

Protein disulfide isomerase–endoplasmic reticulum resident protein 57 regulates allergen-induced airways inflammation, fibrosis, and hyperresponsiveness

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Background: Evidence for association between asthma and the unfolded protein response is emerging. Endoplasmic reticulum resident protein 57 (ERp57) is an endoplasmic reticulum–localized redox chaperone involved in folding and secretion of glycoproteins. We have previously demonstrated that ERp57 is upregulated 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 epithelium–specific ERp57 in the pathogenesis of allergic asthma.

Methods: We examined the expression of ERp57 in human asthmatic airway epithelium and used murine models of allergic asthma to evaluate the relevance of epithelium-specific ERp57. **Results:** Lung biopsy specimens from asthmatic and nonasthmatic patients revealed a predominant increase in ERp57 levels in epithelium of asthmatic patients. Deletion of ERp57 resulted in a significant decrease in inflammatory cell counts and airways resistance in a murine model of allergic asthma. Furthermore, we observed that disulfide bridges in eotaxin, epidermal growth factor, and periostin were also decreased in the lungs of house dust mite–challenged ERp57-deleted mice. Fibrotic markers, such as collagen and α smooth muscle actin, were also significantly decreased in the lungs of ERp57-deleted mice. Furthermore, adaptive immune responses were dispensable for house dust mite–induced endoplasmic reticulum stress and airways fibrosis.

Conclusions: Here we show that ERp57 levels are increased in the airway epithelium of asthmatic patients and in mice with allergic airways disease. The ERp57 level increase is associated with redox modification of proinflammatory, apoptotic, and fibrotic mediators and contributes to airways hyperresponsiveness. The strategies to inhibit ERp57 specifically within the airways epithelium might provide an opportunity to alleviate the allergic asthma phenotype. (*J Allergy Clin Immunol* 2015;■■■■:■■■-■■■.)

Key words: Unfolded protein response, endoplasmic reticulum stress, asthma, house dust mite, protein disulfide isomerase, endoplasmic reticulum protein 57, recombination-activating gene 1, epithelium, airways hyperresponsiveness

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Allergic asthma is characterized by airways inflammation, mucus metaplasia, and peribronchiolar fibrosis, which affect lung structure and function.^{1,2} Airways 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 surfaces of AECs and subsequent secretion of various mediators are required for responses from dendritic cells and subsequent immune responses.⁴⁻⁶ House dust mite (HDM) is one of the most common complex airborne allergens,⁷ inducing an allergic response in approximately 50% to 85% of asthmatic patients.^{7,8} HDM contains numerous antigens, proteases, and ligands for pattern recognition receptors, resulting in activation of AECs and inducing the secretion of growth factors and cytokines that regulate subsequent activation of innate lymphoid cells and T cells, mucus metaplasia, inflammation, airways hyperresponsiveness (AHR), and fibrosis.^{7,9,10}

Abbreviations used

AEC:	Airways epithelial cell
AHR:	Airways hyperresponsiveness
ATF6:	Activating transcription factor 6
CHOP:	CCAAT/enhancer-binding protein homologous protein
CCSP:	Club cell secretory protein promoter
Ctr mice:	Double-transgenic littermates containing either <i>CCSP-rtTA/TetO-Cre</i> or <i>CCSP-rtTA/Erp57^{loxP/loxP}</i>
DTT:	Dithiothreitol
EGF:	Epidermal growth factor
EpCAM:	Epithelial cell adhesion molecule
ER:	Endoplasmic reticulum
Erp57:	Endoplasmic reticulum resident protein 57
HDM:	House dust mite
MPB:	3-(n-maleimido-propionyl) biocytin
PAS:	Periodic acid–Schiff
PDI:	Protein disulfide isomerase
Rag1:	Recombination-activating gene 1
R _n :	Central airways resistance
α-SMA:	α Smooth muscle actin
UCSF:	University of California, San Francisco
UPR:	Unfolded protein response
WT:	Wild-type

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 3 ER transmembrane proteins: inositol-requiring enzyme 1, activating transcription factor 6 (ATF6), and PKR-like ER kinase.¹³ A prolonged UPR can cause CCAAT/enhancer-binding protein homologous protein (CHOP)-induced apoptosis.¹² Additionally, the protein disulfide isomerases (PDIs), which construct disulfide bridges (-S-S-) in the ER, are upregulated to cope with excessive protein folding load.¹⁴ One such PDI, endoplasmic reticulum resident protein 57 (Erp57), mediates misfolded protein-induced apoptosis by means of oligomerization of proapoptotic Bak through the formation of intermolecular disulfide (-S-S-) bridges and the permeabilization of mitochondria.¹⁵ Thus studies from our laboratory and others have shown that ER stress-dependent activation of the transcription factors X-box binding protein 1 or ATF6α are required during mucus metaplasia and proinflammatory responses in the setting of ovalbumin- or HDM-induced allergic airways disease.^{16,17}

Our group has previously demonstrated that along with ATF6α, Erp57 is upregulated in both murine and human epithelial cells challenged with HDM.¹¹ However, the effect 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, peribronchial 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 epithelium-specific upregulation of Erp57 in patients with allergic asthma.

