Protein thiol oxidoreductas and allergic airways disease

CHAPTER 6

Summary and Future Directions

Endoplasmic Reticulum Stress in Allergic Airways Disease

Allergic asthma is characterized by airways inflammation, mucus metaplasia and peri- bronchiolar fibrosis, impacting lung structure and function [1, 2]. Airway epithelial cells (AECs) reside at the intersection of the lung and the external environment [3], and recent studies have demonstrated that activation of a number of receptors on the surface of AECs and subsequent secretion of various mediators are required for responses from dendritic cells (DCs) and subsequent immune responses [1, 4-6]. House Dust Mite (HDM) contains numerous antigens, proteases and ligands for Pattern Recognition Receptors (PRRs) resulting in activation of airway epithelial cells, and inducing the secretion of growth factors and cytokines that regulate subsequent activation of innate lymphoid cells, T cells, mucus metaplasia, inflammation, airways hyperresponsiveness (AHR), and fibrosis [7-9]. As discussed in Chapter 1, during allergen stimuli a demand for increases in protein synthesis and folding (eg. cytokines or mucus production) can create an imbalance in the endoplasmic reticulum (ER). This leads to an increase in misfolded proteins in the ER, causing ER stress and initiating the UPR signaling [10].

Studies thus far from our laboratory and others have shown that ER stress-dependent activation of transcription factors XBP1 and ATF6α are required during mucus metaplasia and pro-inflammatory responses respectively, in ovalbumin or HDM-induced allergic airways disease [11, 12]. As discussed in Chapter 2, both cleavage product of ATF6α and ERp57 protein are up regulated in murine and human epithelial cells in association with caspase-3 activation, following HDM exposure. siRNA-mediated knockdown of ATF6α and ERp57 during HDM administration in mice resulted in a decrease in components of HDM-induced ER stress, disulfide mediated oligomerization of Bak, and activation of caspase-3. However, while siRNA also led to decreased inflammation, airway hyperresponsiveness and airway fibrosis, the impact of UPR-mediated induction of ATF6α or ERp57 specifically has not been characterized in the development of allergic asthma. Furthermore, it is not clear whether allergen-induced ATF6α or ERp57 is directly linked to multiple facets of asthma such as inflammation, apoptosis, peri-bronchiolar fibrosis and impairment in respiratory mechanics.

Substantial evidence suggests that the persistence of asthma is driven by ongoing immune responses that generate mediators driving airway remodeling and airway dysfunction. As has been discussed, the epithelium is both a site of production of these mediators and a source of cells that respond to mediators produced by immune cells and other cells within the airway, and understanding how epithelial cells recognize and respond to allergens and other stimuli is critical. Our laboratory has recently embarked on the projects utilizing ATF6α systemic knock out mice and a doxycyline (Dox)

inducible triple transgenic *CCSP-rTetA/TetO-Cre/ERp57loxp/loxp* (Δ*Epi-ERp57*) mouse to delete ERp57 specifically in lung epithelial cells, to determine whether deletion of ATF6α or ERp57 in mice or specifically in airway epithelial cells respectively attenuates pathophysiology associated with HDM challenge. Being a protein disulfide isomerase with specificity towards glycoproteins [13] (eg. cytokines and growth factors), it can be hypothesized that ERp57 could be involved in formation of disulfide bonds (-S-S-) in epithelial derived cytokine and chemokine production, affecting innate and adaptive immune responses following HDM exposure. Unraveling the pro-inflammatory mediators that are targets of ERp57 represents a major goal of future investigation.

Glutaredoxin-1 and OVA-induced Allergic Airways Disease

Glutaradoxin-1 (Grx1), an oxidoreductase responsible for protein deglutathionylation, is primarily expressed in the lung epithelium and in alveolar macrophages [14, 15]. Alterations in Grx1 expression have been associated with inflammatory diseases of the lung such as COPD and asthma [16, 17], and studies from our laboratory found significantly elevated levels of Grx1 within the airways of mice following OVA immunization and challenge [18]. To further elucidate the function of Grx1 in allergic airway disease, we determined the effects of ablation of the glutaredoxin-1 gene (*Glrx1*$^{-/-}$) in the OVA model. In response to OVA sensitization and challenge, *Glrx1*$^{-/-}$ mice displayed decreased eosinophilic inflammation, mucus metaplasia, as well as enhanced resolution of airway hyperresponsiveness to methacholine. *Glrx1*$^{-/-}$ mice also exhibited increased protein *S*-glutathionylation in the lung [19] following repeated antigen challenge. As discussed in Chapter 1, a study by Kuipers, *et al.,* reported decreased lung function associated with higher sputum Grx1 levels and lower PSSG levels [17], indicating that the Grx1/PSSG redox module may regulate AHR in allergic airways disease. While increases in overall protein *S*-glutathionylation were seen in the lungs of *Glrx1*$^{-/-}$ mice exposed to OVA, the targets of *S*-glutathionylation remain unidentified. Future studies utilizing antibodies that target *S*-glutathionylated proteins or strategies to selectively reduce and label *S*-glutathionylated proteins [20] coupled with mass spectrometry will be required to identify these targets.

