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Protein Disulfide Isomerase-ERp57 regulates allergen-induced airways inflammation, fibrosis and hyperresponsiveness

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Abstract

Background—Evidence for association between asthma and the unfolded protein response (UPR) is emerging. ERp57 is an ER localized redox chaperone involved in folding and secretion of glycoproteins. We have previously demonstrated that ERp57 is up regulated in allergen-challenged human and murine lung epithelial cells. However, the role of ERp57 in asthma pathophysiology is unknown.

Objectives—Here, we sought to examine the contribution of airway epithelial-specific ERp57 in the pathogenesis of allergic asthma.

Methods—We examined the expression of ERp57 in human asthmatic airway epithelium and utilized murine models of allergic asthma to evaluate the relevance of epithelial- specific ERp57.

Results—Lung biopsies from asthmatics and non-asthmatics revealed a predominant increase in ERp57 in asthmatic epithelium. Deletion of ERp57 resulted in a significant decreases in the

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inflammatory cells and airways resistance in a murine model of allergic asthma. We further observed that disulfide bridges in eotaxin, EGF and periostin were also decreased in the lungs of HDM-challenged ERp57 deleted mice. Fibrotic markers such as collagen and αSMA were also significantly decreased in the lungs of ERp57-deleted mice. Furthermore, adaptive immune responses were dispensable for HDM-induced ER stress and airways fibrosis.

Conclusions—Here we show that ERp57 is increased in the airway epithelium of asthmatics and in mice with allergic airways disease. ERp57 increase is associated with redox modification of pro- inflammatory, apoptotic and fibrotic mediators, and contribute to airways hyperresposiveness (AHR). The strategies to inhibit ERp57 specifically within the airways epithelium may provide an opportunity to alleviate allergic asthma phenotype.

Keywords

UPR; ER Stress; Asthma; HDM; PDI; ERp57; Rag1; Epithelium; AHR

Introduction

Allergic asthma is characterized by airways inflammation, mucus metaplasia and peri-bronchiolar fibrosis which impacts lung structure and function[1, 2]. Airway epithelial cells (AECs) reside at the intersection of the lung and the external environment[3]. 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) is one of the most commonly found complex airborne allergens[7], inducing an allergic response in approximately 50–85% of asthmatics[7, 8]. 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, 10].

Complex allergens such as HDM are known to induce the unfolded protein response (UPR)[11]. Demand for increases in protein synthesis and folding (eg. cytokine 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 [12]. In mammalian cells, accumulation of unfolded proteins are sensed by three ER transmembrane proteins: Inositol Requiring Enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and PKR-like ER kinase (PERK) [13]. A prolonged UPR can cause CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP)-induced apoptosis[12]. Additionally, to cope with excessive protein folding load, the protein disulfide isomerases (PDIs), which construct disulfide bridges (-S-S-) in the ER, are upregulated[14]. One such PDI, ERp57, mediates misfolded protein-induced apoptosis by oligomerization of pro-apoptotic Bak through the formation of inter-molecular disulfide (-S-S-) bridges and the permeabilization of mitochondria[15]. Studies thus far from our laboratory and others have shown that ER stress-dependent activation of transcription factors Xbp1 or ATF6α are required during mucus metaplasia and pro-inflammatory responses in ovalbumin or HDM-induced allergic airways disease [16, 17]. Our group has previously demonstrated that along with ATF6α, ERp57 is up

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regulated in both murine and human epithelial cells challenged with HDM[11]. However, the impact of UPR-mediated induction of ERp57 has not been characterized in the development of allergic asthma. Furthermore, it is not clear whether allergen-induced ERp57 is directly linked to multiple facets of asthma such as inflammation, apoptosis, peri-bronchiolar fibrosis and impairment in respiratory mechanics. The objective of this study was to use clinical specimens and mouse models to gain insight into the function of epithelial-specific up regulation of ERp57 in allergic asthma.

Material and Methods

Human Samples

Physician diagnosed asthma, and non-asthmatic lung tissues were obtained from University of California, San Francisco, Department of Medicine and Cleveland Clinic, Department. Of Pathobiology. The Institutional Review Boards of the University of California, and Cleveland Clinic approved provision of de-identified materials for research at the University of Vermont. All subjects were non-smokers defined as never smoker or former smoker with no smoking for at least 1 year prior to enrollment and total pack-years ≤ 15. All asthmatics were refrained from ICS for 6 weeks prior to enrollment into the study. Six (n=6) non-asthmatics, six (n=6) asthmatics lung biopsies were from UCSF airway tissue bank and three (n=3) non-asthmatics and three (n=3) asthmatics lung biopsies were from Cleveland Clinic (Table S1 A and B).

Animals

For all experiments, age matched male and female mice (C57BL6/J) were used. Bi-transgenic mice carrying the rat club cell secretory protein (CCSP) promoter 5′ to the open reading frame for the reverse tetracycline trans activator (*CCSP-rtTA* (Line 1, which in adult lung is expressed in bronchiolar and type II epithelial cells) [46], plus seven tetracycline operon 5′ to the open reading frame for Cre recombinase (*TetOP-Cre*) mice were provided by Dr. Whitsett (Cincinnati Children's Hospital) [47]. *CCSP-rtTA*$^{+}$, *TetOP-Cre*$^{+}$ mice were bred with mice carrying the *ERp57*$^{loxp/loxp}$ alleles[48]. Mice expressing *CCSP-rtTA/TetOP-Cre*/*ERp57*$^{loxp/loxp}$ were used to ablate ERp57 from lung epithelial cells (denoted as Δ*Epi-ERp57*), by feeding doxycycline (Dox) containing chow (6g/kg, Purina Diet Tech, St. Louis, MO) 5 days before exposure to HDM. Mice were maintained on Dox food until the completion of the experiment. Double transgenic littermates either containing *CCSP-rtTA/TetOP-Cre* or *CCSP-rtTA/ERp57*$^{loxp/loxp}$ (labeled as Ctr) fed Dox food were used as controls in the experiments. The *Rag1*$^{-/-}$ mice were maintained in our colony and for the experiments, age matched male and female mice (C57BL6/J) were used as controls (WT).

Statistics

All assays were performed in triplicates. Mice experiments were repeated once (total of 8 to 10 mice in two experiments). Data were analyzed by one-way analysis of variance (ANOVA) and a Tukey's post-hoc test to adjust for multiple comparisons, or student's t test where appropriate. Histopathological scores were analyzed using the Kruskal-Wallis and Dunn's multiple comparison post-hoc tests. Data from multiple experiments were averaged and expressed as mean values ± SEM. Correlations between ERp57 scores and blood

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eosinophils or bronchodilator response were performed using Spearman's rank correlation coefficients. The data were analyzed using JMP® Pro 10 (SAS Institute Inc., Cary, NC, USA). P values <0.05 were regarded as statistically significant.

