the airways and is a frequently observed in settings of asthma.

Figure 2: Schematic depicting classical and alternative NF-κB pathways. In the classical NF-κB pathway (left), activation by various stimuli results in phosphorylation of the upstream kinase IKKβ, which can then phosphorylate inhibitor of kappa B alpha (IκBα). Subsequent ubiquitination of IκBα marks it for degradation by the 26S proteasome, allowing the RelA/p50 heterodimer to enter the nucleus. In the alternative NF-κB pathway (right), activation begins with stabilization of NF-κB-inducing kinase (NIK), which phosphorylates IKKα. Activated IKKα can then phosphorylate p100, which leads to its subsequent ubiquitination and partial degradation by the 26S proteasome to the active p52 form. This then allows entry of RelB/p52 heterodimers to enter the nucleus.
Figure 3: Schematic depicting cysteine oxidations. Reactive cysteines can exist in the reduced thiol form (SH) or more reactive thiolate form (S\(^-\)). After exposure to an oxidant, such as hydrogen peroxide (H\(_2\)O\(_2\)), the reactive cysteine can be oxidized to sulfenic acid (SOH). In the presence of abundant oxidants, the SOH form of cysteine can be further oxidized to sulfinic (SO\(_2\)H) and sulfonic (SO\(_3\)H) acids. The SO\(_3\)H form is irreversible, resulting in permanent protein inactivation and usually degradation. The SO\(_2\)H form, however, can be reduced in some instances (specified in the text) by sulfiredoxin (Srx). SOH forms of reactive cysteines are also thought to be the precursor step to S-glutathionylation (PSSG), which can be catalyzed by glutathione S transferase Pi (GSTP) and reversed by glutaredoxin 1 (Grx1).
Figures

Figure 1:

Allergens, viruses, bacteria

REPAIR

INJURY

CHRONIC INJURY

Conducting epithelium

Basal cells

Fibroblasts/Smooth Muscle

Mucus

↑VEGF Vascular remodeling

Collagen
Figure 2:
Figure 3:
List of Abbreviations:

GWAS: Genome-wide association study
TSLP: Thymic stromal lymphopoietin
IL-25: Interleukin-25
IL-33: Interleukin-33
IL-4: Interleukin-4
IL-5: Interleukin-5
IL-13: Interleukin-13
IL-17A: Interleukin-17A
IL-17F: Interleukin-17F
IL-17C: Interleukin-17C
IL-17RA: Interleukin-17 receptor A
IL-17RC: Interleukin-17 receptor C
IL-17RE: Interleukin-17 receptor E
IL-22: Interleukin-22
IgE: Immunoglobulin E
Th1: T helper type 1
Th2: T helper type 2
Th17: T helper type 17
ILC2: Type 2 innate lymphoid cell
ILC3: Type 3 innate lymphoid cell
iNKT cell: Invariant natural killer T cell
KC/CXCL1: Keratinocyte chemoattractant/chemokine (C-X-C motif) ligand 1
CCL20: Chemokine (C-C motif) ligand 20
G-CSF: Granulocyte colony stimulating factor
GM-CSF: Granulocyte macrophage colony stimulating factor
mRNA: Messenger RNA
AHR: Airway hyperresponsiveness
CCSP/CC10: Club cell secretory protein
DPPC: dipalmitoylphosphatidylcholine
PRR: Pattern recognition receptor
TLR: Toll-like receptor
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
TRAF6: Tumor necrosis factor receptor-associated factor 6
TRAF5: Tumor necrosis factor receptor-associated factor 5
TRAF3: Tumor necrosis factor receptor-associated factor 3
MCP-1: Monocyte Chemotactic Protein 1
IL-13: Interleukin-13
Alum: Aluminum hydroxide
Ova: Ovalbumin
LPS: Lipopolysaccharide
HDM: House dust mite
αSMA: Alpha smooth muscle actin
TGFβ: Transforming growth factor beta
IL-13Rα1: Interleukin-13 receptor alpha 1
Muc5AC: Mucin 5AC
JAK: Janus kinase
STAT6: Signal transducer and activator of transcription 6
ASM: Airway smooth muscle
LTRA: Leukotriene receptor antagonist
RANTES: Regulated on activation, normal T cell expressed and secreted
IL-8: Interleukin-8
MMP2: Matrix metalloproteinase 2
VEGF: Vascular endothelial growth factor
HIF: Hypoxia inducible factor
mir-1: micro-RNA 1
ICS: Inhaled corticosteroid
FDA: Federal Drug Administration
TAK1: TGFβ-associated kinase 1
IKKa: Inhibitor of nuclear factor kappa B kinase, alpha
IKKB: Inhibitor of nuclear factor kappa B kinase, beta
IKKγ/NEMO: Inhibitor of nuclear factor kappa B kinase, gamma/ Nuclear factor-kappa B essential modulator
IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha
NIK: Nuclear factor-kappa B inducing kinase
PTM: Post-translational modification
ROS: Reactive oxygen species
H₂O₂: Hydrogen peroxide
O₂⁻: Superoxide
NADPH: Nicotinamide adenine dinucleotide phosphate
NOX: Nicotinamide adenine dinucleotide phosphate-oxidase
DUOX1: Dual oxidase 1
DUOX2: Dual oxidase 2
NOS: Nitric oxide synthase
nNOS/NOS1: neuronal nitric oxide synthase/nitric oxide synthase 1
iNOS/NOS2: inducible nitric oxide synthase/nitric oxide synthase 2
eNOS/NOS3: endothelial nitric oxide synthase/nitric oxide synthase 3
NO: Nitric oxide
cGMP: Cyclic guanosine monophosphate
RNS: Reactive nitrogen species
ONOO⁻: Peroxynitrite
GSH: Glutathione
GSNO: S-nitrosoglutathione
GSNOR: S-nitrosoglutathione
ER: Endoplasmic reticulum
SOD2/MnSOD: Superoxide dismutase 2/Manganese superoxide dismutase
UPR: Unfolded protein response
PDI: Protein disulfide isomerase
Ero1: Endoplasmic reticulum oxidoreductase 1
Prx4: Peroxiredoxin 4
MPO: Myeloperoxidase
Cl⁻: Chloride anion
HOCl: Hypochlorous acid
EPO: Eosinophil peroxidase
Br⁻: Bromide anion
HOBr: Hypobromous acid
Trx: Thioredoxin
A. alternata: Alternaria alternate
Cys: Cysteine
SH: sulfhydryl/thiol
S': Thiolate anion
SOH: Sulfenic acid
SO$_2$H: Sulfinic acid
SO$_3$H: Sulfonic acid
ICAT: Isotope-coded affinity tag
PrxI-IV: Peroxiredoxin I-IV
Srx: Sulfiredoxin
SSG: S-glutathionylation
SNO: S-nitrosylation
GSTP: Glutathione S transferase Pi
GSTM: Glutathione S transferase Mu
GSTO: Glutathione S transferase Omega
S-S: Disulfide bond
Glrx: Glutaredoxin
PICOT: Protein kinase C-interacting cousin of thioredoxin
JNK: c-Jun N terminal kinase
QSOX1: Quiescin sulfhydryl oxidase 1
Glrx1-/-: Glutaredoxin 1 knock-out mouse
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CHAPTER 2

THE GLUTAREDOXIN/S-GLUTATHIONYLATION AXIS REGULATES INTERLEUKIN 17A-INDUCED PRO-INFLAMMATORY RESPONSES IN LUNG EPITHELIAL CELLS IN ASSOCIATION WITH S-GLUTATHIONYLATION OF NUCLEAR FACTOR-κB FAMILY PROTEINS

Abstract

Interleukin-17A (IL-17A) is a newly emerging player in the pathogenesis of chronic lung diseases that amplifies inflammatory responses and promotes tissue remodeling. Stimulation of lung epithelial cells with IL-17A leads to activation of the transcription factor, nuclear factor kappa B (NF-κB), a key player in the orchestration of lung inflammation. We have previously demonstrated the importance of the redox-dependent post-translational modification, S-glutathionylation, in limiting activation of NF-κB and downstream gene induction. Under physiological conditions, the enzyme glutaredoxin 1 (Grx1) acts to deglutathionylate NF-κB proteins, which restores functional activity. In this study, we sought to determine the impacts of S-glutathionylation on IL-17A-induced NF-κB activation and expression of pro-inflammatory mediators. C10 mouse lung alveolar epithelial cells, or primary mouse tracheal epithelial cells exposed to IL-17A show rapid activation of NF-κB, and the induction of pro-inflammatory genes. Upon IL-17A exposure, sulfenic acid formation and S-glutathionylated proteins increased. Assessment of S-glutathionylation of NF-κB pathway components revealed S-glutathionylation of RelA (RelA-SSG) and inhibitory kappa B kinase alpha (IKKα-SSG) after stimulation with IL-17A. SiRNA-mediated ablation of Grx1 increased both RelA-SSG and IKKα-SSG and acutely increased nuclear content of RelA, and tended to
decrease nuclear RelB. SiRNA mediated ablation or genetic ablation of *Glrx1* decreased the expression of NF-κB regulated genes, KC and CCL20, in response to IL-17A, but conversely increased the expression of IL-6. Lastly, siRNA-mediated ablation of IKKα attenuated nuclear RelA and RelB content and decreased expression of KC and CCL20 in response to IL-17A. Together, these data demonstrate a critical role for the S-glutathionylation/Grx1 redox axis in regulating IKKα-SSG and the responsiveness of epithelial cells to IL-17A.
Introduction

The Interleukin-17 (IL-17) family is a newly emerging class of cytokines that plays a critical role in a variety of biological processes, including innate host defense and inflammation. This cytokine family consists of six members, IL-17A-F, with IL-17A the most extensively studied member to date. Upon binding of IL-17A to its heterodimeric receptors (IL-17RA/RC), various adaptor proteins containing unique interaction domains are recruited to the receptor complex, activating multiple pathways, which include mitogen activated protein kinases (MAPK) and nuclear factor kappa B (NF-κB). Aberrant IL-17 signaling has been functionally linked to the pathogenesis of a variety of autoimmune and inflammatory diseases, including multiple sclerosis, systemic lupus erythematosus, psoriasis, rheumatoid arthritis and asthma [1-5]. Although T lymphocytes are the main source of IL-17A, the expression of IL-17Rs appears to be more ubiquitous [6], and the role of structural cells, including epithelial cells, as major responders to IL-17A has been well documented [7-9]. Airway epithelial cells have been shown to upregulate many pro-inflammatory mediators and genes involved in host-defense following stimulation with IL-17A. These include beta defensins, mucin genes and dendritic cell and neutrophil chemoattractants [7, 10, 11], in part due to the activation of the transcription factor NF-κB.

Previous studies in our laboratory have demonstrated the importance of NF-κB activation within lung epithelial cells in the pathogenesis of allergic airways disease in mouse models [12-14]. Two parallel NF-κB activation pathways have been described to date: the classical and alternative pathways. In the classical pathway, inhibitor of kappa B
kinase β (IKKβ) becomes activated via phosphorylation, allowing it to then phosphorylate inhibitor of kappa B α (IκBα), marking it for ubiquitination and subsequent proteasomal degradation. This event facilitates nuclear entry of the transcription factor heterodimer RelA/p50, and subsequent gene induction. In the alternative NF-κB pathway, IKKα activation leads to the phosphorylation of p100 and its resultant processing to the active p52 form, and allows nuclear entry of the RelB/p52 heterodimer [15, 16]. The alternative pathway has typically been considered to function primarily in cells of the adaptive immune system and lymphoid organs, but more recent studies have demonstrated that both NF-κB pathways can cooperate to control pro-inflammatory responses in lung epithelial cells [17].