While the OVA model is widely used to study allergic airways disease, controversy exists regarding its physiological relevance. Both ovalbumin and the adjuvant, alum, are administered intraperitoneally in this model, but asthmatic patients are believed to be sensitized via the airway. Our laboratory therefore sought to utilize an alternative and perhaps more clinically relevant model of

allergic airways disease to further study protein *S*-glutathionylation and Grx1 in the context of allergic airways disease, as described in Chapter 3.

Epithelial NF-κB in HDM-Induced Allergic Airways Disease

House dust mite (HDM) is a multifaceted allergen to which 50-85% of asthmatics are allergic [7]. HDM extract contains many components including fecal matter, LPS, chitin, and proteins such as Derp1, all of which are hypothesized to be involved in the allergic response. Previous studies with HDM demonstrated strong hyperresponsiveness to methacholine, inflammation, mucus metaplasia, and fibrotic remodeling in the mouse lung [21]. Additionally, HDM-induced allergic airway disease in mice via TLR4 triggering of airway structural cells, as well as production of pro-inflammatory cytokines TSLP, GM-CSF, IL-25, and IL-33 [4]. Utilization of the HDM model has enabled the investigation of early innate immune responses in the lung which are thought to contribute to the development of allergic airway disease.

Nuclear Factor-κB (NF-κB) is a family of transcription factors involved in the regulation of pro-survival, pro-inflammatory, and immune pathways. Dysregulation of NF-κB has been linked to a variety of chronic inflammatory diseases including cancer, sepsis, and asthma. NF-κB activity is elevated in lung epithelial cells of asthmatic patients in comparison to healthy controls [22], and activation of classical NF-κB in the lung epithelium is both sufficient and necessary to regulate allergic airway inflammation in mice [6, 23, 24]. The data presented in Chapter 3 demonstrate that HDM administration via the airways induces activation of both classical and alternative NF-κB, and that NF-κB inhibition in the epithelium protects against HDM-induced inflammation, hyperresponsiveness, and remodeling. Additionally, inhibition of NF-κB decreased the expression of IL-33, a cytokine known to promote T_H2 differentiation activation of both the NF-κB and MAP Kinase pathways. Increased IL-33 expression has been demonstrated in the lungs of patients refractory to classic anti-inflammatory treatments, highlighting the potential therapeutic relevance of epithelial driven allergic airways disease.

As previously mentioned in Chapter 2, both classical and alternative NF-κB are inhibited in the epithelium following expression of the NF-κB_{SR} transgene. Given the knowledge that NF-κB Rel proteins bind to ankyrin repeat domains on IκB's, this was is not surprising. Further experiments were necessary to define the independent contributions of the classical and alternative pathways in HDM-driven allergic disease. Utilizing RelB siRNA, we demonstrated that attenuation of the alternative pathway conveyed partial protection against HDM-induced pro-inflammatory cytokine expression. siRNA administration is

not specific to the epithelium, however, and future studies might involve specific genetic models to further address the role of alternative NF-κB in the lung epithelium. Crossing a NIK/IKKα/RelB$^{flox/flox}$ mouse with the CC10-rtta tetOP/Cre mouse, for example, might help clarify how activation of alternative NF-κB within epithelial cells regulates HDM-driven allergic airways disease.

Oxidant-induced post-translational modifications strongly influence NF-κB signaling, and key enzymes such as Grx1 control the extent of cysteine oxidation. NF-κB proteins are targets for *S*-glutathionylation in part via Grx1 [25], so mice which either inducibly overexpress Grx1 in CC10 positive epithelial cells, or which harbor a floxed Grx1 allele, could be used to selectively decrease Grx1 expression in the airway epithelium. These experiments would address the epithelial cell-specific effects of Grx1 for NF-kB activation, NF-κB driven allergic inflammation, and subsequent downstream effects such as inflammation, mucus production and AHR. Studies using chimeric mice in which wild-type and *Glrx1*-deficient animals are irradiated and their bone marrow repopulated with marrow of the opposite genotype could also demonstrate whether Grx1 expressed in structural cells of the lung, including epithelial cells, plays a role in allergic disease, and further identify potential targets for PSSG in epithelial cells and redox-based regulation in the orchestration of inflammatory responses.