A more detailed information on material and methods is available in the article's on line data repository (supplementary material and methods section).

Results

ERp57 is increased in humans with asthma and mice with allergic airways disease

To investigate whether ERp57 expression is altered in the lung during asthma pathogenesis, we stained bronchial biopsy samples from non-asthmatics and asthmatics (refrained from inhaled corticosteroid (ICS) for 6 weeks prior to the study) for ERp57 by immunohistochemistry. Marked increases in ERp57 was observed in asthmatic lung samples (n=9) as compared to non-asthmatics (n=9) (Fig 1 A, supplementary Fig S 1 A & C and supplementary table S1 A & B). Furthermore, these increases were found to be predominantly in the airway epithelium of the asthmatics by semi-quantitative scoring (Figs 1 A, S1 A & C). Increase in ERp57 also showed a positive correlation with increases in levels of eosinophils in blood (n=9/group) and bronchodilator response (% baseline FEV1) in asthma patients (n=8-data not available for 1 of the patients) as compared to non-asthmatic (n=9) subjects (Fig S 1 D & E).

To explore alterations in ERp57 expression and to investigate the impact on downstream mechanisms of asthma pathogenesis, we used a model of HDM-induced allergic airways inflammation in mice. Evaluation of whole lung homogenates by western blots showed a marked increase in ERp57 as well as other ER stress markers such as GRP94, cleaved 50 kD fragment of ATF6 ($ATF6^{50}$) and CHOP in HDM challenged mice as compared to those challenged with PBS (Fig 1 B). To examine whether ERp57 was increased in specific cell types of the lung as observed in humans, we stained for ERp57 from both PBS and HDM challenged lungs. Immunohistochemistry of the lungs showed that there was a dramatic increase in ERp57 in the epithelium of the HDM treated lungs as compared to PBS treated lungs (Fig 1 C). These results illustrate that both humans with asthma and mice sensitized and challenged with the allergen, HDM show increases in ER stress, associated with an increase in ERp57 predominantly in the lung epithelium.

Ablation of ERp57 in lung epithelium attenuates allergen-induced asthma-like responses in mice

To determine whether increased ERp57 in airway epithelial cells may contribute to allergic airways disease in mice, we investigated whether specific deletion of ERp57 in airway epithelial cells attenuated pathophysiology associated with HDM challenge. To answer this question, we generated a doxycyline (Dox) inducible triple transgenic *CCSP-rTetA/TetO-Cre/ERp57*$^{loxp/loxp}$ (Δ*Epi-ERp57*) mouse to delete ERp57 specifically in lung epithelial cells. The mice carrying *TetO-Cre/ERp57*$^{loxp/loxp}$ or *CCSP-rTetA/TetO-Cre* were used as controls (Ctr). Our analysis showed that there was a clear decrease in ERp57 in the Epcam positive cells of lungs of the Dox treated Δ*Epi-ERp57* mice as compared to Ctr mice (Fig. 2

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A). For the experiments with allergen challenge, all mice were maintained on Dox for the length of the experiment beginning three days prior to initial sensitization (Fig. 2 B). Analysis of total cells in the BALF showed that there was an increased influx in cells in both HDM treated groups (Ctr and Δ*Epi-ERp57)* (Fig 2 C). Analysis of specific inflammatory and immune cell types indicated a significant attenuation of eosinophils, neutrophils and lymphocytes in Δ*Epi-ERp57* mice challenged with HDM as compared to Ctr mice challenged with HDM. We did not observe any statistically significant changes in macrophage counts in any groups (Fig 2 D–G).

We next determined the consequence of epithelial-specific ablation of ERp57 on airways hyperresponsiveness (AHR) to increasing doses of inhaled methacholine (12.5, 25 and 50mg/ml). These measurements revealed a significant decrease in AHR as measured by changes in central airways resistance (R_n) in Δ*Epi-ERp57* mice challenged with HDM as compared to Ctr mice challenged with HDM (Fig 2 H). We did not observe any significant changes within the PBS treated mice from both genotypes (Fig 2 H).

Ablation of ERp57 in airways epithelium attenuates allergen-induced cytokine and chemokine responses in the lung

Deletion of ERp57 in the airways epithelium showed significant decreases in HDM induced eosinophils, neutrophils, lymphocytes and AHR. Therefore we investigated whether these decreases could be explained by decreases in epithelial derived cytokines and chemokines. Our analysis from whole lung tissue lysates from Ctr and Δ*Epi-ERp57* mice showed a slight but significant decrease in eotaxin and highly significant decreases in CCL20, IL-33 and IL-6 in HDM challenged Δ*Epi-ERp57* mice as compared to Ctr HDM challenged mice (Fig 3 A–D). Although there were decreased neutrophils in the Δ*Epi-ERp57* mice, we did not find any significant alterations in production of epithelial derived neutrophil-chemoattractant, G-CSF in PBS or HDM challenged mice in both genetic backgrounds (data not shown).

ERp57 is a protein disulfide isomerase that specifically facilitates disulfide (-S-S-) bond formation on glycoproteins being processed and secreted by the ER[18]. We next determined whether deletion of ERp57 in the airways epithelium had any effect on cytokines such as eotaxin, a glycoprotein containing two disulfide bonds (http://www.uniprot.org/uniprot/P48298). Our analysis using labeling of cysteine sulfhydyl (-SH) groups of (Fig 3 E) eotaxin and subsequent immunoprecipitation revealed considerably less disulfide bonds in eotaxin in the lungs of Δ*Epi-ERp57* mice compared to Ctr mice (Fig 3 F & G) challenged with HDM. Collectively these results suggest that ERp57 deletion attenuates HDM-induced cytokine production and may also be affecting the function due to the lack of disulfide bonds.

Epithelial-specific ablation of ERp57 does not alter allergen-induced mucin production

To examine the consequence of ERp57 ablation in airway mucus production we quantified Muc5AC and Gob5 levels in the total lung lysates. Our results show that mRNA for Muc5AC or Gob5 were not decreased in Δ*Epi-ERp57* mice challenged with HDM compared to Ctr mice challenged with HDM (Fig. S 2 A & B). Staining for mucus in the airways by Periodic Acid Schiff (PAS) also showed similar mucus production in airway epithelial cells

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of Δ*Epi-ERp57* and Ctr mice challenged with HDM (Fig. S 2 C & D). These results indicate that airway epithelial cell specific deletion of ERp57 has no effect on HDM induced mucus production.