Given the central role of NF-κB in driving inflammatory responses, its activation is tightly regulated via protein-protein interactions as well as several post-translational modifications. Reversible oxidative modifications of cysteines, including sulfenic acid formation and protein S-glutathionylation (PSSG), have been shown to affect protein function [18-20]. PSSG represents the conjugation of the tripeptide antioxidant, glutathione (GSH), to reactive, oxidizable cysteines within proteins. This modification has considerable implications for biological processes, as it can alter the function of various proteins, and both PSSG-induced activation and inactivation have been reported, depending on the protein target [21]. Work by our laboratory and others has demonstrated that the NF-κB pathway, as well as upstream adaptor molecules such as TRAF6, are negatively regulated via PSSG [22-26]. Under physiological conditions, the thiol oxidoreductase glutaredoxin-1 (Grx1) can deglutathionylate proteins, thus restoring the
native thiol form of the target cysteine residue [27]. Numerous studies initially focused on oxidant-induced PSSG, but more recent studies have demonstrated the role of this modification in the context of biologically relevant stimuli [22, 28-30]. Although IKKα acts as a positive regulator of IL-17A production in T-cells [31] and has also been shown to regulate IL-17A responses in synoviocytes, kidney cells and astrocytes [32], the role of epithelial IKKα in response to IL-17A is unclear. To date, it is also unknown whether IL-17A elicits redox perturbations in lung epithelial cells, and whether redox modifications alter pro-inflammatory responses to IL-17A. Because of the known regulation of the NF-κB pathway through PSSG, the ability of Grx1 to reverse this modification, and the possible link between IKKα and IL-17A, we focused herein on the role of the Grx1/PSSG redox axis in regulating the IKKα-driven responsiveness of lung epithelial cells to IL-17A.

Materials and Methods

Antibodies: The following antibodies were used in this study: rabbit anti-RelA, rabbit anti-RelB (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Histone H3, rabbit anti-IκBα (Cell Signaling Technology, Danvers, MA), mouse anti-GSH (Virogen, Watertown, MA), mouse anti-IKKα (Upstate, Millipore, Billerica, MA) Streptavidin conjugated-HRP (Jackson, West Grove, PA), goat anti-Grx1 (American Diagnostica, Stamford, CT) and mouse anti-β-actin (Sigma-Aldrich, Saint Louis, MO). The secondary HRP conjugated anti-rabbit and anti-mouse antibodies were from Amersham
(Piscataway, NJ), anti-goat was from Jackson Laboratories (West Grove, PA). All fluorophore-conjugated antibodies were from Invitrogen (Carlsbad, CA).

**Cell Culture:** Primary mouse tracheal epithelial cells (MTECs) were isolated from WT mice or mice lacking the glutaredoxin-1 gene (referred herein as Glrx1 -/-), and cultured as previously described [33, 34]. A spontaneously transformed type II mouse lung alveolar epithelial cell line [35] (C10) was cultured as described previously [33]. Unless otherwise noted, 16 h prior to stimulation, C10 cells were switched to medium containing 0.5% FBS; MTECs were incubated for 16h in serum-free medium. Cells were stimulated with indicated concentrations of Interleukin-17A (IL-17A; R&D Systems, Minneapolis, MN) and harvested as previously described [36].

**Transfection of siRNA:** C10 cells were incubated with scrambled, non-targeting siRNA or Grx1 siRNA (Ambion, Carlsbad, CA) (all at 100 nM). C10 cells were incubated with SMARTpool scrambled non-targeting siRNA or SMARTpool IKKα siRNA (Dharmacon, Lafayette, CO) (all at 100nM). Cells were subsequently stimulated, harvested and analyzed as indicated.

**Labeling of sulfenic acids using dimedone:** Cells were switched to serum-free medium for 2 hours; in the final hour, cells were pre-incubated with 1 mM DCP-Bio1 (Kerafast, Boston, MA) in serum-free medium for 1h at 37° C. Medium containing DCP-Bio1 was removed, and fresh serum-free medium containing 100 ng/ml IL-17A was added to cells
for indicated times. Cells were then fixed in 4% formalin and permeabilized with 0.2% Triton X100 in PBS for 10 min. The permeabilized cells were blocked in 5% BSA in PBS for 1h. The cells were then incubated with fluorescently labeled streptavidin (1:2000, SA alexa fluor 647) and the nucleus was counterstained with DAPI (1:4000). Cell images were acquired using a Zeiss LSM 510 META Confocal Laser Scanning Imaging System. All images were taken at 20X magnification. The image files were converted to tiff format and brightness and contrast were adjusted equally in all images.

Alternatively, cells were harvested in the presence of dimedone to detect oxidized cysteines in cell lysates according to a previously described method [37]. In brief, cells were serum-deprived for 2h, and then stimulated with IL-17A and lysed in buffer containing 1mM dimedone. Excess dimedone was removed using Micro Bio-spin 6 columns (Bio-Rad, Hercules, CA). Samples were analyzed by SDS-PAGE, and extent of sulfenic acid labeling was assessed using anti-dimedone antibody (Millipore, Darmstadt, Germany).

Detection of S-glutathionylated proteins (PSSG): Cells were exposed to IL-17A as indicated, and at the selected times lysates were prepared as described before [38]. In brief, samples were immunoprecipitated (IP) using GSH antibody (2μg/ml) and precipitated proteins were subjected to polyacrylamide gel electrophoresis and subsequent immunoblotting for IKKα [38]. As a reagent control, samples were treated with 50mM DTT prior to IP, to decompose PSSG. In selected experiments, cells were incubated with 250μM biotinylated glutathione ethyl ester (Bio-GEE) for 1h. Bio-GEE
was prepared as previously described [39], and Bio-GEE-conjugated proteins were determined following non-reducing SDS-PAGE and Western blotting [39]. Western Blots were analyzed via densitometry, and

Total levels of PSSG in cell lysates were determined using the glutathione/glutathione reductase/NADPH/5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) recycling assay, according to procedures described previously [40]. Briefly, lung tissue was homogenized in buffer containing 137 mM Tris·HCl, pH 8.0, 130 mM NaCl, and 1% NP-40. After determination of protein concentration, protein content was equalized and 500μg of protein was precipitated with acetone. Following centrifugation, the pellet was resuspended in buffer containing 0.1% Triton X-100 and 0.6% sulfasalicylic acid and freeze-thawed twice. Protein-associated glutathione was released with sodium borohydride, and total GSH levels were determined. The sodium borohydride-sensitive fraction of GSH released from proteins was calculated and expressed as nanomoles GSH per milligram of protein.

**mRNA analyses:** Total RNA was isolated from cells and purified using the RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed to cDNA for taqman gene analysis using SYBR green (Bio-Rad, Hercules, CA). Expression values were normalized to the housekeeping gene cyclophilin. Primers used were: KC; forward GCTGGATTCACCTCAAGA, reverse TGGGGACACCTTTTAGCATC, IL-6; forward CTGATGCTGGTGACAACCAC, reverse CAGAATTGCCATTGCACAAC, CCL20; forward AAGACAGATGGCCGATGAAG, reverse
AGCCCTTTTCACCCAGTTCT, cyclophilin; forward 
TTCCTCCTTCACAGAATTATTCCA, reverse CCAGTGCCATTATGG.

*Enzyme-Linked Immunosorbent Assays (ELISA):* Cells were stimulated, and medium was assayed for KC, CCL-20 and IL-6 (R & D Systems, Minneapolis, MN) according to manufacturer’s instructions.

*Statistical analyses:* Statistical analyses were performed using Graphpad Prism software (GraphPad, San Diego, CA) using one- or two-way ANOVA with Bonferroni correction for multiple comparisons. All experiments were conducted at least three times and data are presented as mean values plus the standard error of the mean.

**Results**

*IL-17A activates classical and alternative NF-κB pathways*

Recent work in our laboratory has provided insights into the interaction of the classical and alternative NF-κB pathways in controlling inflammatory responses in airway epithelial cells [17]. IL-17A-induced classical NF-κB activation in airway epithelial cells has been well documented [7, 10, 41, 42], but only recently was it demonstrated that IL-17A can also activate the alternative NF-κB pathway [17]. Results in Figure 1A and B demonstrate activation of the classical NF-κB pathway, as indicated by phosphorylated forms of RelA and IκBα (Fig. 1A) as well as IκBα degradation and increases in nuclear RelA content (Fig. 1B). Expression of IκBα and A20, negative
regulators of NF-κB which are induced upon activation of NF-κB, was enhanced following IL-17A exposure (Fig. 1C). In response to IL-17A, slight increases in nuclear RelB content occurred over time, indicative of alternative NF-κB activation (Fig. 1B). These data demonstrate that IL-17A activates both classical and alternative NF-κB in lung epithelial cells.

_Cysteine oxidation and protein S-glutathionylation are increased following IL-17A exposure_

Given that modifications of cysteines play an important role in protein regulation, we next investigated whether IL-17A stimulation leads to perturbations in the cellular redox environment through determination of cysteine redox modifications. We utilized dimedone or a dimedone-based derivative (DCP-Bio1) to measure the extent of sulfenic acid formation following stimulation with IL-17A. Cells were either lysed in the presence of dimedone to trap sulfenic acid moieties or pre-incubated with DCP-Bio1 and then stimulated with IL-17A to visualize oxidation of cysteines via immunofluorescence. Results in Figure 2 demonstrate a rapid increase in sulfenic acid formation of numerous proteins based upon dimedone labeling and analysis via western blot (Fig. 2A) and immunofluorescence analysis (Fig. 2B). In cells stimulated with IL-17A, sulfenic acid residues were detectable predominantly in the periphery of the cell, suggestive of compartmentalization of sulfhydryl oxidations.

Because sulfenic acid formation is believed to be a gateway for further cysteine modifications, we next asked if S-glutathionylation (PSSG) was also increased in
response to stimulation of cells with IL-17A. Glutathione-conjugated proteins from cell lysates were assessed by non-reducing SDS-PAGE analysis. Incubation with an anti-GSH antibody revealed a time-dependent increase in overall glutathione-adducted proteins in response to IL-17A (Fig. 3A). Overall PSSG content measured following release of GSH from precipitated proteins also showed trends towards increases in PSSG in epithelial cells stimulated with IL-17A at early time points (Fig. 3B). Extensive data exists to support a functional role for PSSG of NF-κB components in controlling the activity of this pathway [22-25], including work in our laboratory demonstrating the importance of IKKβ S-glutathionylation (IKKβ-SSG) [23, 43]. Therefore, we next examined the glutathionylation status of specific proteins in the NF-κB pathway after IL-17A stimulation. Although we were unable to consistently detect IKKβ-SSG in response to IL-17A (data not shown), we did detect increases in RelA-SSG and IKKα-SSG (Fig. 3C), which occurred in a biphasic manner. The bi-phasic changes in RelA-SSG and IKKα-SSG could reflect alterations in expression of Grxl. However, assessment of Grxl protein content revealed no differences in expression (Fig. 3C) suggesting that time-dependent alterations of PSSG in response to IL-17A are not a mere reflection of differences in Grxl content. Overall, these data indicate that IL-17A stimulation leads to a rapid increase in sulfenic acid and PSSG in lung epithelial cells, and that RelA and IKKα are targets for PSSG.
Glutaredoxin 1 plays an important role in controlling epithelial cell responsiveness to IL-17A

Under physiological conditions, the oxidoreductase Grx1 can catalyze the deglutathionylation of proteins, restoring their native structure and function, and IKKβ is known to be a substrate for this enzyme [23, 43]. Therefore, we hypothesized that Grx1 plays a role in IL-17A signaling by de glutathionylating NF-κB proteins, namely RelA and IKKα. First, we evaluated the effects of siRNA-mediated ablation of Grx1 on NF-κB activation by assessing nuclear content of RelA and RelB in response to IL-17A. RelA nuclear content was increased acutely after ablation of Grx1, compared to siRNA control cells exposed to IL-17A (Fig. 4A). In contrast, while nuclear content of RelB increased in response to IL-17A in siRNA control transfected cells, nuclear content of RelB in cells subjected to Grx1 ablation did not appear to change (Fig. 4A), indicating differential regulation of both NF-κB pathways by Grx1. The kinetics of nuclear RelA and RelB accumulation in response to IL-17A were slightly different as compared to Fig. 1B, likely the result of transfection of the cells. Interestingly, ablation of Grx1 also led to a baseline increase in mRNA expression of A20, which was further increased in response to IL-17A, compared to siRNA controls (Fig. 4B), indicating that certain facets of the NF-κB signaling pathway may be intrinsically dampened in the absence of Grx1.