METHODS**Human samples**

Lung tissues from patients with physician-diagnosed asthma and non-asthmatic subjects were obtained from the Department of Medicine of the University of California, San Francisco (UCSF), and the Department of Pathobiology of the Cleveland Clinic. The Institutional Review Boards of the University of California and Cleveland Clinic approved provision of deidentified materials for research at the University of Vermont. All subjects were nonsmokers defined as never smokers or former smokers with no smoking for at least 1 year before enrollment and total pack years of 15 or less. All asthmatic patients refrained from inhaled corticosteroids for 6 weeks before enrollment in the study. Lung biopsy specimens from 6 nonasthmatic subjects and 6 asthmatic patients were from the UCSF airway tissue bank, and lung biopsy specimens from 3 nonasthmatic subjects and 3 asthmatic patients were from Cleveland Clinic (see [Tables E1 and E2](#) in this article's Online Repository at www.jacionline.org).

Animals

Age-matched male and female mice (C57BL6/J) were used for all experiments. Bitransgenic mice carrying the rat club cell secretory protein (CCSP) promoter 5' to the open reading frame for the reverse tetracycline transactivator (*CCSP-rtTA*; line 1, which in adult lungs is expressed in bronchiolar and type II epithelial cells)¹⁸ plus 7 tetracycline operon 5' to the open reading frame for Cre recombinase (*TetO-Cre*) mice were provided by Dr Whitsett (Cincinnati Children's Hospital).¹⁹ *CCSP-rtTA⁺, TetO-Cre⁺* mice were bred with mice carrying the *Erp57^{loxP/loxP}* alleles.²⁰ Mice expressing *CCSP-rtTA/TetO-Cre/Erp57^{loxP/loxP}* were used to ablate Erp57 from lung epithelial cells (denoted as ΔEpi-Erp57) by feeding doxycycline-containing chow (6 g/kg; Purina Diet Tech, St Louis, Mo) 5 days before exposure to HDM. Mice were maintained on doxycycline-containing food until completion of the experiment. Double-transgenic littermates containing either *CCSP-rtTA/TetO-Cre* or *CCSP-rtTA/Erp57^{loxP/loxP}* (Ctr mice) and fed doxycycline-containing food were used as controls in the experiments. The recombination-activating gene 1 (*Rag1*)^{-/-} mice were maintained in our colony, and for the experiments, age-matched male and female mice (C57BL6/J) were used as controls (wild-type [WT]).

Statistics

All assays were performed in triplicates. Mouse experiments were repeated once (total of 8–10 mice in 2 experiments). Data were analyzed by using 1-way ANOVA and a Tukey *post hoc* test to adjust for multiple comparisons or the Student *t* test where appropriate. Histopathologic scores were analyzed by using the Kruskal-Wallis and Dunn multiple comparison *post hoc* tests. Data from multiple experiments were averaged and expressed as mean values ± SEMs. Correlations between Erp57 scores and blood eosinophil counts or bronchodilator response were performed by using Spearman rank correlation coefficients. The data were analyzed with JMP Pro 10 software (SAS Institute, Cary, NC). *P* values of less than .05 were regarded as statistically significant.

More detailed information on the materials and methods in this study is available in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS**Erp57 levels are increased in human subjects 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 nonasthmatic and asthmatic subjects (who refrained from inhaled corticosteroids for 6 weeks before the study) for Erp57 by means of immunohistochemistry. Marked increases in Erp57