Lung epithelial-specific ablation of ERp57 decreases proapoptotic Bak oligomerization, and caspase-3 activity in mice

ER stress mediated induction of ERp57 leads to interaction with Bak and forms disulfide (-S-S-) mediated Bak oligomerization, promoting intrinsic apoptosis[11, 15, 19]. Therefore, we next determined whether ERp57 deletion in mice decreases HDM-induced -S-S- mediated oligomerization of Bak and activation of caspase-3 (apoptotic marker). We examined the redox modification of Bak using non-reducing (-DTT) denaturing (+SDS) polyacrylamide gel electrophoresis (PAGE). Our results in figure S3 demonstrate that ER stress mediated increases in ERp57 were associated with the promotion of -S-S- mediated oligomers of Bak as evidenced by DTT mediated decomposition of oligomers in HDM challenged lungs of Ctr mice compared to Δ*Epi-ERp57* mice (Fig. S 3 A). As a consequence HDM-induced caspase-3 activity was also attenuated in ERp57 deleted Δ*Epi-ERp57* mice as compared to HDM-challenged Ctr mice (Fig S 3 B). Furthermore, staining for both ERp57 and active caspase-3 in serial sections (5μm apart) also revealed a dramatic decreases in active casepase-3 in the same regions of the airway epithelium that corresponds to decreases in ERp57 in Δ*Epi-ERp57* mice as compared to HDM-challenged Ctr mice (Fig S 3 C). These results indicate that ablation of ERp57 in lung epithelial cells results in decreased -S-S- mediated oligomerization of Bak and decreased allergen-induced apoptosis in the lung compared to non-ablated (Ctr) mice.

Lung epithelial-specific ablation of ERp57 decreases allergen-induced airways fibrotic alterations

To examine the role of ERp57 in structural airway remodelling, we assessed airways smooth muscle and collagen content following HDM exposure in Ctr and Δ*Epi-ERp57* mice. HDM challenge led to increases in alpha-smooth muscle actin (αSMA) in the peri-bronchiolar region of HDM-challenged Ctr mice. Semi-quantitative scoring by three independent scientists blinded to the identity of the samples revealed significant decreases in αSMA staining in HDM challenged Δ*Epi-ERp57* mice compared to Ctr mice (Fig. 4 A & B). Similarly, biochemical analysis of collagen deposition showed a reduction in collagen following HDM-challenge in Δ*Epi-ERp57* mice compared to Ctr mice (Fig 4 C).

Analysis of pro-fibrotic growth factors, TGFβ and periostin, produced by epithelial cells during injury[20, 21] did not show significant differences in expression (Fig. 5 A & B). Glycoproteins such as periostin and EGF (also produced by airway epithelial cells)[1] have glycosylated moieties and a number of disulfide bonds in their receptor binding domain (http://www.uniprot.org/uniprot/P01132; http://www.uniprot.org/uniprot/Q62009). Our sulfhydryl labeling experiments indicated that periostin and EGF showed more exposed sulfhydryls (-SH) in the absence of epithelial ERp57. In other words, there were less disulfide bonds (-S-S-) in EGF and periostin from Δ*Epi-ERp57* mice as compared to Ctr mice challenged with HDM (Fig 5 D–G). Collectively these results indicate that ER stress mediators, specifically ERp57 controls allergen- induced airways fibrosis in the lung.

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B and T lymphocytes are dispensable in induction of HDM induced UPR and fibrosis

To test whether HDM-induced ER stress and increases in ERp57 were induced as a consequence of the strong adaptive immune responses, we compared the response to HDM challenge in wild type (WT) mice and mice deficient in Recombination-Activating Gene1 (*Rag1*). *Rag1* encodes enzyme involved in the recombination of the immunoglobulin and T cell receptor genes and are essential for the generation of mature B and T lymphocytes[22] two essential components of the adaptive immune system. The results (Fig 6) demonstrate that although HDM increased ER stress markers such as ATF6[50] and CHOP in both WT and *Rag1*$^{-/-}$ mice, there was no difference in up-regulation of ER stress markers between the groups. Furthermore, we also observed similar increases in ERp57 in HDM challenged WT and *Rag1*$^{-/-}$ mice (Fig 6 A). As expected *Rag1*$^{-/-}$ mice exhibited dramatic decreases in Total BALF cell counts, eosinophils, PMNs, lymphocytes and immunoglobulin production (IgG & IgE) (Fig 6 BE). Interestingly, we did not see any significant alterations in collagen content in HDM-challenged *Rag1*$^{-/-}$ mice as compared to WT mice (Fig. 6 F). These results suggested that HDM induced T helper cell and B cell responses are not required for HDM-induced UPR and fibrosis in the lungs of HDM-challenged mice.

Discussion

Our results show that asthmatics exhibit a marked increases in ERp57 as compared to non-asthmatics, predominantly in the lung epithelium. Using a murine model of allergic asthma, we demonstrated that epithelial specific up-regulation of protein disulfide isomerase-ERp57 modulates allergen induced inflammation, AHR and airways fibrosis in the lung. Furthermore we also report that adaptive immune responses are dispensable for the development of allergen induced UPR, up-regulation of ERp57 and airways fibrosis.

Perturbations in ER homeostasis can cause unfolded protein response (UPR), leading to inflammation and, when unresolved, cell death [23]. Recent reports suggest that UPR mediated activation of transcription factor X-box binding protein 1 (XBP-1) is required to induce mucus metaplasia in the lungs of mice challenged with ovalbumin[16, 17]. These reports did not address the implications of UPR in other facets of allergic asthma, such as inflammation, epithelial apoptosis, AHR and peri-bronchiolar fibrosis. In our earlier work, we demonstrated that *in vivo*, a complex allergen, HDM induces higher levels of UPR markers and upregulation of ERp57 as compared to murine models of LPS or Ovalbumin +LPS challenge[11]. Additionally, in contrast to the recent reports[16, 17], our studies with human epithelial cells (*in vitro*) demonstrated the activation of ATF6α and up regulation of ERp57[11]. We believe that the differences in the activation of specific UPR transducers may be due to the complex signaling pathways activated (by multiple PRR agonists, Uric acid and proteases) in the epithelium by HDM, compared to the simple antigen ovalbumin or TLR4 agonist, LPS [7, 9]. Thus, our results indicate a multifaceted mechanism of allergen-specific activation of ER stress mediators in mouse and human airway epithelial cells.