We next analyzed the impact of siRNA-mediated ablation of Grx1 on the expression of pro-inflammatory cytokines induced by IL-17A. IL-17A-induced mRNA expression of KC and CCL20 was substantially decreased following ablation of Grx1 (Fig. 5A), and similar decreases in content of these cytokines were apparent subsequent
to Grx1 ablation (Fig. 5B), pointing to a requirement of Grx1 in augmenting the production of these cytokines. Intriguingly, the ability of IL-17A to increase expression of IL-6 mRNA and protein was markedly increased in C10s following siRNA mediated ablation of Grx1, in contrast to siRNA control transfected cells which demonstrated only small increases in IL-6 upon stimulation with IL-17A (Fig. 5A & B). Similar patterns of expression of KC, CCL20 and IL-6 were seen in primary mouse tracheal epithelial cells (MTECs) lacking the glutaredoxin-1 gene (Glrxl) following stimulation with IL-17A (Fig. 5C), further solidifying the importance of Grx1 in controlling epithelial cell responses to IL-17A. These data suggest an important functional role for Grx1 in controlling IL-17A-induced pro-inflammatory signaling. These findings also illuminate the unique impact of Grx1 in regulating pro-inflammatory responses to IL-17A in lung epithelial cells, which are highly dependent on the target gene.

*S-glutathionylation of RelA and IKKα is controlled by glutaredoxin 1*

In order to further understand the mechanism by which Grx1 controls IL-17A-induced pro-inflammatory responses, we first evaluated the overall patterns of PSSG following ablation of Grx1. Cells were incubated with a biotinylated form of GSH (Bio-GEE) 1h prior to stimulation with IL-17A, and harvested at various time points. Results in Fig. 6A demonstrate a time-dependent increase in numerous Bio-GEE-conjugated proteins in response to IL-17A, some of which are further increased following Grx1 ablation, even in unstimulated control cells. We next determined whether RelA-SSG and IKKα-SSG are potential substrates for Grx1-mediated deglutathionylation, which may act
to control IL-17A-induced pro-inflammatory signaling. Immunoprecipitation of S-glutathionylated proteins revealed a biphasic pattern of both RelA-SSG and IKKα-SSG in siRNA control transfected cells stimulated with IL-17A (Fig. 6B). The kinetics of increases in RelA-SSG and IKKα-SSG are somewhat different to earlier observations in Fig. 3C, likely due to the transfection protocol used. Nonetheless, IL-17A-mediated increases in RelA-SSG and IKKα-SSG were more pronounced subsequent to Grx1 ablation (Fig. 6B). Taken together, these data suggest a direct role for Grx1 in controlling the extent of both RelA-SSG and IKKα-SSG.

Loss of IKKα results in dampened IL-17A responses in airway epithelial cells

The relationship between IKKα and IL-17A has been established in various cell types, but the role of epithelial IKKα in controlling IL-17A responses is unknown. Therefore, we investigated the functional link between IKKα and IL-17A signaling in airway epithelial cells. Ablation of IKKα using siRNA resulted in small reductions in nuclear content of both RelA and RelB in response to IL-17A stimulation (Fig. 7A), in conjunction with diminished expression of KC and CCL20 (Fig. 7B). Ablation of IKKα using siRNA did not alter IL-6 expression in response to IL-17A (data not shown). These data collectively indicate a role for both Grx1 and IKKα in controlling airway epithelial cell responses to IL-17A stimulation, and that IKKα-SSG correlates with the magnitude and pattern of IL-17A-induced pro-inflammatory responses.
Discussion

The Interleukin-17 (IL-17) family of cytokines has garnered much recent attention. For example, a protective role of IL-17A has been demonstrated in host defense against bacterial infections [44-47]. Conversely, a pathogenic role of IL-17A has emerged in settings of autoimmune diseases, neutrophilic inflammation, cancer, and fibrotic tissue remodeling [48-51]. Therefore, an understanding of the molecular mechanisms whereby IL-17 family members elicit biological responses is of critical importance towards the development of therapeutics aimed at targeting this family of cytokines [6, 52, 53]. IL-17A is the most well characterized member, and proximal signaling events induced at the IL-17RA/C complex have been well characterized [6]. Thiol/redox perturbations are emerging as cardinal regulators of many biological pathways, yet it remains unclear whether IL17A elicits any redox changes and whether these events affect the cellular responses to IL-17A. In the present study we demonstrate that exposure to IL-17A induced rapid protein thiol oxidations, evidenced by increases in sulfenic acid formation and S-glutathionylated proteins. We also demonstrated that RelA and IKKα are both targets for IL-17A-induced PSSG, and that IKKα contributes to pro-inflammatory signaling in lung epithelial cells. Furthermore, Glutaredoxin-1 (Grx1) controlled both RelA-SSG and IKKα-SSG, and regulated the profile and magnitude of pro-inflammatory responses. Overall these findings illuminate a new dimension of IL-17A-induced biological responses that involve a thiol/redox control mechanism.

Studies herein have focused on the response of airway epithelial cells to IL-17A. The airway epithelium is the first line of defense against inhaled insults and stimuli, and
proper functioning of airway epithelial cells is necessary to maintain proper homeostasis in the lung [54, 55]. The airway epithelium also plays a vital role in responding to both external and internal signals to control the extent of inflammatory responses. Thus, airway epithelial cells are a major target of IL-17A, and have previously been shown to produce various host defense and pro-inflammatory mediators upon IL-17A exposure [7, 8, 10, 11]. IKKα plays a role in both classical and alternative NF-κB pathways, but also has NF-κB independent functions [56]. IKKα was originally demonstrated to play a role in adaptive immune responses and lymphoid organ development [57]. More recently, a functional role for IKKα in regulating polarization of IL-17-producing T helper 17 lymphocytes has been demonstrated [31] and IKKα has also been shown to regulate the responsiveness of synoviocytes, kidney cells and astrocytes to IL-17A [32]. The role of IKKα in regulating the responsiveness of epithelial cells to IL-17A had not been previously demonstrated. Results from the present study not only show the functional importance of IKKα in regulating the responsiveness of epithelial cells to IL-17A, but also show that the outcome of IL-17A signaling in epithelium is controlled by the Grx1/PSSG axis, in association with IKKα-SSG. These observations have broad implications not only for IL-17A-induced pro-inflammatory responses, but also for other stimuli that signal through IKKα, as these may also be regulated by Grx1-mediated PSSG. However, additional studies are needed to map the reactive cysteine within IKKα that is the target for PSSG induced by IL-17A, and to determine its functional importance.
The functional impact of RelA-SSG had been established previously, as studies have shown both inhibition of RelA nuclear entry [22, 58] and DNA binding [59] following RelA-SSG. Paradoxically, we demonstrate here that IL-17A-induced RelA-SSG corresponded to increases in nuclear RelA, which were further enhanced following ablation of Grx1. These results indicate that, in response to IL-17A, nuclear translocation of RelA may not be affected by its S-glutathionylation, but that RelA-SSG could interfere with DNA binding, as has been shown previously following oxidant-triggered PSSG in vitro [59]. Further investigation is needed to determine the functional role of RelA-SSG in response to IL-17A, which was beyond the scope of the current study.

It is plausible that other components of the IL-17 pathway, in addition to RelA and IKKα, may be targets for thiol/redox control. In this regard, TRAF6 was recently described as a target for Grx1-mediated deglutathionylation in the context of TLR/IL1R signaling [26]. Since the IL-17R complex also utilizes this adaptor molecule for the activation of NF-κB [6], it is plausible that TRAF6 is also a target for PSSG in cells stimulated with IL-17A. A recent study demonstrated the importance of A20 in negative feedback regulation of IL-17A-induced cellular responses [60]. A20 is one of several de-ubiquitinases that has been shown to be a target for reversible oxidations which regulate its activity [61]. The active site cysteine of A20 in particular was demonstrated as a target of hydrogen peroxide-induced oxidation, resulting in inhibition of its deubiquitinase activity [61]. It is plausible that PSSG also inhibits de-ubiquitinase activity of A20, thereby regulating the responsiveness of epithelial cells to IL-17A, scenarios that will require further investigation. It is worthy to mention that Grx1 status regulates mRNA
expression of A20, and that absence of Grx1 led to a two fold increase of A20 mRNA. Despite this apparent link between A20 and Grx1, the specific mechanisms whereby Grx1 regulates A20 remain to be further elucidated.

The opposing outcome of IL-17A-induced KC and CCL20 expression compared to IL-6 expression following ablation of Grx1 is intriguing. While ablation of Grx1 attenuated IL-17A-induced expression of KC and CCL20, ablation of Grx1 augmented the ability of IL-17A to induce IL-6, and similar effects were observed in two distinct epithelial cell cultures. These results clearly demonstrate that ablation of Grx1 does not dampen the overall pro-inflammatory response, but rather that ablation of Grx1 modifies the nature of the pro-inflammatory response of epithelial cells to IL-17A. It remains unclear whether the altered cellular responses to IL-17A following ablation of Grx1 are indeed due to altered function of NF-κB, and whether this involves RelA/p50 or RelB/p52 heterodimers, the latter of which are known to bind to a broader spectrum of kappaB sites [62]. Following ablation of Grx1, the overall nuclear content of RelA was acutely increased, whereas nuclear RelB content tended to decrease in response to IL-17A, as compared to respective siRNA controls. Thus, an altered balance of RelA/p50 or RelB/p52 dimers could account for the observed changes of pro-inflammatory responses to IL-17A. In this regard, prior studies have indicated that active RelB dimers can repress transcription of *IL1B* and *IL12B* genes [63], and that in association with decreased RelB, IL-6 levels increased [64]. C-Rel is important in the transcriptional regulation of *IL6* [65], which raises the possibility that Grx1 may alter *IL6* gene expression via c-Rel. Alternatively, it is possible that other transcription factors modulate pro-inflammatory
gene expression following modulation of Grx1 which could account for the differential responses of IL-6 expression as compared to KC and CCL20. Future studies mapping transcription factor occupancy at the promoters of these cytokine genes will be required to fully understand these scenarios.

Results from the present study demonstrate increases in sulfenic acid formation and PSSG in response to IL-17A. Numerous studies have previously demonstrated these oxidative events under conditions of overt stress, or in response to direct exposure to an oxidant. The subtle patterns of oxidations demonstrated herein are reflective of a biological stimulus that elicits a cellular response without creating overt cellular stress, nor inducing cell death. The source of oxidants following IL-17A stimulation remains unknown. Increased sulfenic acid formation was observed as early as five minutes following IL-17A stimulation, and appeared to be localized primarily near the cell periphery (Fig. 2B). In the context of TLR/IL-1R signaling, previous studies have observed an oxidant-dependent recruitment of TRAF6 to the receptor complex, via non-phagocytic NADPH oxidase 2 (Nox2), which is necessary for signal propagation [66]. The pattern of sulfenic acid formation observed herein could potentially reflect a similar NADPH oxidase-induced signaling event that is localized to the IL-17R complex. Activation of Nox2 following engagement of the IL17R may potentially be responsible for the production of oxidants, which in turn lead to sulfenic-acid containing proteins. Indeed, further analyses will be required to fully determine the source of oxidants and the specific subcellular localization of these oxidative events.
The biochemical links between sulfenic acid intermediates and PSSG in biological settings remain poorly understood. Sulfenic acid intermediates can be unstable and give rise to further oxidations [67]. PSSG of sulfenic acid intermediates is believed to be important in the protection of cysteines against overoxidation [68]; therefore, it is plausible that sulfenic acid intermediates observed in response to IL-17A are the precursor to PSSG. The mechanisms that govern PSSG in response to IL-17A also remain unknown. PSSG can occur spontaneously, notably under conditions of oxidative stress when an abundance of oxidized glutathione (GSSG) is present [19]. Enzymatic catalysis of PSSG by glutathione S-transferase Pi (GSTP) has been demonstrated under conditions of oxidative stress [69], or in response to the death inducing ligand FasL [70]. It remains unknown at this time whether GSTP, a protein that is highly expressed in lung epithelial cells [71], plays a role in the catalysis of IKKα-SSG in response to IL-17A.