Glutaredoxin-1 and T_H17 Driven Allergic Asthma

Given the beneficial effects of global Grx1 knockout on mucus metaplasia and resolution of airway hyperresponsiveness observed in Chapter 2, and the role of NF-κB in HDM-induced allergic airways disease discussed in chapter 3, our group next investigated the role of Grx1 and protein *S*-glutathionylation in the pathophysiology of HDM-driven allergic airways disease. WT and *Glrx1*$^{-/-}$ mice were challenged intranasally with 50μg of HDM five times per week for three consecutive weeks. 72h following the final challenge, WT mice exhibited prominent T_H2-type inflammation, increased mucus metaplasia and AHR. Repeated HDM exposure increased Grx1 expression in the whole lung, and led to robust increases in PSSG in WT mice. Although we have not yet investigated the role of other deglutathionylating enzymes like Srx, it is clear that the level to which HDM induced PSSG could still not be overcome by endogenous antioxidants and reducing enzymes. As expected, in the absence of Grx1, overall PSSG was further increased as compared to WT mice. Analytical detection and quantification of glutathionylated proteins has been made easier by advances in proteomics and mass spectrometry. The results from Fig. 1C show prominent glutathionylation of proteins in the lung ranging from 100-150kDa, and mass spectrometry may be used to identify these proteins and provide further insight into the role of oxidative modifications in the pathogenesis of allergic airways disease.

One of the most striking results from this study was the apparent shift away from the T_H2-type response seen in WT mice towards a T_H17-like phenotype in the absence of *Glrx1*. Neutrophils were significantly elevated in *Glrx1*$^{-/-}$ mice in response to HDM challenge as compared to WT mice, while eosinophils were markedly decreased. Moreover, IL-17A mRNA expression in the whole lung as well as cytokine production from single lung cell suspensions was increased in the absence of *Glrx1*, while IL-5 production in the lung was significantly dampened. Severe asthma differs from mild or moderate persistant asthma, as it is characterized by neutrophilic inflammation either in the presence or absence of classical T_H2-induced eosinophilic inflammation. Additionally, T_H17 cells, which mediate neutrophil recruitment, are thought to have an influential role in asthma pathogenesis, especially in asthmatics who do not respond to glucocorticoid therapy [26]. Neutrophils are known to be largely steroid-insensitive and glucocorticoids (GC) inhibit neutrophil apoptosis [27] however, it is still unclear which cellular and molecular mechanisms contribute to this steroid insensitivity. Apart from T_H17 cells, multiple cell types such as macrophages, γδ T cells, as well as CD1d-restricted (i)NKT and innate lymphoid 3 cells (ILC3s) have previously been shown to be important sources of IL-17 in the lung [28-30], and future experiments will attempt to identify the cellular source of IL-17 in response to HDM in *Glrx1*$^{-/-}$ mice. Furthermore, IL-17A has been shown to drive AHR in mice [31] and although hyperresponsiveness was dampened in the distal airways of *Glrx1*$^{-/-}$ mice, central airway resistance (R_N) was increased to a magnitude similar to HDM-challenged WT mice. An IL-17A neutralizing antibody may therefore provide insight into the functional role of IL-17A in the absence of Grx1. If IL-17A does in fact play a significant role in HDM-induced allergic airways inflammation and AHR in *Glrx1*$^{-/-}$ mice, it would be interesting to address whether or not Grx1 ablation confers steroid insensivity, thus potentially illuminating a novel mechanism by which Grx1 and PSSG regulates disease type and severity. Interestingly, IL-17A is known to enhance chemotaxis of neutrophils by inducing IL-8 production in human bronchial epithelial cells [32] and airway smooth muscle cells [33]. Therefore, additional *in vitro* experiments might pinpoint a role for Grx1 in T_H17-mediated glucocorticoid-insensitive inflammation.

Finally, as previously mentioned, *Glrx1*$^{-/-}$ mice demonstrated signifiantly decreased tissue resistance (G) and elastance (H), as well as decreased Muc5ac mRNA expression compared to HDM-treated WT mice. These data, combined with results described in Chapter 2, highlight a potential functional role for Grx1 and PSSG in regulating mechanisms underlying airway hyperreactivity. Further immunohistological evaluation of the small and large airways may reveal regional variations in Grx1 expression and PSSG reactivity, and the predominant cell type(s) in which Grx1 is expressed. All

together, these experiments may implicate a potential role for Grx1 and protein thiol oxidation, notably *S*-glutathionylation, in the pathogenesis of allergic asthma.