Interestingly ORMDL sphingolipid biosynthesis regulator 3 (ORMDL3) a protein linked to asthma susceptibility, contributes to alterations in ceramide and calcium homeostasis[24–26] and can induce ATF6α activation in epithelial cells[26]. How ATF6α activation contributes to asthma pathophysiology remains to be determined. Previous work performed by our

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laboratory demonstrated that ATF6α knockdown in human bronchiolar epithelial cells decreased HDM-induced up-regulation of ERp57 and apoptosis[11]. However, these studies did not address the role of ERp57 as a regulator of HDM-induced pro-inflammatory/pro-fibrotic response from epithelial cells in vivo. Our experiments here demonstrate that lung epithelial-specific deletion of ERp57 significantly decreased HDM-induced influx of neutrophils and, lymphocytes, and modestly decreased eosinophilic infiltration into the lung. To elucidate the mechanism by which ERp57 acts to regulate immune responses, we analyzed epithelial derived cytokine and chemokine production[1, 27]. Our results show that deletion of ERp57 in lung epithelial cells dramatically decreased IL-33, IL-6, and CCL20 production, and slightly, but significantly decreased eotaxin production following HDM challenge in ERp57 deleted mice. These results indicate that deletion of ERp57 in epithelial cells could directly affect the production of the above epithelial-derived, innate lymphoid cell, T cell and dendritic cell activators[1] that are involved in innate and adaptive immune responses following HDM exposure.

We hypothesized that being a protein disulfide isomerase with specificity towards glycoproteins (eg. cytokines and growth factors), ERp57 could be involved in formation of disulfide bonds (-S-S-) in specific cytokines. Furthermore, ablation of ERp57 would likely both decrease the secretion of cytokines and also affect their function. We tested this hypothesis on eotaxin since its levels were not dramatically altered yet we observed significant attenuation of eosinophils in ERp57 deleted mice. Our results showed that deleting ERp57 in epithelial cells exposed more –SH groups in eotaxin compared to Ctr mice challenged with HDM. Based on our results we speculate that although eotaxin is produced in copious amounts, deficiency in disulfides may be affecting the function of eotaxin in ERp57 deleted mice. ERp57 is also known to modulate the activity of a redox active transcription factor Ref-1[28]. At this juncture we can only speculate that the decrease in cytokines such as IL-33, IL-6 and chemokine CCL20 could be due to inactivity of Ref-1 in ERp57 deleted airway epithelial cells. However, additional targeted experiments are required to substantiate the role of the ERp57 in oxidative folding of eotaxin and Ref-1 regulation in HDM-induced allergic airways disease.

Mucins are large glycosylated proteins encompassing multiple disulfide bonds[29]. However, it is known that mucins are folded and secreted by epithelial cells through PDI-AGR2[16, 29, 30], and not ERp57. Therefore, there was no significant decreases in mucin production in HDM challenged Δ*Epi-ERp57* mice compared to Ctr mice.

Allergen-induced structural remodeling of the lung was thought to be the consequence of damage to the airways epithelium and subsequent cross talk between airways epithelial cells (AECs), mesenchymal and immune cells[31, 32]. Recent studies on ER stress-mediated apoptosis have also shown involvement of ERp57 in disulfide-mediated oligomerization of proapoptotic Bak on the ER and mitochondria associated membranes[11, 15, 19, 33]. Based on the results presented herein indicating that ERp57 deleted mice showed decreased oligomerization of Bak and apoptosis marker active caspase-3, it is reasonable to speculate that ERp57 could be regulating apoptosis of epithelial cells during HDM challenge.

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Growth factors such as EGF, TGFβ and periostin, released by injured airway epithelium are thought to be the potential pro-fibrotic growth factors involved in airways structural remodeling in asthma[1, 34]. The allergen dependent chronic activation of ER stress and apoptosis can cause repeated injury to the airway epithelium. In fact, injured epithelium in human asthmatics as well as in mouse models up regulate pro fibrotic growth factors, stimulating proliferation of the underlying smooth muscle cells, and subsequently leading to the deposition of collagen [34]. Measurement of profibrotic-growth factor mRNAs in our study did not yield any significant differences between the two genotypes challenged with HDM. This suggests that ERp57 deficiency in the epithelial cells does not alter the transcription of these mRNAs. However, our analysis of sulfhydryl groups in EGF and periostin (both are primarily secreted by epithelial cells in response to damage and repair)[20, 35–37] revealed more sulhydryl groups exposed in ERp57 deleted HDM challenged mice as compared to Ctr mice challenged with HDM. These results suggest that ERp57 may also be controlling the oxidative disulfide mediated (-S-S-) folding of EGF and periostin (http://www.uniprot.org/uniprot/P01132; http://www.uniprot.org/uniprot/Q62009) (cysteine rich-glycosylated growth factors). Therefore deletion of ERp57 in epithelial cells may not affect the production but rather inhibits their function due to lack of disulfide bonds which are required for tertiary structure and of the functional ligands.

ER stress transducers, such as ATF6 and CHOP, play a prominent role in apoptosis of alveolar type II epithelial cells in fibrotic interstitial lung diseases[38, 39]. Recent studies have suggested that asthmatics and HDM based mouse models of asthma develop sub-epithelial thickening marked by αSMA (smooth muscle hyperplasia) and increased collagen deposition[40–42], resulting in peri-bronchiolar fibrosis. Accordingly results presented here show that HDM induces severe ER stress, leading to apoptosis of airway epithelial cells and subsequent fibrosis.

Deletion of ERp57 in airways epithelial cells also resulted in a significant decrease in HDM-induced central airway resistance (R_n). We did not observe statistically significant differences in tissue resistance (G) and tissue stiffness (H) in HDM-challenged, Ctr mice as compared to HDM-challenged, ERp57 deleted mice (data not shown). This suggests that the effect of ERp57 on AHR is mediated through effects on central but not peripheral, airway function. Indeed it is difficult to compare functional measures of respiratory impedance with exact anatomical locations. Nonetheless, we believe that an effect on only central airway function is consistent with our findings of an effect of ERp57 on airway smooth muscle content (α-SMA staining) but not on mucus production (PAS staining, MUC5AC mRNA, Gob5 mRNA). This is consistent with the increase in airway smooth muscle, which would be expected to predominantly increase central airway narrowing in response to methacholine. In contrast, we have previously shown that increased peripheral airway responses to methacholine are predominantly due to increased airway closure, which is likely due to mucus metaplasia [43]. Furthermore, enhanced apoptosis of epithelial cells likely decreased the protective barrier layer of the airways. Increased permeability of larger airways could perhaps allow increased access of methacholine to smooth muscle cells [44, 45], resulting in increased central airway narrowing. However, the mechanisms by which ERp57 increase AHR of central, but not peripheral airways are yet to be determined.