In summary, results from the present study demonstrate that the pro-inflammatory response elicited by IL-17A is controlled by the S-glutathionylation/Grx1 redox axis in association with RelA-SSG and IKKα-SSG. Therefore, avenues that target the protein thiol redox status can be exploited to harness the cellular response to IL-17A and have the potential to impact host defense and diseases associated with aberrant IL-17 signaling.
**Figure Legends**

*Figure 1: Activation of classical and alternative NF-κB pathways in lung epithelial cells in response to IL-17A.* (A) Mouse alveolar type II cells (C10) were stimulated with IL-17A (100ng/ml) and NF-κB was assessed in whole cell lysates by measuring phosphorylated forms of RelA (pRelA) and IκBα (pIκBα). β-actin; loading control. Right panels: densitometric evaluation of pRelA and pIκBα. Data reflect arbitrary units, following normalization to loading control, β-actin. (B) Assessment of nuclear RelA and RelB content and degradation of cytosolic IκBα following stimulation with IL-17A (50ng/ml) in C10 cells. β-actin; loading control for cytosolic lysates, (labeled as C); Histone H3 (H3) was used as a loading control for nuclear lysates, (labeled as N). Right and bottom panels: densitometric evaluation of nuclear RelA, nuclear RelB and IκBα. Data reflect arbitrary units, following normalization to respective loading controls. (C) Evaluation of mRNA expression of NF-κB-dependent negative regulators A20 and IκBα via real-time PCR in C10 cells stimulated with IL-17A (50ng/ml). Results were normalized to the housekeeping gene cyclophilin and are presented as relative expression. *p≤0.05 (ANOVA), compared to untreated controls (Sh).

*Figure 2: IL-17A increases cysteine oxidation in lung epithelial cells.* (A) C10 cells were stimulated with IL-17A (50ng/ml) for indicated times and subsequently harvested in the presence of dimedone (1mM) to trap the sulfenic acid form (SOH) of cysteines. Samples were analyzed via reducing SDS-PAGE, and overall sulfenic acid formation was determined using an anti-dimedone antibody (1:1000). -Dim, minus dimedone control.
(B) Assessment of SOH formation (red) via immunofluorescence in C10 cells pre-incubated with DCP-Bio1 (1mM) for 1h and stimulated with 100ng/ml IL-17A for indicated times. An anti-biotin Alexafluor-647 secondary antibody was used to visualize DCP-Bio1-bound proteins. Nuclei are counterstained with DAPI (blue). No DCP-Bio1; omission of DCP-Bio1 label.

Figure 3: Increases in protein S-glutathionylation in C10 cells stimulated with IL-17A.

(A) C10 cells were stimulated with 50ng/ml IL-17A, and whole cell lysates were harvested at indicated times. SDS-PAGE analysis was performed under non-reducing conditions and GSH-conjugated proteins were assessed using an anti-GSH antibody (1:1000). A separate sample was incubated with (+DTT) to decompose S-glutathionylated proteins. (B) Following stimulation with 50ng/ml IL-17A, C10 cells were harvested at indicated times and overall S-glutathionylation was evaluated using the DTNB recycling assay. (C) C10 cells were stimulated with 50ng/ml IL-17A and harvested as indicated. S-glutathionylated proteins (200μg protein/sample) were immunoprecipitated (IP) using an anti-GSH antibody (2μg/ml) overnight at 4°C. Following IP, samples were subjected to SDS-PAGE and Western Blot analysis using RelA and IKKα antibodies. WCL: Whole cell lysates to confirm equal content of RelA and IKKα, or Grx1. +DTT, control sample incubated with DTT to decompose S-glutathionylated proteins. β-actin: loading control.

Bottom panel: densitometric evaluation of IKKα-SSG and RelA-SSG. Data reflect arbitrary units, following normalization to total IKKα or RelA.
Figure 4: Ablation of Grx1 alters NF-κB activation and mRNA expression of A20. (A) Assessment of NF-κB activation following siRNA-mediated ablation of Grx1 or control siRNA and stimulation with IL-17A (50ng/ml). Nuclear (N) and cytosolic (C) lysates were harvested from C10 cells and nuclear content of RelA and RelB (top panels) was assessed. Histone H3 (H3); loading control. Bottom panels: Evaluation of Grx1 in cytosolic lysates to confirm siRNA-mediated ablation. β-actin; loading control. Graphs represent densitometric evaluation of nuclear RelA and RelB. Data reflect arbitrary units, following normalization to the loading Histone H3. (B) C10 cells were subjected to siRNA-mediated ablation of Grx1 or control siRNA, and stimulated with IL-17A for indicated times, and A20 mRNA expression was evaluated. Results were normalized to the housekeeping gene cyclophilin and are presented as relative expression.

Figure 5: Grx1 SiRNA or genetic ablation of Glrx1 alters pro-inflammatory responses in lung epithelial cells. C10 cells stimulated with IL-17A (50ng/ml) for indicated times were analyzed for mRNA expression (A) and protein levels (B) of KC, CCL20 and IL-6 following siRNA-mediated ablation of Grx1 or control siRNA. (C) Expression of KC, CCL20 and IL-6 in WT or Glrx1/- primary mouse tracheal epithelial cells (MTECs) following stimulation with IL-17A (20ng/ml). Expression values were normalized to the housekeeping gene cyclophilin, and data are presented as relative expression. * p≤0.05 (ANOVA) compared to untreated controls (Sh); † p≤0.05 (ANOVA) compared to scr siRNA groups at the same time point.
Figure 6: Grx1 ablation promotes S-glutathionylation of RelA and IKKα. C10 cells were subjected to siRNA-mediated ablation of Grx1 or control siRNA. (A) Cells were pre-incubated with Bio-GEE (250μM) for 1h prior to stimulation with IL-17A (50ng/ml), and subsequently harvested at indicated times. SDS-PAGE performed under non-reducing conditions, followed by Western Blot analysis of Bio-GEE-conjugated proteins assessed via streptavidin-HRP. –BG; control wherein the Bio-GEE label was omitted. ns; non-specific bands. (B and C) Following siRNA-mediated ablation of Grx1, C10 cells were stimulated with IL-17A (50ng/ml) and harvested at indicated times. S-glutathionylated proteins were immunoprecipitated with GSH antibody (2μg/ml) and resolved via SDS-PAGE and western blotting for IKKα (B) or RelA (C). Bottom panel: western blot showing confirmation of Grx1 ablation. Right panels: densitometric evaluation of IKKα-SSG and RelA-SSG. Data reflect arbitrary units, following normalization to total IKKα or RelA.

Figure 7: SiRNA-mediated ablation of IKKα modulates expression of pro-inflammatory mediators in lung epithelial cells. (A) Assessment of nuclear RelA and RelB following siRNA-mediated ablation of IKKα or control siRNA and stimulation with IL-17A (50ng/ml). Nuclear (N) and cytosolic (C) lysates were harvested from C10 cells and nuclear content of RelA and RelB was assessed. Histone H3 (H3) was used as a loading control. Bottom panels: Assessment of IKKα in cytosolic lysates to confirm siRNA-mediated ablation. β-actin; loading control. Graphs represent densitometric evaluation of
nuclear RelA and RelB. Data reflect arbitrary units, following normalization to the loading Histone H3. (B) Assessment of KC and CCL20 mRNA expression following siRNA-mediated ablation of IKKα and stimulation with IL-17A (50ng/ml) at indicated times. Values were normalized to the housekeeping gene cyclophilin, and data presented and relative expression. * p≤0.05 (ANOVA) compared to untreated controls (Sh); † p≤0.05 (ANOVA) compared to scr siRNA groups at the same time point.
Figures

Figure 1:
Figure 2:
Figure 3:
Figure 4:
Figure 5:
Figure 6:

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B.

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Figure 7:
References


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CHAPTER 3

THE THIOL TRANSFERASE GLUTAREDOXIN-1 ATTENUATES HOUSE DUST MITE-INDUCED FIBROTIC AIRWAY REMODELING

Abstract

Allergic airways disease is accompanied by changes in the redox environment, although the exact functional role of redox changes in disease pathogenesis remains unknown. Protein S-glutathionylation (PSSG) represents an oxidative modification of reactive protein cysteines and has emerged as a key regulatory event in biological processes. Under physiological conditions, the thiol transferase, glutaredoxin-1 (Glrx1), can de-glutathionylate protein cysteines, restoring the reduced protein thiol group. In the present study we addressed the functional role of Glrx1 in house dust mite (HDM)-induced allergic airways disease using mice that globally lack the Glrx1 gene (Glrx1−/−) or Bitransgenic mice that inducibly overexpress Glrx1 in airway epithelial cells (Epi-Glrx1) upon administration of Doxycycline (Dox). Glrx1−/− mice exposed to HDM had increased airway hyperresponsiveness (AHR), increased α-SMA reactivity and increased sub-epithelial collagen deposition, compared to respective WT mice. Conversely, Epi-Glrx1 mice exposed to HDM displayed decreased AHR and substantial protection against sub-epithelial collagen deposition, compared to respective controls. E-cadherin expression, a marker of epithelial barrier function, was decreased in Glrx1−/− mice and in primary tracheal epithelial cells obtained from Glrx1−/− mice. Lastly, genetic ablation or knockdown of Glrx1 augmented TGFβ1- or HDM-induced decreases in E-cadherin and increases in α-SMA. Together, these findings demonstrate a cardinal role
for Glrx1 in protecting against HDM-induced fibrotic airways remodeling, in association with increasing epithelial barrier function.
Introduction

Asthma is a multifaceted disease characterized by chronic inflammation, airway hyperreactivity and airway remodeling. Multiple cell types have been implicated in the pathogenesis of asthma, and a role of epithelial cells has been clearly established. Epithelial cells directly respond to components in allergens, leading to the release of mediators, such as IL1α, IL33, and TSLP that in turn control adaptive immune responses [1, 2]. Mouse models of allergic airways disease, such as house dust mite (HDM) or ovalbumin (Ova), have yielded important insights into the roles that airway epithelial cells play in the etiology of allergic airways disease.

Changes in the redox environment have been demonstrated in a variety of pulmonary diseases, including asthma. Increased oxidant production [3, 4], oxidation of glutathione [5, 6] and inactivation of antioxidants [7] have been observed in airways of asthmatics. It is now becoming widely accepted that oxidants and subsequent modifications of various targets may be central players in (patho-) physiological processes. Redox homeostasis in the lung is controlled by an extensive network of enzymes and small molecules that act in concert to tightly regulate the oxidation of proteins and other macromolecules. Oxidation of cysteine thiol groups within proteins serves as a sensor that converts an oxidant signal into a biological response. Low pKa cysteines are targets for oxidation and can form sulfenic acid, S-nitrosylated, and S-glutathionylated intermediates, among others. S-glutathionylation is the conjugation of glutathione to protein cysteines and may play a critical role in regulating biological processes, as the addition of glutathione alters the charge and conformation of the target...
protein and subsequently can alter protein function. The process of S-glutathionylation is regulated by glutaredoxins, a family of thiol transferases that primarily acts to de-glutathionylate proteins under physiological conditions, restoring native cysteine sulfhydryl groups [8, 9]. Of particular interest are findings demonstrating that S-glutathionylation of proteins within the NF-κB pathway, notably IKKβ, IKKα, RelA, and p50 inhibit NF-κB function and dampen downstream NF-κB dependent signaling [10, 11].

Using the Ova model of allergic airways disease, our laboratory has previously demonstrated that global ablation of glutaredoxin-1 (Glrx1−/−) leads to attenuated airway hyperreactivity in mice of the Balb/C background [12]. Furthermore, elevated Glrx1 as well as decreased protein S-glutathionylation has been observed in the airways of patients with asthma [13]. In settings of HDM-induced allergic airway disease, the potential role of Glrx1 has not been addressed. We therefore sought to examine whether Glrx1 status controls inflammation, airway hyperresponsiveness and airways remodeling induced by HDM. We utilized mice that globally lack the Glrx1 gene, and mice that inducibly overexpress Glrx1 in airway epithelial cells.