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Conclusion

Collectively, our work illuminates a previously unexplored mechanism of HDM-induced UPR and epithelial ERp57. We demonstrate that ERp57 up-regulation in the epithelial cells play a regulatory role in airway inflammation, peri-bronchiolar fibrosis and AHR. Based on our results we believe that strategies to inhibit ERp57 in airway epithelial cells may offer beneficial effects in treating allergic asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AECs	Airways Epithelial Cells
AHR	Airways Hyperresponsiveness
ATF6	Activating Transcription Factor 6
BAL	Bronchoalveolar lavage
CHOP	C/EBP homologous protein
CCSP	Club cell secretory protein promoter
ER	Endoplasmic Reticulum
ERp57	Endoplasmic Reticulum protein 57
GRP78	Glucose Regulated Protein 78
GRP94	Glucose Regulated Protein 94
HDM	House Dust Mite
IRE	Inositol requiring enzyme
LPS	Lipopolysaccharide
LoxP	Locus of cross-over in P1 sites
OVA	Ovalbumin
PERK	PKR-like ER kinase
PDI	Protein Disulfide Isomerase

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Rn	Central airway resistance
rTetA	Reverse tetracycline trans activator
Teto	Tetracycline operon promoter
UPR	Unfolded Protein Response
XBP-1	X-box binding protein

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Key Messages

- Asthmatics exhibit induction of ERp57 predominantly in airways epithelium.
- Allergen exposure also induces ERp57 in the airways epithelium of mice.
- ERp57 increase is associated with redox modification of key mediators of asthma phenotype.
- Strategies to inhibit ERp57 specifically within the airways epithelium may provide an opportunity to alleviate allergic inflammation and airways fibrosis associated with asthma.

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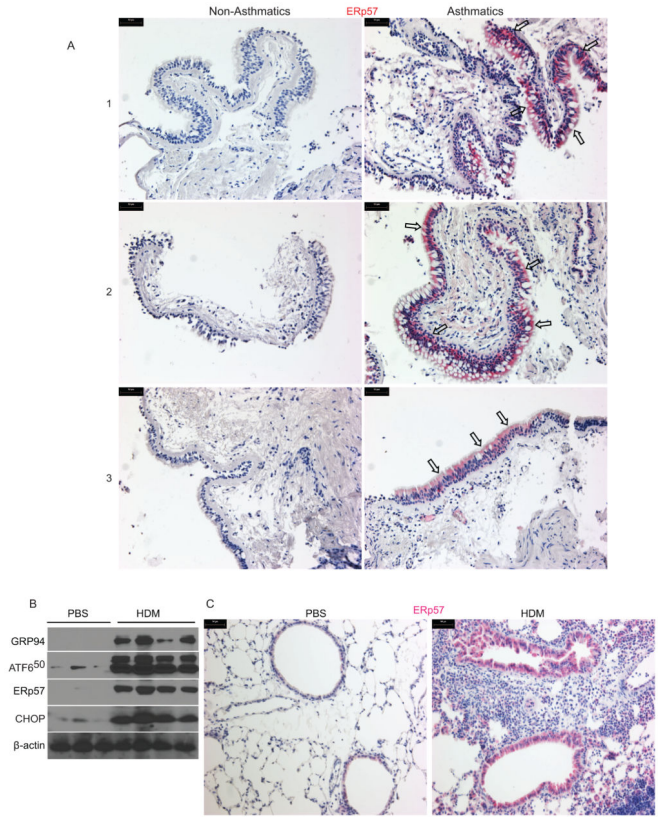

Figure 1.

ERp57 is increased in asthmatics and allergen challenged mouse epithelium. **A:** Representative images of human lung tissue samples obtained from UCSF airway tissue bank stained for ERp57 (red). **B**: Western blots of whole lung lysates from PBS or HDM challenged mice probed for various UPR markers, and ERp57. β-actin was used as control. **C:** Representative images of the lungs of mice challenged with PBS or HDM stained for ERp57 (red). Scale bars represents 50μm.

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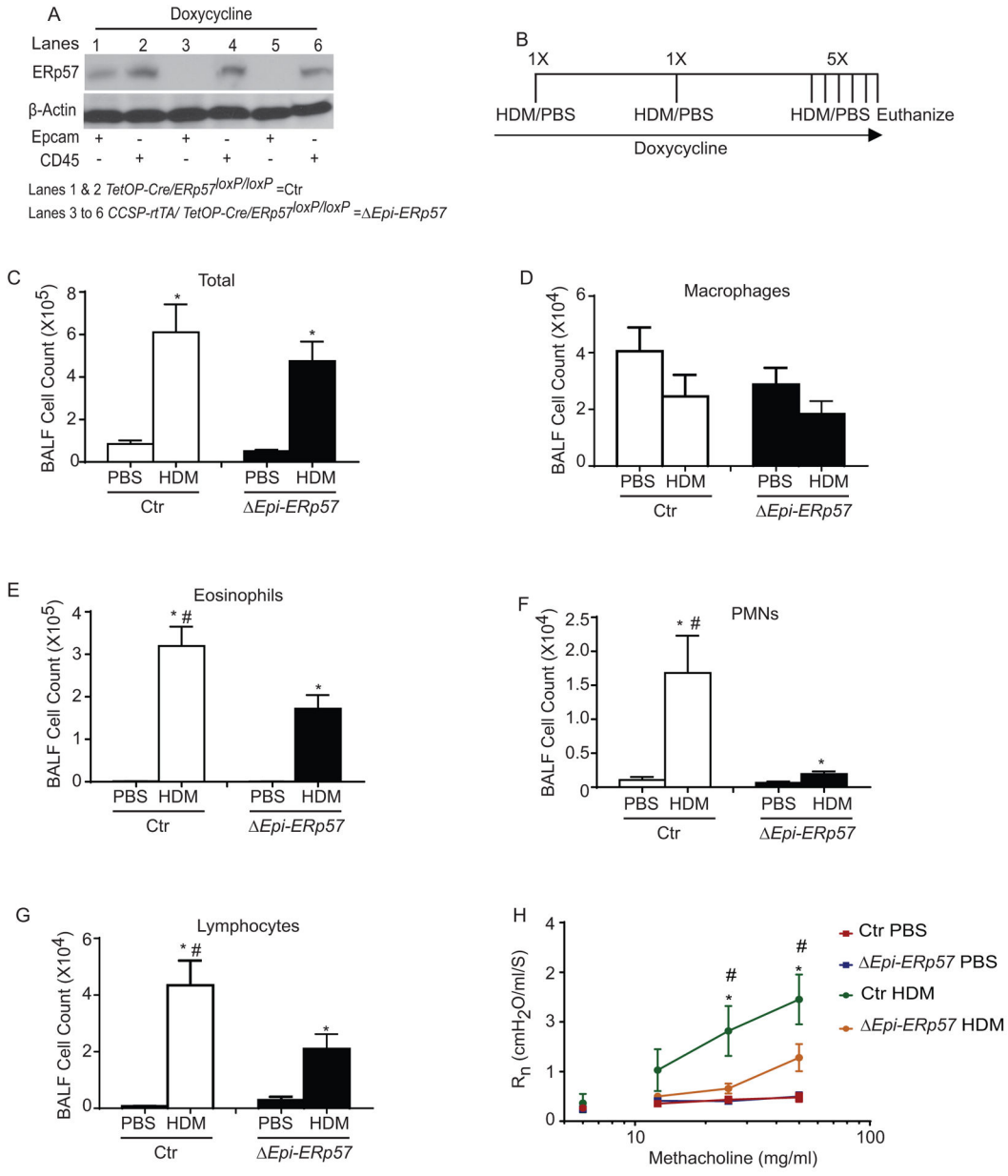

Figure 2.

HDM-induced experimental asthma is attenuated in ERp57 ablated mice. **A:** Ablation of ERp57 from Epcam^{+} epithelial cells in mice containing *CCSP-rtTA/TetOP-Cre/ERp57$^{loxP/loxP}$* allele. β-actin was used as loading control. **B:** HDM or PBS instillation regimen. **C–G:** Analysis of inflammatory and immune cells in the BALF. **H:** Analysis of methacholine induced AHR in mice. *p<0.05 denotes significant differences compared with PBS groups. # p<0.05 denotes significant differences compared with the HDM groups.

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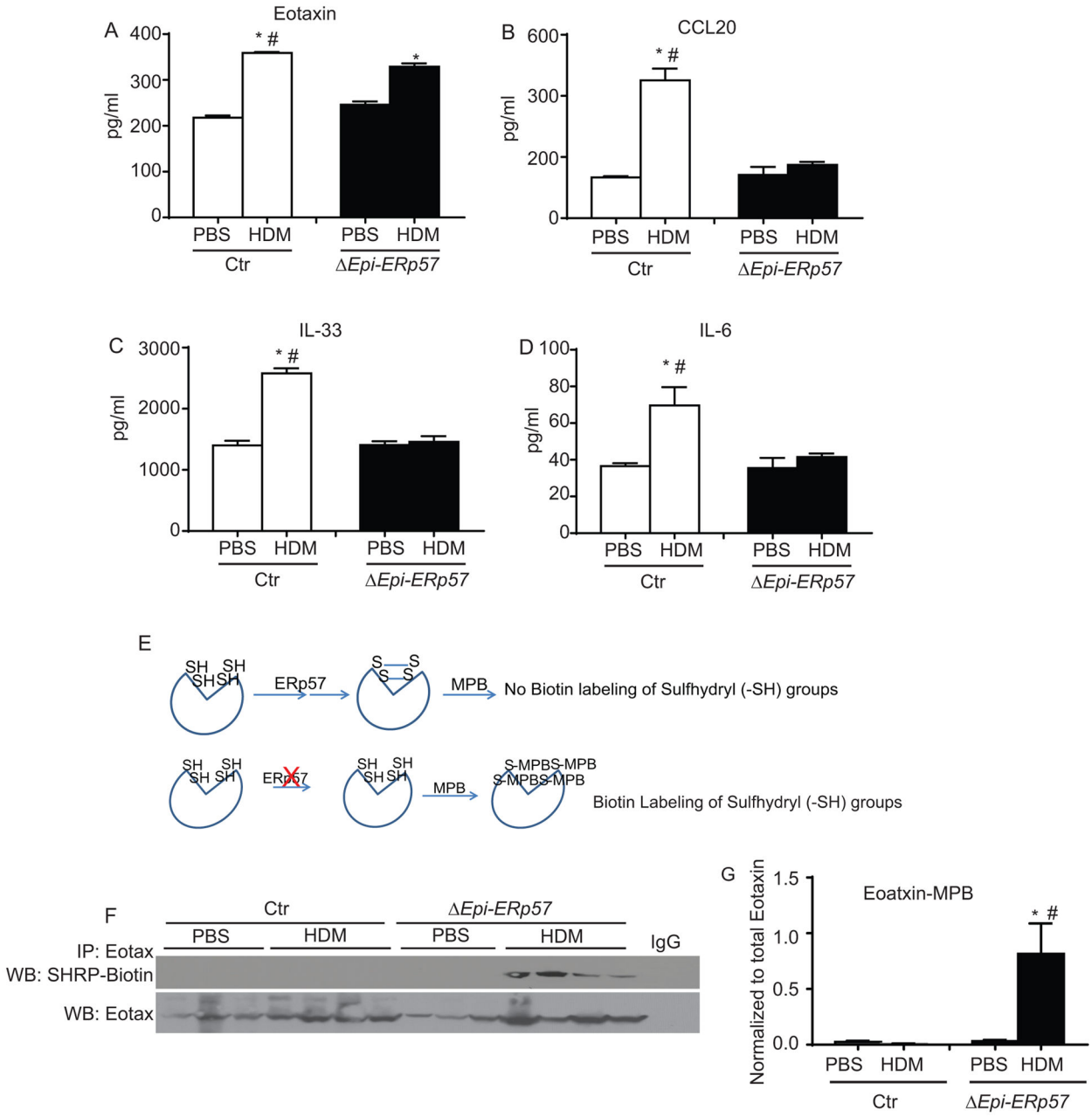

Figure 3.