Materials and Methods

Mice: For all experiments, 8-12 week old C57B/6NJ mice were used (The Jackson Laboratory, Bar Harbor, ME). Lung epithelial specific overexpression of Glrx1 was achieved by crossing mice expressing the Club cell secretory protein (CC10) promoter 5’ to the open reading frame of the reverse tetracycline transactivator (CC10-rtTA) with
mice expressing the TetOP-FLAG Glrx1 construct [14]. Transgenic mice expressing CCSP-rtTA/TetOP-FLAG Glrx1 were fed doxycycline (Dox) containing food (6g/kg, Purina Diet Tech, St. Louis, MO) starting 7 days before the first administration of HDM and were maintained on Dox food until the completion of the experiment. Bi-transgenic mice not fed Dox food, or single transgenic mice expressing only the CCSP-rtTA construct were fed Dox food and used as controls. Glrx1<sup>−/−</sup> mice were described previously, and WT littermates served as controls [15]. All studies were approved by the Institutional Animal Care and Use Committee at the University of Vermont. Experiments were performed at least twice, and animal numbers in each group are noted in each figure.

**HDM-induced allergic airways disease** and assessment of airway hyperresponsiveness: WT, Glrx1<sup>−/−</sup> and Glrx1 TG mice were intra-nasally instilled with HDM extract containing 1μg protein (GREER, Lenoir, NC) on days 1 and 8, and on days 15-19 mice were instilled with 10μg HDM each day and euthanized 24h after the last HDM administration (Fig. 1A). HDM extract contained 35 endotoxin units/mg normalized to protein content. Control mice were administered PBS as a vehicle control in each experiment. Following completion of the HDM protocol, mice were anesthetized with intraperitoneal pentobarbital sodium (90 mg/kg), tracheotomized, and mechanically ventilated at 200 breaths/min. Mice were subjected to increasing doses of methacholine (0, 12.5 mg, 25 mg and 50 mg) and respiratory mechanics were assessed using a forced oscillation technique on a computer-controlled small animal ventilator (SCIREQ, QC,
Canada), as previously described [16, 17]. Parameters of Newtonian resistance (Rn), tissue resistance (G) and elastance (H) were calculated and quantified by averaging the three highest measurements obtained at each incremental methacholine dose for each mouse.

*Bronchoalveolar lavage:* After mice were euthanized, bronchoalveolar lavage (BAL) was performed using 1 ml PBS. BAL was collected and total cell counts were determined using an Advia 120 Automated Hematology Analyzer. BAL was spun down at 1200xg for 5 min. Cells were transferred to slides using a cytospin, fixed in methanol and stained using the Hema3 kit (Fisher Scientific, Kalamazoo, MI) and analyzed by counting a minimum of 300 cells per mouse, as described elsewhere [16]. Supernatants were flash frozen in liquid nitrogen and stored at -80°C until analysis via ELISA.

*Measurement of airways fibrosis:* The right lung was fixed in 4% PFA and embedded in paraffin. Lung sections (5μm) were stained using Masson’s Trichrome to visualize collagen. Staining intensity in peribronchiolar regions was analyzed by two independent investigators using a scale of 0-3, where 0 indicates no collagen positivity and 3 indicates highest levels of collagen staining. Values for each group are presented as averaged scores from both investigators. Images were acquired using an Olympus BX50 Light Microscope with QImaging Retiga 2000R digital camera. Collagen content was determined by isolating the superior lobe of the right lung and measuring the total content of hydroxyproline, as previously described [18].
Cell culture and siRNA transfections: Primary mouse tracheal epithelial cells, isolated as described previously [19, 20] and the C10 type II alveolar epithelial cell line [21] were exposed to TGFβ (R&D Systems, Minneapolis, MN) or HDM (GREER) where indicated. Cells were harvested for western blot analysis or qPCR as noted. For siRNA experiments, a spontaneously transformed type II mouse lung alveolar epithelial cell line [21] (C10) was incubated with non-targeting siRNA (Scr siRNA) or siRNA targeting Glrx1 (Ambion, Carlsbad, CA) using Dharmafect reagent (Thermo Scientific, Waltham, MA) according to manufacturer’s instructions. All siRNA was used at 100nM final concentration.

Assessment of mucus metaplasia: Periodic acid-Schiff staining was performed and the extent of mucus metaplasia was evaluated by two independent investigators. Lung sections were blinded and ranked on a scale from 0 to 3: 0, no reactivity; 1, minimal staining; 2, moderate staining; 3, prominent staining. Data represented are averages of the cumulative score from each mouse according to treatment group.

mRNA analyses: RNA was isolated from lung tissue using the RNeasy Kit (Qiagen, Valencia, CA) and reverse transcribed for analysis using SYBR green (Bio-Rad, Hercules, CA). Primers used were: TGFβ, FW: TGCTTCAGCTCCACAGAGAA, RV: TGGTTGTAGAGGGCAAGGAC; αSMA, FW: CGCTGTCAGGAACCCTGAGA, RV: CGAAGCCGGCCTTACAGA; Muc5AC, FW: GCTACACCCAGGTTGAGAAGTG,
RV: TCCTCACTTTCCCTGGACTTGA. Expression was normalized to β-actin, FW: CTGAATGGCCCAGGTCTGA, RV: CCCTCCCAGGGAGACCAA.

Statistical Analyses: Data for differential cell counts, Muc5AC expression and hydroxyproline were analyzed by ANOVA. All experiments were repeated at least twice and data from combined experiments are represented as mean +/- S.E.M. Scoring of histological staining was analyzed by Kruskal-Wallis test and Dunn’s post hoc test. Data for AHR were analyzed via least squares mean test. For all analyses, P values ≤0.05 were considered significant.

Results
Glrx1−/− mice display elevated subepithelial collagen deposition in response to house dust mite (HDM) exposure.

The function of glutaredoxin 1 (Glrx1) has not been thoroughly investigated in settings of allergic airways disease, particularly in response to HDM. We have previously shown that the antigen ovalbumin (Ova) results in increased Glrx1 in the airways of mice and that Glrx1−/− mice have altered resolution of Ova-induced inflammation and airway hyperresponsiveness (AHR) [12, 22]. However, a direct role for Glrx1 in the pathogenesis of HDM-induced disease has yet to be determined. To address this, we first utilized mice globally lacking Glrx1 (Glrx1−/−) on the C57BL/6 background to determine the impact on HDM-induced AHR, cellular influx and fibrotic remodeling. In mice subjected to HDM (Fig. 1A), assessment of cellular influx into the airways in
bronchoalveolar lavage (BAL) fluid revealed comparable significant increases in total cell counts in WT and Glrxl−/− mice (Fig. 1B). Statistically significant increases in neutrophils, eosinophils and lymphocytes were observed in WT HDM-exposed mice compared to PBS controls (Fig. 1C). In contrast, in Glrx1−/− mice, statistically significant increases in BAL eosinophils and lymphocytes, but not neutrophils, were observed following HDM exposure (Fig. 1C). No significant differences were observed in macrophages in any of the groups (Fig. 1C) and no significant differences were observed between HDM-exposed WT mice and HDM-exposed Glrx1−/− mice. Western blot analysis demonstrated increases in Glrx1 protein in lung tissue in WT mice in response to HDM, consistent with prior observations following Ova [22] and HDM [23], whereas, as expected, no detectable Glrx1 immunoreactivity was observed in Glrx1−/− mice (Fig. 1D).

Next we investigated the impact of Glrx1 ablation on HDM-induced airway remodeling. Manifestations of airway remodeling include mucus metaplasia, increased alpha smooth muscle actin content (αSMA), and elevated subepithelial collagen deposition. Mucus metaplasia was significantly increased following exposure to HDM in WT and Glrx1−/− mice compared to respective PBS controls(Fig. 2A and B), in association with increased expression of the mucin gene, Muc5AC (Fig. 2C). Analysis of αSMA immunoreactivity revealed significant HDM-induced increases in both WT mice and Glrx1−/− mice (Fig. 2A & B). PAS reactivity and αSMA reactivity tended to be enhanced in Glrx1−/− mice as compared to WT animals, although these trends did not reach statistical significance. Despite elevated αSMA observed via immunohistochemical
analysis, expression of αSMA mRNA was not significantly increased in response to HDM in WT or Glrx1−/− mice (data not shown).

In order to assess the extent of subepithelial fibrosis following HDM exposure, immunohistochemical analysis of collagen deposition was investigated. Subepithelial collagen was increased in the lungs of HDM-exposed WT mice and was further elevated in Glrx1−/− mice (Fig. 2A & B). Hydroxyproline content, indicative of total lung collagen, was significantly increased in WT mice exposed to HDM (Fig. 2D). HDM-induced increases in hydroxyproline were significantly elevated in Glrx1−/− mice compared to WT mice (Fig. 2D). Lastly, we evaluated HDM-induced AHR. In response to HDM, both WT mice and Glrx1−/− mice showed significant increases in tissue resistance (G) compared to respective PBS groups, whereas airway resistance (Rn) was only significantly increased over PBS in Glrx1−/− mice (Fig. 2E). HDM-exposed Glrx1−/− mice displayed significantly higher AHR in parameters Rn and G compared to WT mice exposed to HDM (Fig. 2E), but no significant differences were observed in elastance (H) (Fig. 2E). Together, these data suggest that Glrx1−/− mice exhibit enhanced airway remodeling and slight increases in AHR compared to WT mice.

*Decreases in HDM-induced peri-bronchiolar collagen deposition in transgenic mice expressing Glrx1 in airway epithelial cells*

As a complimentary approach, we used a tissue specific, inducible system (CC10-rtTA/TetOP-FLAG Glrx1) in which mice express Glrx1 specifically in CC10-positive airway epithelial cells in the presence of doxycycline (Dox; hereafter referred to as Epi-
The transgene was activated 7 days prior to the start of experiments and HDM was administered as described in Fig. 3A. As controls, mice harboring the CC10-rtTA transgene but lacking the TetOP-FLAG Glrlx1 transgene (hereafter referred to as rtTA mice) were given Dox, or CC10-rtTA/TetOP-FLAG Glrlx1 mice not receiving Dox (hereafter referred to as Bitransgenic mice) were used. HDM exposure resulted in significantly elevated total cells in Bitransgenic mice not given Dox and Epi-Glrlx1 mice compared to respective PBS groups (Fig. 3B). HDM-exposed Epi-Glrlx1 mice exhibited significantly lower total cells compared to HDM-exposed rtTA mice receiving Dox (Fig 3B). Furthermore, Epi-Glrlx1 mice also displayed a significant decrease in eosinophils compared to rtTA mice receiving Dox (Fig. 3C), likely accounting for the observed decreases in totals cell counts. Airway neutrophils, eosinophils and lymphocytes were all significantly elevated in Bitransgenic mice and Epi-Glrlx1 mice compared to respective PBS controls, but no significant differences were observed between HDM-exposed Bitransgenic mice and HDM-exposed Epi-Glrlx1 mice (Fig. 3C). No significant differences were observed in macrophages in any group (Fig. 3B). Western blotting of lung lysates confirmed Dox-induced transgene activation in Epi-Glrlx1 mice (Fig. 3D).

Next, we investigated the extent of remodeling in the lungs of Epi-Glrlx1 mice. Analysis of mucus metaplasia and αSMA immunoreactivity showed increased HDM-induced mucus in all groups of animals, but no clear impact following expression of the Epi-Glrlx1 transgene (Fig. 4A). A statistically significant HDM-induced increase in Muc5AC mRNA was apparent in both Bitransgenic mice and Epi-Glrlx1 mice compared to PBS controls (Fig. 4B). In contrast, HDM-mediated increases in hydroxyproline
content were significantly attenuated in Epi-*Glrx1* mice compared to both Bitransgenic mice and rtTA control mice (Fig. 4C), consistent with attenuation of peri-bronchiolar Masson’s trichrome reactivity in Epi-*Glrx1* mice (Fig 4A). Analysis of AHR revealed significant HDM-induced increases in Rn, G and H in Bitransgenic mice not given Dox and Epi-*Glrx1* mice compared to respective PBS groups (Fig. 4D). A significant decrease in elastance (H) was observed in HDM-exposed Epi-Glrx1 mice compared to both Bitransgenic mice not given Dox and rtTA mice given Dox, indicating overexpression of *Glrx1* may protect against certain aspects of AHR (Fig. 4D). Similarly, Epi-Glrx1 mice exhibited significantly lower tissue resistance (G) compared to rtTA mice given Dox (Fig. 4D). Furthermore, a significant attenuation in airway resistance (Rn) was observed in rtTA mice receiving Dox compared to Epi-Glrx1 mice, indicating a potential confounding effect of Dox administration itself (Fig. 4D). Overall, the current data suggest that overexpression of *Glrx1* in airway epithelial cells protects against fibrotic airway remodeling and development of AHR.