Deletion of ERp57 in airway epithelial cells decreases various cytokines and chemokines secreted from epithelial cells. **A–D:** ELISA for cytokines and chemokines. **E:** Biotin labeling of free sulfhydryl (-SH) groups by MPB. **F:** Western blot analysis of sulfhydryl (-SH) content of eotaxin by MPB labeling and immunoprecipitation. **G:** Densitometry of the eotaxin-MPB in F. *p<0.05 denotes significant differences as compared with PBS groups. # p<0.05 denotes significant differences compared with the HDM groups.

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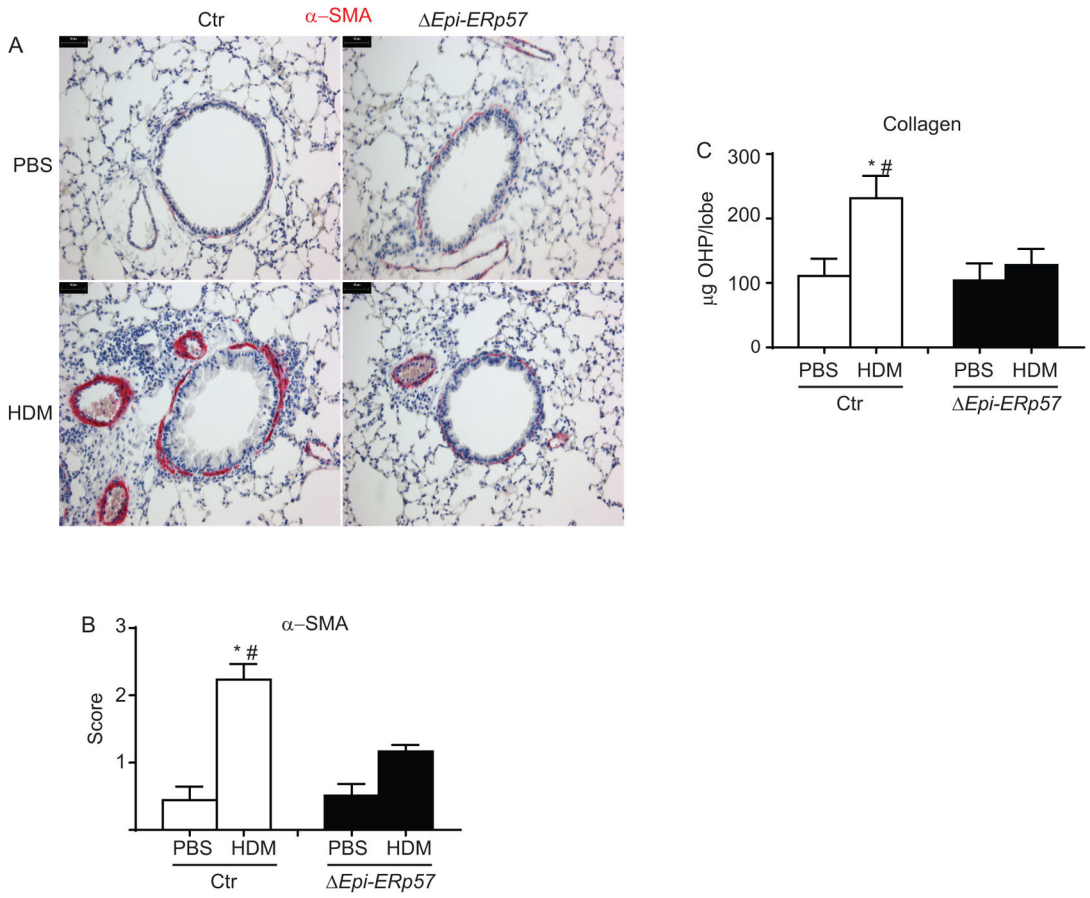

Figure 4.

Ablation of ERp57 in lung epithelial cells decreases smooth muscle hypertrophy and collagen deposition. **A:** IHC staining for α-SMA in PBS and HDM challenged lungs from Ctr and Δ*ERp57* mice. **B:** Histological scores for α-SMA. **C:** Analysis of collagen deposition. *p<0.05 denotes significant differences as compared with PBS groups. # p<0.05 denotes significant differences as compared with the HDM groups. Scale bars represents 50μm.

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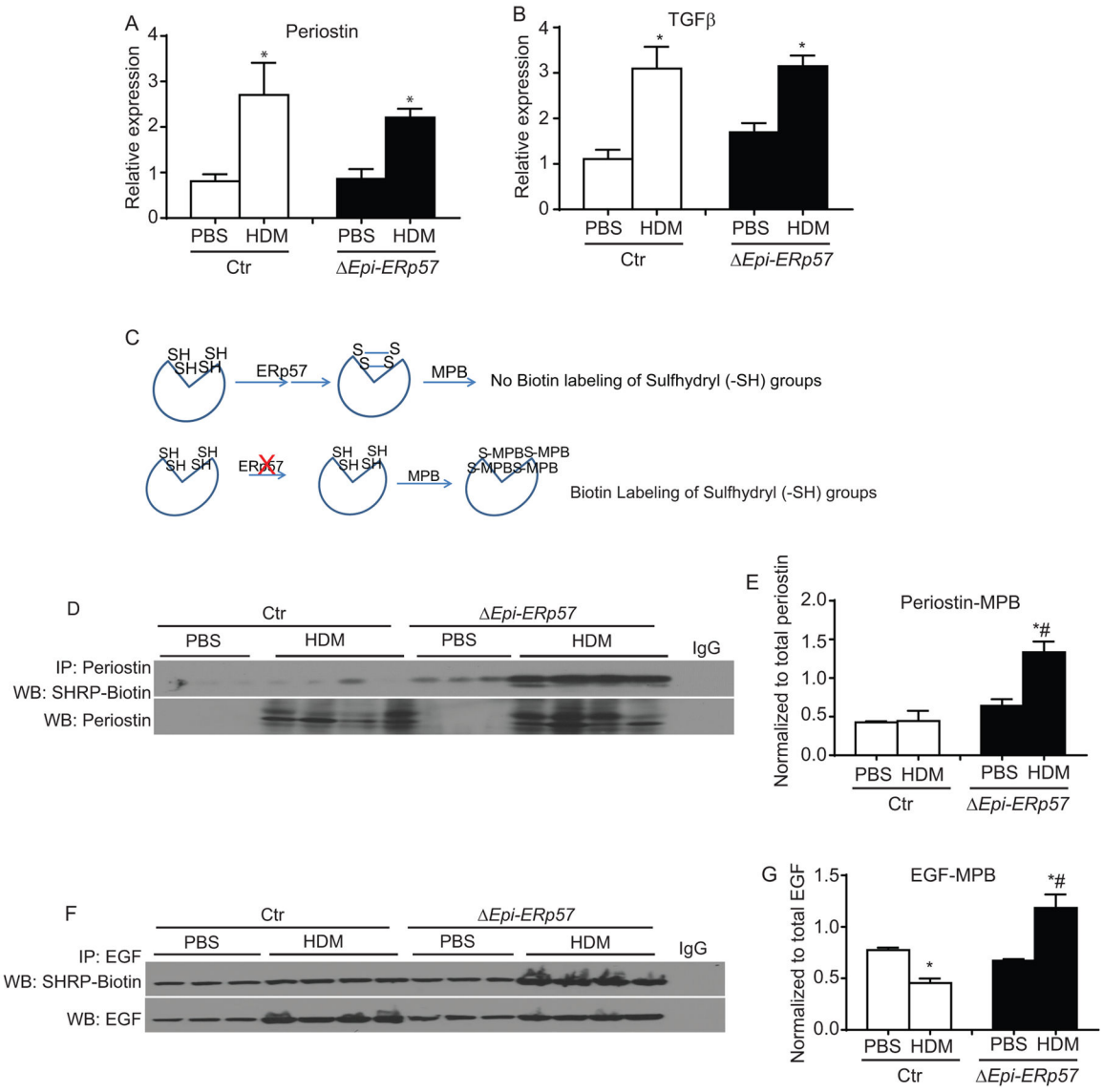