**Absence of Glrx1 promotes mesenchymal transition of epithelial cells**

Due to the importance of Glrx1 in controlling fibrotic airway remodeling *in vivo*, we next analyzed expression of the epithelial marker, E-cadherin, in the lungs of mice. Loss of E-cadherin is generally associated with cancer metastasis and epithelial-to-mesenchymal transition (EMT), indicating loss of cell-cell contacts [24]. Greater E-cadherin depletion has also been reported in airway epithelial cell cultures from asthmatics exposed to TGFβ, compared to healthy control cells stimulated with TGFβ, pointing to a
compromised ability of the asthmatic epithelium to self-repair [25]. In response to HDM, WT mice showed decreased E-cadherin mRNA expression and protein content in lung tissue compared to control animals (Fig. 5A and B). Interestingly, Glrx1−/− mice already exhibited a decrease in basal expression of E-cadherin that was not significantly altered following exposure to HDM (Fig. 5A and B). These data indicate that Glrx1−/− mice may have impaired epithelial barrier function prior to allergen exposure. Because we observed decreased E-cadherin in Glrx1−/− mice, we next sought to determine the impact of Glrx1 status on epithelial cell responses to HDM. Similar to the data observed in mice, E-cadherin was decreased in WT epithelial cells exposed to HDM for 7 days, but was already lower in control Glrx1−/− epithelial cells, and exposure to HDM did not result in a further loss of E-cadherin (Fig. 5C).

A prominent feature of remodeling and fibrotic diseases is enhanced TGFβ production and signaling, potentially leading to elevated expression of αSMA and collagen. Therefore, we sought to further investigate the importance of Glrx1 in mediating aspects of remodeling by stimulating cells with TGFβ and assessing αSMA production. In WT cells, no detectable baseline αSMA reactivity was apparent. Upon stimulation of cells with TGFβ for 72h, αSMA increased substantially (Fig. 5D, left). Interestingly, marked increases in αSMA were observed in unstimulated Glrx1−/− cells and were further enhanced following TGFβ stimulation (Fig. 5D, right). Comparable results were observed utilizing siRNA targeted against Glrx1. TGFβ-dependent increases in αSMA were observed in C10 cells transfected with non-targeting scrambled (Scr) siRNA (Fig. 5E, left), but following siRNA-mediated ablation of Glrx1, baseline αSMA was
already detectable and further elevated in response to TGFβ exposure (Fig. 5E, right). These data indicate a central role for Glrx1 in controlling the extent of fibrotic airways remodeling induced by HDM, in association with controlling epithelial barrier function and EMT.

Discussion:

In the current study, we investigated the impact of an altered redox environment in a house dust mite (HDM) model of allergic airways disease by utilizing mice globally lacking glutaredoxin-1 (Glrx1−/−) and mice overexpressing Glrx1 specifically in the airway epithelium (Epi-Glrx1). Glrx1 is an oxidoreductase that is primarily responsible for deglutathionylation of proteins. An imbalanced redox environment in the lung is known to contribute to asthma, with increased lung and blood GSH levels having previously been reported [26]. Evidence suggests a role for Glrx1 in mouse models of allergic airways disease [12, 22], as well as in patients with asthma, wherein increases in airway Glrx1 have been demonstrated in the airways of asthmatics, and lower Glrx1 correlated with better lung function in these patients [13]. Findings from the present study suggest that absence of Glrx1 results in enhanced fibrotic airway remodeling in allergen-exposed mice. Furthermore, overexpression of Glrx1 can attenuate fibrotic airway remodeling, indicating a potential novel role of Glrx1 not yet described in the setting of allergic airways disease.

Previous data from our group indicated that Glrx1−/− mice in the Balb/C background exposed to the antigen ovalbumin (Ova) displayed enhanced resolution of
airway hyperresponsiveness (AHR) and attenuated mucus metaplasia, demonstrating that absence of Glrx1 may be beneficial in controlling certain aspects of the disease [12]. In the current study utilizing the relevant allergen HDM, we observed significantly higher AHR (parameters Rn and G) in Glrx1−/− mice and significant protection against the development of AHR in mice overexpressing Glrx1 in airway epithelial cells (Epi-Glrx1) (Fig. 2E and 4E), in comparison to the respective control groups. The apparent discrepancy with the previous study may be linked to the strain of mice used, as experimental animals used in this study were in the C57Bl/6 background, compared to mice in a Balb/C background used in the previous study. Furthermore, the model of allergic airways disease used herein, and the timing of analysis relative to the last challenge with antigen was also different and could have accounted for apparent differences with respect to the impact of Glrx1 on AHR.

Analysis of cell differentials indicated that Glrx1 may be involved in controlling influx of eosinophils and to a lesser extent, neutrophils. Loss of Glrx1 resulted in a trend towards fewer neutrophils that was not significant (Fig. 1C), whereas overexpression of Glrx1 did not affect neutrophils, but instead significantly decreased eosinophil recruitment to the lung (Fig. 3C). However assessment of the impact of Glrx1 expression on airway inflammation was confounded by the effects of Dox itself on HDM-induced inflammation, findings that are consistent with prior observations [27]. Unraveling the exact impact of epithelial Glrx1 on allergic airways inflammation therefore will necessitate additional analyses or alternative approaches to induce the Glrx1 transgene.
No information exists about Glrx1 and eosinophil biology, but a prior publication has shown that Glrx1 is important for the proper functioning of neutrophils by reducing dehydroascorbate to ascorbate, allowing the quenching of diffusible oxidants [28]. Interestingly, another study demonstrated that neutrophils isolated from Glrx1<sup>−/−</sup> mice demonstrated impaired chemotaxis, partially due to altered actin S-glutathionylation [29]. This resulted in fewer total neutrophils at the site of infection and impaired neutrophil function, findings that may indicate the possible impact on neutrophilia in the lungs of Glrx1<sup>−/−</sup> mice compared to WT animals, although these data did not reach significance (Fig. 1C). The potential ramifications of S-glutathionylation/Glrx1 for neutrophil and eosinophil function, and the function of other inflammatory cells in settings of allergic airway disease will require further investigation.

The most striking observation from the current study was the impact of Glrx1 manipulation on airway remodeling, particularly collagen production. HDM-exposed Glrx1<sup>−/−</sup> mice displayed significantly elevated lung collagen compared to WT mice (Fig. 2A, B & D), whereas HDM-exposed Epi-Glrx1 mice exhibited significantly lower collagen deposition compared to HDM-exposed Bitransgenic mice and rtTA mice (Fig. 4A & C). C57/BL6 mice are known to be more prone to developing fibrosis than are other strains, including Balb/C [30, 31], which is a plausible explanation for the observations in the present study. The role of Glrx1 in airways fibrosis was not previously reported, and our data demonstrates a critical role for Glrx1 in controlling fibrotic airway remodeling in response to allergen exposure. An imbalanced redox environment can impact the development of experimental fibrosis [32, 33] and has also
been observed in patients with fibrotic lung diseases, such as idiopathic pulmonary fibrosis [34-37]. To our knowledge, this is the first report of Glrx1 playing a role in fibrotic airway remodeling in response to allergen challenge and may suggest a new approach for targeted therapy.

Airway remodeling is believed to result from an imbalanced epithelial injury/repair response, ultimately resulting in subepithelial fibrosis. It is well-established that airway remodeling is a key marker of asthma severity, but recent studies also support a more central role for remodeling in the pathogenesis of asthma [38]. Further illustrating the importance of Glrx1 in allergic airways remodeling are the data showing decreased E-cadherin and increased αSMA in stimulated lung epithelial cells lacking Glrx1 (Fig. 5) or in Glrx1−/− mice (Fig. 2A and B). These phenotypic changes are consistent with those commonly observed in epithelial to mesenchymal transition (EMT). A role of Glrx1 in attenuating TGFβ-induced EMT in A549 cells has been previously demonstrated [39], consistent with the observations herein. Although the existence of EMT in asthma is still a highly debated topic [25, 40-43], our data indicate an important role for Glrx1 in controlling airway remodeling and epithelial cell integrity, as Glrx1 absence results in loss of epithelial markers, increased expression of mesenchymal markers and enhanced collagen production.

Treatments targeting established remodeling of the airways, particularly fibrosis, have been largely unsuccessful [44]. Early therapies for airway remodeling include treatment with the inhaled corticosteroid, flunisolide, which was shown to aid in reversing smooth muscle mass but had no efficacy in reversing established fibrosis [45].
Other drugs that are used to treat idiopathic pulmonary fibrosis with some success, such as pirfenidone [46], could possibly be repurposed for use in asthmatics who present with extensive airways fibrosis. In fact, early studies indicated that pirfenidone may aid in reversing established fibrosis in the lungs of asthmatics, although no further studies were performed [47]. The increases in understanding of how redox perturbations affect disease pathogenesis, which encompasses increased knowledge about sources of oxidants and their targets, have illuminated new potential avenues for treatment. Data from the present study suggest that Glrx1 may be a promising target for therapeutic intervention in settings of airways fibrosis associated with allergic asthma, in association with improved epithelial barrier function.
Figure Legends

**Figure 1:** Analysis of HDM-induced cellular influx in the lungs of WT and Glrx1\(^{-/-}\) mice. (A) Scheme depicting the HDM-exposure regimen for *in vivo* experiments with Glrx1\(^{-/-}\) mice. On days 0 and 7, mice were given 1ug (protein) HDM and on subsequent challenges beginning on day 14, mice were given 10ug (protein) HDM, indicated by arrows. Mice were euthanized on day 19, 24h following the last challenge. Mice (n = at least 8 mice/group) were exposed to PBS or HDM as indicated in (A) and BAL was assessed for total lung cell counts (B), macrophages, neutrophils, eosinophils and lymphocytes (C). (D) Western blot for Glrx1. *p≤0.05 (ANOVA) compared to respective PBS group.

**Figure 2:** Assessment of HDM-induced airway remodeling in Glrx1\(^{-/-}\) mice. HDM protocol described in Fig. 1A was followed and periodic acid Schiff (PAS; mucus), α smooth muscle actin (αSMA) and Masson Trichrome (MT; collagen) stainings on lung sections were performed (A). Scale bar, 50 μm. (B) Quantification of mucus metaplasia (PAS); αSMA positivity; and MT staining. mRNA expression of mucin gene Muc5AC (C). (D) Collagen content in the right superior lung lobe as measured by hydroxyproline assay. *p≤0.05 (ANOVA) WT HDM compared to respective PBS group; †p≤0.05 (ANOVA) compared to WT HDM group. (E) Assessment of AHR using forced oscillation in response to increasing doses of methacholine (12.5, 25, 50 mg/ml). Shown are parameters Rn (Newtonian resistance), G (tissue resistance) and H (Elastance). Statistics for (E), $p<0.05$ (Least square mean) WT HDM compared to WT PBS; *p≤0.05
(Least squares mean) \( \text{Glrx1}^{+/} \) HDM compared to \( \text{Glrx1}^{+-} \) PBS; †p≤0.05 (Least squares mean) \( \text{Glrx1}^{+/} \) HDM compared to WT HDM.

**Figure 3:** Analysis of HDM-induced cellular influx in the lungs of mice expressing Glrx1 in airway epithelial cells. (A) Scheme for Doxycycline (Dox) and HDM exposure regimen for experiments involving Glrx1 transgenic (Epi-\( \text{Glrx1} \)) mice. Mice were fed Dox-containing chow starting 7 days before the first HDM exposure and maintained on Dox chow for the duration of the experiment. Mice were given 1ug (protein) HDM on days 0 and 7, and 10ug (protein) HDM on days 14-19, and euthanized 24h after the last challenge. Mice (n = at least 10 mice/group) were exposed to PBS or HDM as indicated in (A) and BAL was collected and analyzed for total lung cell counts (B), macrophages, neutrophils, eosinophils and lymphocytes (C). (D) Western blot analysis of Glrx1 in homogenized lung tissue. *p≤0.05 (ANOVA) compared to respective PBS group; †p≤0.05 (ANOVA) compared to Epi-\( \text{Glrx1} \) HDM group (grey bar).