Figure 5.

Ablation of ERp57 in lung epithelial cells decreases disulfide bonds (-S-S-) in pro-fibrotic growth factors. **A & B:** Analysis of mRNA for epithelial derived growth factors. **C:** Biotin labeling of free sulfhydryl (-SH) by MPB. **D:** Western blot analysis of sulfhydryl (-SH) content of perisotin. **E:** Densitometry of the periostin-MPB in D. **F:** Western blot analysis of sulfhydryl (-SH) content of EGF. **G:** Densitometry of the EGF-MPB in F. *p<0.05 denotes significant differences as compared with PBS groups. # p<0.05 denotes significant differences compared with the HDM groups.

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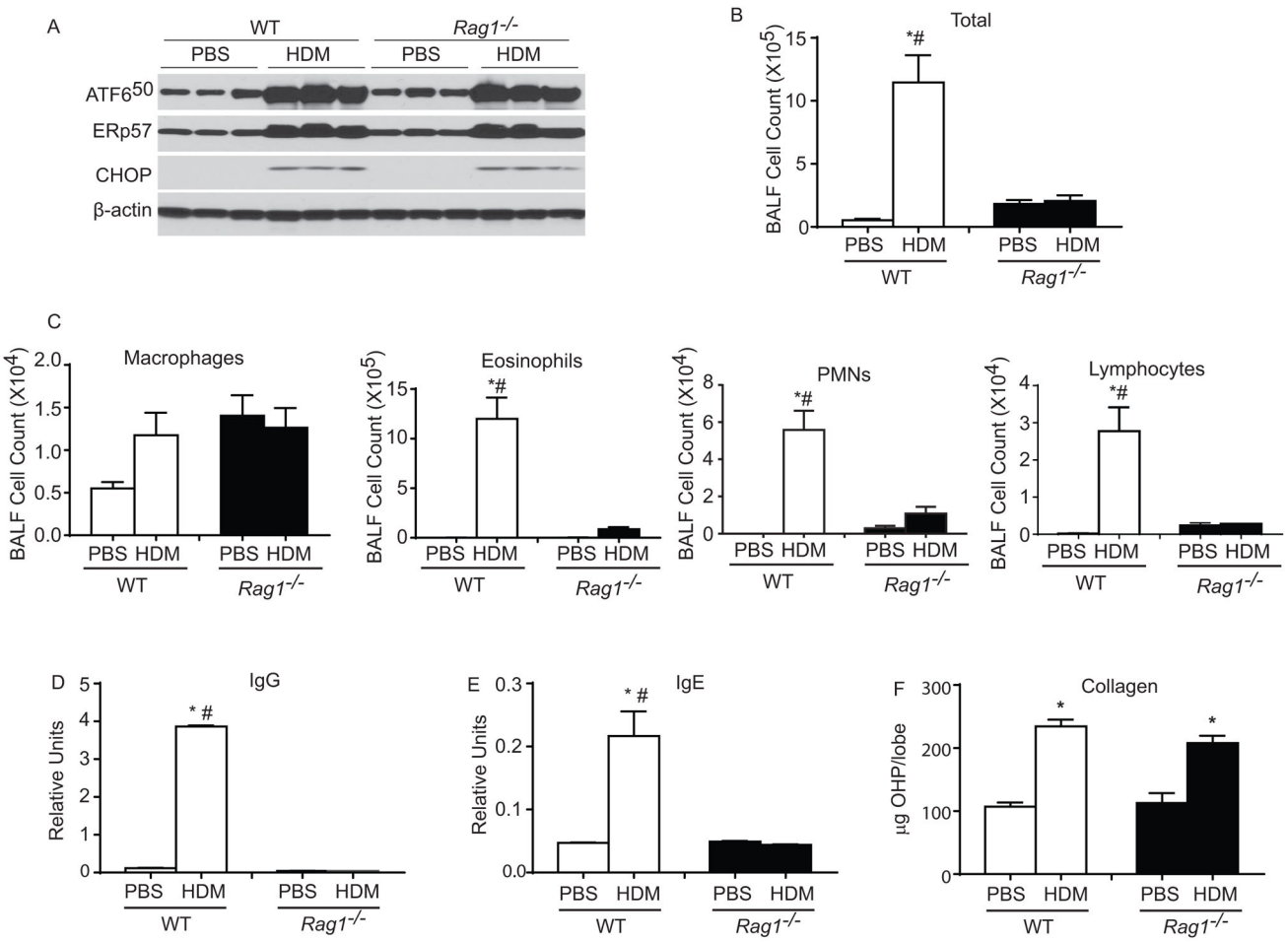

Figure 6.

T or B cells are not required for HDM induced ER stress activation and collagen deposition. **A:** Western blot analysis for various ER stress markers and ERp57 in WT and *Rag1*$^{-/-}$ mice, β-actin was used as loading control. **B & C:** Analysis of inflammatory and immune cells in the BALF. **D & E:** Assay for production of IgG and IgE. **F:** Hydroxyproline assay. $p<0.05$ denotes significant differences as compared with PBS groups. # $p<0.05$ denotes significant differences compared with the HDM groups.