**Figure 4:** Assessment of HDM-induced airway remodeling in mice expressing Glrx1 in airway epithelial cells. Following HDM protocol in Fig. 3A, lung sections were stained as in Fig 1A for PAS, \( \alpha \text{SMA} \) and MT (A). Scale bar, 50 μm. (B) RNA was isolated from whole lung and analyzed for Muc5AC. (C) Collagen content in the lung as measured by hydroxyproline assay. *p≤0.05 (ANOVA) compared to respective PBS group; †p≤0.05 (ANOVA) compared to Bitransgenic -Dox HDM group (black bar); ‡p<0.05 (ANOVA) compared to Epi-\( \text{Glrx1} \) HDM group (grey bar). (D) Assessment of AHR using forced
oscillation in response to increasing doses of methacholine (12.5, 25, 50 mg/ml). Parameters Rn, G and H are shown as in Fig 2F. Statistics for (D), $p<0.05$ (Least square mean) Bitransgenic HDM compared to Bitransgenic PBS; *$p≤0.05$ (Least squares mean) Epi-GLrx1 HDM compared to Epi-GLrx1 PBS; †$p≤0.05$ (Least squares mean) Epi-GLrx1 HDM compared to Bitransgenic HDM; #$p<0.05$ (Least squares mean) rtTA HDM compared to Epi-GLrx HDM.

Figure 5: Evaluation of E-cadherin in lung tissue and epithelial cells lacking Glrx1. Analysis of E-cadherin mRNA expression (A) and protein content (B) from the lungs of WT and Glrx1−/− mice following HDM protocol in Fig. 1A. WLL: whole lung lysates. (C) WT and Glrx1−/− MTECs were stimulated with 50μg/ml HDM (protein) on days 1, 3 & 5 and cells were harvested on day 7 for Western blot analysis for E-cadherin. Cyto, cytosolic lysates. (D) WT and Glrx1−/− MTECs were stimulated with 5ng/ml TGFβ for 72h and whole cell lysates (WCL) were harvested for analysis of αSMA content. (F) C10 lung epithelial cells were transfected with scrambled (Scr) or Glrx1 siRNA and subsequently stimulated with TGFβ for 24h, after which WCL were harvested for analysis of αSMA via Western blot. *$p≤0.05$ (ANOVA), compared to PBS controls; ns, not significant.

Figure S1: Schematic of the CCSP-rtTA and TetO-FLAG-Glrx1 constructs. In lung epithelial cells expressing Club cell secretory protein (CCSP), rtTA is constitutively expressed under the CCSP promoter. In the presence of Doxycycline (Dox), the rtTA
protein can bind to the Tet-Operon (Tet-O), driving expression of the \textit{FLAG-Grx1} transgene (bottom). In the absence of Dox, rtTA is unable to bind the Tet-O, thus preventing the expression of the \textit{FLAG-Grx1} transgene (top).
Figures

Figure 1:
Figure 2:

A. Histological staining of lung sections from WT, Glrx1−/−, and Glrx1−/− mice exposed to PBS or HDM. PAS, cSMA, and MT staining were used to evaluate changes in lung structure.

B. Bar graphs showing the quantitation of PAS, αSMA, and MT staining scores in WT and Glrx1−/− mice exposed to PBS or HDM.

C. Graphs showing relative expression of Muc5AC in WT and Glrx1−/− mice exposed to PBS or HDM.

D. Graphs showing collagen content in WT and Glrx1−/− mice exposed to PBS or HDM.

E. Graphs showing the effect of MCh on Rn, G, and H in WT, WT PBS, Glrx1−/− PBS, Glrx1−/− HDM mice.
Figure 3:

A.

B.

C.

D.

![Bar charts and graphs showing data for different cell types under various conditions.](image-url)
Figure 4:
Figure 5:
Figure S1:
References


of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-beta1. *Am J Respir Crit Care Med* **180**:122-133; 2009.


CHAPTER 4

SUMMARY AND CONCLUDING REMARKS

Summary of Intents

Prior work by our group has provided insight into the pathogenesis of asthma by investigating the role of an imbalanced redox environment in settings of allergic airway disease [1-4]. Through these studies, glutaredoxin-1 (Glrx1) has emerged as a crucial player in disease pathogenesis by regulating inflammation, airway hyperresponsiveness (AHR) and protein S-glutathionylation in mouse models of disease. In addition, in-depth studies evaluating the impact of Glrx1 in airway epithelial cells have illuminated an important role for Glrx1 in controlling NF-κB activation and subsequent inflammation, in part by controlling the S-glutathionylation of the upstream NF-κB kinase, IKKβ [5]. Despite these findings, little was known regarding the function of Glrx1 in cytokine-induced NF-κB activation, downstream gene induction and S-glutathionylation of other NF-κB family members in airway epithelial cells. We therefore set out to examine airway epithelial cell responses to interleukin-17A (IL-17A), a cytokine commonly upregulated in the lungs of asthmatic patients, following manipulation of Glrx1 content to determine downstream impacts on NF-κB-dependent inflammatory cytokine production and glutathionylation of NF-κB family members (Chapter 2). Additionally, absence of Glrx1 has been shown to decrease inflammation and enhance resolution of AHR in vivo [2], but to date minimal data exists regarding the extent of airway remodeling and fibrosis following chronic allergen exposure in settings of Glrx1 absence or overexpression. As a result, we next focused on house dust mite (HDM)-induced allergic airway disease in
mice globally lacking Glrx1 \((\text{Glrx1}^{-/-})\) or in mice inducibly overexpressing \(\text{Glrx1}\) specifically in CCSP positive airway epithelial cells (Epi-\(\text{Glrx1}\)) in order to determine impacts on AHR, inflammation and markers of airway remodeling (Chapter 3).

**Glrx1 Control of Inflammation in Lung Epithelial Cells**

Results presented in this thesis expand upon the role of glutaredoxin-1 (Glrx1) in controlling NF-κB activation and downstream pro-inflammatory gene induction in lung epithelial cells. We demonstrate a novel facet of IL-17A signaling associated with cysteine oxidation and protein S-glutathionylation (PSSG), offering new insights into the complex network of signaling events leading to inflammation (Ch. 4, Fig. 1 & 2). Previous work by our group has established that stimulation of epithelial cells with the oxidant, hydrogen peroxide (\(\text{H}_2\text{O}_2\)), results in inhibition of the canonical NF-κB pathway via reversible oxidation and S-glutathionylation of a critical cysteine in IKKβ (IKKβ-SSG) [5]. Importantly, IKKβ-SSG can be reversed by Glrx1, effectively restoring IKKβ kinase function and downstream canonical NF-κB activation [5]. An additional study performed by our group showed that overexpression of Glrx1 enhances lipopolysaccharide (LPS)-induced NF-κB activity in lung epithelial cells in part by deglutathionylating IKKβ [6], consistent with previous findings. The latter study also demonstrated a novel feedback loop in which NF-κB positively regulates Glrx1 expression, illustrating a redox-dependent feedback loop that enhances NF-κB signaling. In this thesis, a major goal was to establish a role for Glrx1 in controlling lung epithelial cell responses to cytokines relevant to asthma. For this reason, we utilized IL-17A, a
potent pro-inflammatory cytokine frequently observed in asthmatic airways that activates NF-κB in lung epithelial cells, resulting in heightened production of pro-inflammatory mediators and host defense proteins [7-12].

The results presented in this thesis suggest that lung epithelial cell responses to IL-17A are governed in part by Glrx1, as absence of Glrx1 altered NF-κB nuclear content and downstream gene induction. Furthermore, stimulation of lung epithelial cells with IL-17A resulted in increased oxidative stress and S-glutathionylation of NF-κB proteins, the latter of which was mediated by Glrx1. For the first time, we demonstrated that IL-17A stimulation results in increased oxidative modifications of reactive cysteines, including sulfenic acid (SOH) formation (Ch. 2, Fig. 2) and S-glutathionylation (Ch. 2, Fig. 3). As shown in previous work by our group [5, 6], these data also suggest that Glrx1 mediates the extent of S-glutathionylation of NF-κB proteins, namely IL-17A-induced RelA-SSG and IKKα-SSG (Ch. 2, Fig. 6), although IKKβ-SSG was not consistently detected in response to IL-17A. It should be noted that there are limitations to the detection of S-glutathionylated proteins. Therefore, utilization of various complementary techniques in the future, including mass spectrometry, may help identify S-glutathionylated proteins that could be exploited as therapeutic targets. IL-17A can activate various downstream pathways, including p38 MAPK, ERK and NF-κB; to maintain focus, our studies focused on the impact of Glrx1 on IL-17A-induced activation of NF-κB. IL-17A-induced activation of NF-κB in the lung epithelium has previously been demonstrated, but until now, a role for Glrx1 in this process was not known (Ch. 2, Fig. 4 & 5). As described in the previous section, the major function of Glrx1 is deglutathionylation of target proteins,
a function supported by the findings of this thesis. Other functions of Glrx1 may exist, however, and these will be discussed later.

IKKα is known to play a role in regulating IL-17A responses in various cell types [13], as well as translocating to the nucleus in T-cells and stabilizing the Th17 phenotype by promoting activation of the il17a gene, independent of its role in NF-κB signaling [14]. Our findings demonstrate a clear role for IKKα in contributing to IL-17A induced inflammation in lung epithelial cells, as knockdown of IKKα results in blunted inflammatory responses (Ch. 2, Fig. 7). Furthermore, IKKα was shown to be a target of Glrx1-dependent de-glutathionylation following IL-17A exposure, a process that likely inhibits its kinase function. This is a tentative conclusion based on existing data regarding its close relative IKKβ, although S-glutathionylation of IKKα may also have an impact on its nuclear function, independent of NF-κB. Previous work in our laboratory revealed that cysteine-179 of IKKβ is the target for inhibitory S-glutathionylation, as mutating this residue to alanine no longer resulted in IKKβ-SSG [5]. Given this knowledge, as well as the sequence similarity between IKKα and IKKβ, we sought to determine whether the homologous cysteine residue in IKKα, cysteine-178, is the target for S-glutathionylation. Surprisingly, mutation of this cysteine to alanine did not diminish IKKα-SSG, but instead resulted in increased IKKα-SSG (data not shown). Further studies are needed to determine the S-glutathionylated cysteine in IKKα and the precise impact of IKKα-SSG as this may represent a promising target in multiple cell types. Investigation of IKKα nuclear content in cells and lung tissues could aid in determining its subcellular localization following exposure to various stimuli, including IL-17A. Additionally,
analysis of potential interactions between IKKα and transcription factors or possible DNA binding analysis via ChIP or EMSA may lead to more in-depth understanding of the precise function of IKKα.

Our *in vitro* findings link two important features of asthma: an altered redox environment, with a specific role for Glrx1 and IL-17A-induced pro-inflammatory signaling in epithelial cells. Loss of Glrx1 in lung epithelial cells dampens IL-17A-induced expression of the neutrophil chemoattractant, KC. Given the strong evidence supporting an altered redox environment in asthma, and the specific role of Glrx1 in lung epithelial cells, these data represent a possible therapeutic avenue not yet exploited in asthma. Due to the heterogeneous effects of oxidants on proteins, targeting specific proteins known to contribute to disease pathogenesis may be a more appealing option compared to broad-spectrum approaches which alter the global redox balance of the lung. To further evaluate the possibility that Glrx1 may indeed be one of these potential targets, we performed *in vivo* studies to assess the effects of manipulating Glrx1 in an allergen model of allergic airway disease.

*Glrx1 Manipulation in HDM-Induced Allergic Airway Disease*

Glrx1 has been shown to be increased *in vivo* in response to challenge with ovalbumin (Ova) [1] likely to counterbalance the increased oxidative stress encountered in allergen-induced inflammation. Furthermore, Balb/C mice lacking Glrx1 displayed improved respiratory mechanics compared to WT counterparts in response to Ova, while clinical data showed that airway function in human asthmatics negatively correlated with
Glrx1 content in the lung [2, 15]. These data indicate that less Glrx1 in the lung appears to be beneficial in settings of allergic airway diseases, including asthma. The limitations of studies conducted in animal models to date are multifactorial. Due in large part to the use of Ova as the allergen (which has limited relevance to asthma) and the atypical route of immunization (intraperitoneally), limited comparisons can be made to human disease. Alternatively, in vivo studies in this thesis utilize house dust mite (HDM), a common allergen to which approximately 80-85% of asthmatics are allergic [16]. The route of sensitization in these models is directly via the airways, simulating the events thought to occur in the development of asthma. The physiologically relevant initiation of allergic airway disease has allowed for more pertinent comparisons to human disease, although limitations still exist.

Due to the lack of data regarding Glrx1 manipulation in a model of allergic airway disease utilizing a physiologically-relevant allergen, a major goal of this thesis was to investigate the impact of Glrx1 ablation or overexpression on disease pathogenesis in response to HDM. In contrast to previous studies, we did not observe improved airway function in Glrx1−/− mice; in fact, we observed significant increases in Rn in Glrx1−/− mice (Ch. 3, Fig. 2). We also observed decreased neutrophils in Glrx1−/− mice (Ch. 3, Fig. 1), contrasting with data obtained from Glrx1−/− mice on the Balb/C background exposed to Ova. It is important to note that all mice used in these experiments were on the C57/BL6 background, so direct comparisons to previous experiments which utilized Balb/C mice are tentative at best. Strain-dependent differences in various settings, including inflammation, have been explored, including one such study by our group which
demonstrated profound differences in NF-κB activation in allergic airway inflammation [17]. Specifically, Balb/C mice showed elevated production of TNFα, driving NF-κB activation, a finding not mirrored in C57/BL6 mice [17]. This may offer a possible explanation for the results shown here, although further investigation is required to determine precise strain-specific effects of Glrx1. As a converse approach, we utilized mice overexpressing Glrx1 specifically in CCSP-positive lung epithelial cells when fed doxycycline (Dox)-containing chow. Overexpression of Glrx1 appeared to decrease eosinophils in response to HDM, but did not significantly affect the influx of other cell types to the lung (Ch. 3, Fig. 3). There was an apparent effect of Dox itself on neutrophil and eosinophil counts (Ch. 3, Fig. 3) and respiratory mechanics (Ch. 3, Fig. 4), which is a possible confounding factor in the interpretation of these data. A shorter timeframe of Dox administration or lower doses of Dox can be considered in future experiments in order to overcome these limitations. In addition, other models of targeted overexpression can be considered that do not rely on the administration of Dox or other compounds with potential off-target effects could be utilized in future studies.

Due to the mixed Th2/Th17 response often observed in settings of HDM-induced allergic airway disease, as well as our previous study evaluating epithelial cell responses to IL-17A, we evaluated the extent of IL-17A production in our studies. Surprisingly, we did not observe any increases in IL-17A gene expression or protein content in the lungs of HDM-exposed mice (data not shown). Furthermore, there were no differences in IL-17A mRNA or protein that were dependent upon Glrx1 content in these mice, as absence or overexpression of Glrx1 had no additional effect on IL-17A production in the lungs.
These data may indicate that although Glrx1 status in lung epithelial cells has an apparent role in controlling IL-17A driven responses, the production of IL-17A following HDM exposure *in vivo* may not be dependent upon Glrx1 status. This still remains unclear, and further experiments are required to address this issue. It is also important to note that several factors, including the time course of the experiment or the dose of HDM, the genetic background of the mice or the lack of a mixed Th2/Th17 response in this particular model, could offer a possible explanation for our results. Further investigation of the importance of Glrx1 status in other cell types involved in HDM-driven disease, particularly in C57/BL6 mice, will aid in addressing these questions.

The most striking impact in these experiments was on fibrotic airway remodeling following HDM exposure (Ch. 4, Fig. 3 & 4). Here, we observed a clear role for overexpression of Glrx1 in preventing the development of collagen deposition, effectively limiting subepithelial fibrosis (Ch. 3, Fig. 4), whereas a global lack of Glrx1 resulted in enhanced fibrotic airway remodeling (Ch. 3, Fig. 2). These data provide a promising new therapeutic target, Glrx1, for fibrotic remodeling associated with allergic asthma. It is clear that Glrx1 overexpression aids in preventing collagen deposition; however, these studies did not address the impact of increased Glrx1 expression on established disease. Glrx1 is primarily a cytosolic enzyme, but findings also suggest that it can traffic to both the mitochondria and the nucleus [18, 19], as well as be secreted [20], adding another layer of complexity in trying to determine the specific function of Glrx1 in allergic airway disease. The function of extracellular Glrx1 still remains unknown, but given the extracellular function of other redox enzymes, such as QSOX
it is possible that Glrx1 may also be involved in modifying extracellular protein targets.

Future studies are necessary to determine the efficacy of Glrx1 in not only preventing fibrosis, but more importantly reversing established disease. Results from these studies may also have implications for other fibrotic lung diseases, including idiopathic pulmonary fibrosis, the pathogenesis of which is very poorly understood. Some treatments for idiopathic pulmonary fibrosis now exist (pirfenidone and nintedanib), but the limited efficacy of these drugs warrants the development of alternative strategies to halt disease progression, or even reverse existing disease [22]. If proven effective in reversing established fibrosis in animal models, Glrx1 may offer the opportunity for clinicians to use an endogenously occurring therapeutic for lung fibrosis, circumventing the toxic effects commonly seen with xenobiotic compounds and related metabolites.

Evidence of airway remodeling following HDM exposure was observed following analysis of E-cadherin, an epithelial cell marker (Ch. 3, Fig. 5). Surprisingly, both E-cadherin mRNA and protein were markedly decreased in Glrx1−/− mice exposed to PBS. Epithelial cells from Glrx1−/− mice also demonstrated baseline decreases in E-cadherin and enhanced responsiveness to TGFβ as evidenced by upregulation of the mesenchymal marker αSMA. Lung epithelial cells from Glrx1−/− mice appear to be predisposed to a more mesenchymal phenotype, potentially contributing to the extensive collagen deposition observed in the lungs of these mice (Ch. 3, Fig. 2). Because these mice globally lack Glrx1, it is likely that the absence of Glrx1 in other cell types, such as
neutrophils or macrophages, also has a substantial impact on the outcomes measured. Absence of Glrx1 in neutrophils impairs migration [23], a possible explanation for the decrease in neutrophils observed in HDM-exposed Glrx1−/− mice. Our group has also demonstrated that Glrx1 content is increased in macrophages in response to LPS and macrophages from Glrx1−/− mice are smaller, less abundant and display impaired NF-κB function [24]. Future studies assessing the importance of Glrx1 in various cell types in settings of HDM-induced allergic airway disease may aid in revealing cell-specific functions of Glrx1 in this model.

Despite these in vivo data, very little evidence supporting a role for Glrx1 in human manifestations of the disease exist, likely due to the fact that its function in the lung is still not well understood. Our studies utilizing global Glrx1−/− mice present some limitations and there are likely compensatory mechanisms involving other oxidoreductase enzymes. A more ideal system would involve mice expressing a LoxP-flanked Glrx1 gene which could be crossed with a mouse expressing CCSP-Cre to specifically delete the Glrx1 gene in lung epithelial cells. Further refining of this system could also allow for tissue-specific, inducible deletion of the Glrx1 gene as well, similar to the tissue-specific, inducible overexpressing Glrx1 mouse used in this thesis (Epi-Glrx1). Despite emerging data supporting a role for Glrx1 in mouse models of allergic airway disease, very little clinical data exists regarding Glrx1. The only evidence of Glrx1 alterations in human asthma show that less Glrx1 in sputum, and increased S-glutathionylation correlates with improved airway function [15], but the underlying mechanism involved in this functional impact is still unknown. To date, no association between Glrx1 and airway remodeling
has been evaluated in human patients and more in depth studies are required to fully determine the clinical impact of possible mutations or absence of Glrx1.

Concluding Remarks

The role of oxidants and the subsequent impact on disease pathogenesis is a complex process. Cysteine oxidations, including S-glutathionylation, are emerging as critical mediators of protein function in a variety of diseases, including asthma. Glrx1 is a deglutathionylating enzyme that has been associated with asthma, but its exact role in the development of asthma is still not well understood. The findings presented in this thesis demonstrate a novel role for Glrx1 in controlling IL-17A-induced inflammation and S-glutathionylation of NF-κB proteins, RelA and IKKα in lung epithelial cells, pointing to a central role for epithelial Glrx1 in controlling inflammation. In addition, a role for IKKα in controlling IL-17A responses was demonstrated for the first time in lung epithelial cells. Furthermore, in vivo data from this thesis represent a possible role for increased Glrx1 expression in preventing the development of HDM-induced fibrosis. These findings prompt future studies to determine if increased expression of Glrx1 is effective at not only preventing fibrosis, but more importantly, reversing established disease. Finally, although the exact mechanism behind these findings remains to be fully explained, Glrx1 may be a viable option for therapy in fibrotic lung diseases beyond settings of asthma.
Figure Legends

Figure 1: IL-17A-induced inflammation and S-glutathionylation in lung epithelial cells. IL-17A stimulation of lung epithelial cells leads to increased activation of NF-κB and expression of pro-inflammatory genes. IL-17A also causes increased oxidative stress, resulting in elevated levels of oxidized and S-glutathionylated proteins in lung epithelial cells. In particular, NF-κB family members RelA and IKKα are targets for IL-17A-induced protein S-glutathionylation, resulting in altered NF-κB-dependent pro-inflammatory cytokine production. RelA-SSG and IKKα-SSG can be reversed by Glrx1, restoring cysteines back to their native thiol state (SH) and promoting activation of NF-κB.

Figure 2: Summary of findings from IL-17A-stimulation in lung epithelial cells.

Figure 3: Impact of Glrx1 status on allergic airway remodeling. In response to HDM, the airway epithelium produces cytokines and chemokines that recruit and activate inflammatory and immune cells, such as neutrophils, eosinophils, T-cells and dendritic cells (DC). Dendritic cells also present antigens to naïve T-cells, further enhancing the immune response. Production of growth factors, cytokines and other compounds, in part by the lung epithelium, contributes to airway remodeling as measured by increased smooth muscle mass, mucus metaplasia, increased collagen deposition and vascular remodeling. In Glrx1−/− mice, allergic airway remodeling is enhanced, compared to Epi-Glrx1 mice which are partially protected from extensive allergic airway remodeling.
Figure 4: Summary of findings in Glrx1−/− mice and Glrx1 transgenic mice.

Figure 5: Implications and future directions. Implications of the impact of findings from this thesis, as well as future directions for this project.
Figures

Figure 1:
**Figure 2:**

**SUMMARY I**

- Stimulation of lung epithelial cells with IL-17A leads to rapid cysteine oxidation and S-glutathionylation of RelA and IKKα.

- Glrx1 controls IL-17A-induced S-glutathionylation of RelA and IKKα.

- IL-17A-induced expression of pro-inflammatory genes is altered following siRNA-mediated Glrx1 ablation and in Glrx1<sup>−/−</sup> primary tracheal epithelial cells.

- siRNA-mediated ablation of IKKα dampens downstream pro-inflammatory gene induction.

*These findings demonstrate a novel role for the S-glutathionylation/Glrx1 redox axis in controlling RelA-SSG, IKKα-SSG and epithelial cell responses to IL-17A.*
Summary II

- *Girx1<sup>−/−</sup> mice display enhanced mucus production, αSMA content and significantly increased collagen deposition compared to WT mice

- Epi-*Girx1* mice display significantly decreased collagen deposition compared to Bitransgenic mice not given Dox and rtTA control mice

These findings indicate a central role for *Girx1* in controlling the extent of allergen-induced fibrotic airway remodeling
Figure 5:

**Implications and Future Directions**

- Our cell culture results implicate a role for Gtx1 in controlling IL-17A-induced pro-inflammatory gene expression and S-glutathionylation of RelA and IKKα, as well as a role for IKKα in driving IL-17A-induced pro-inflammatory signaling in lung epithelial cells.

- Findings in mouse models suggest a possible role for increased Gtx1 protein in preventing the development of HDM-induced fibrosis.

- Future studies in which (1) the Gtx1 transgene is turned on after established disease or (2) Gtx1 protein is given therapeutically are needed to determine if increased expression of Gtx1 can reverse established fibrosis.

- Finally, although the exact mechanism behind these findings remains to be fully explained, Gtx1 may be a viable option for therapy in fibrotic lung diseases beyond settings of asthma.
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