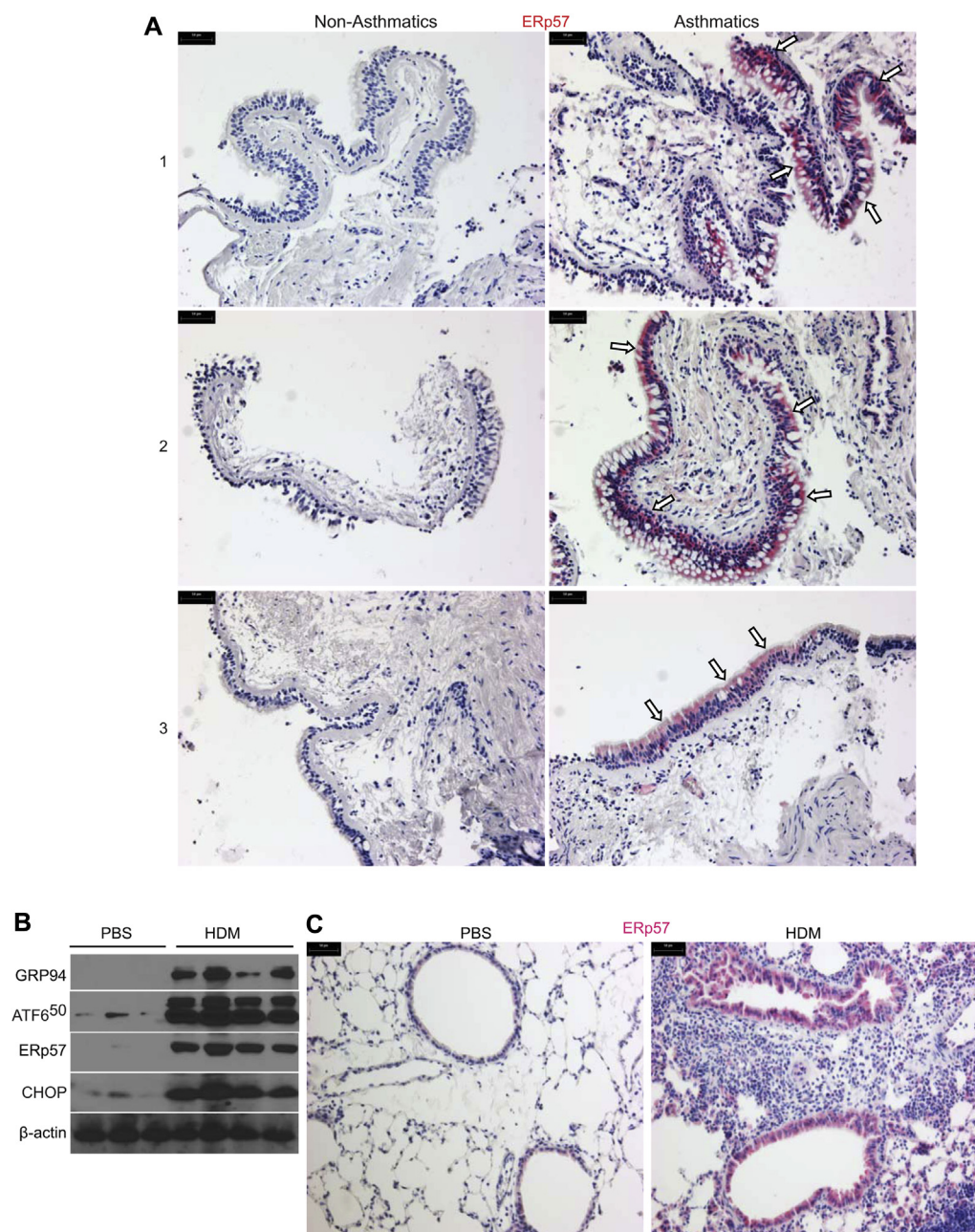


FIG 1. ERp57 levels are increased in asthmatic patients and allergen-challenged mouse epithelium. **A**, Representative images of human lung tissue samples obtained from the UCSF Airway Tissue Bank stained for ERp57 (red). **B**, Western blots of whole-lung lysates from PBS- or HDM-challenged mice probed for various UPR markers and ERp57. β -Actin was used as a control. **C**, Representative images of the lungs of mice challenged with PBS or HDM stained for ERp57 (red). Scale bars = 50 μ m.

levels were observed in lung samples from asthmatic patients ($n = 9$) compared with nonasthmatic subjects ($n = 9$; Fig 1, A, and see Fig E1, A and C, and Tables E1 and E2 in this article's Online Repository at www.jacionline.org). Furthermore, these increases were found to be predominantly in the airway epithelium of the asthmatic patients by using semiquantitative scoring (Fig 1, A, and see Fig E1, A and C). The increase in ERp57 levels also showed a positive correlation with increases in blood eosinophil counts ($n = 9$ per group) and bronchodilator responses (percentage baseline FEV₁) in asthmatic patients ($n = 8$, data

not available for 1 of the patients) compared with those in nonasthmatic ($n = 9$) subjects (see Fig E1, D and E).

To explore alterations in ERp57 expression and to investigate the effect on downstream mechanisms of asthma pathogenesis, we used a model of HDM-induced allergic airways inflammation in mice. Evaluation of whole-lung homogenates by means of Western blotting showed a marked increase in ERp57 levels, as well as levels of other ER stress markers, such as glucose-regulated protein 94, the cleaved 50-kDa fragment of ATF6 (ATF6⁵⁰), and CHOP in HDM-challenged mice compared with

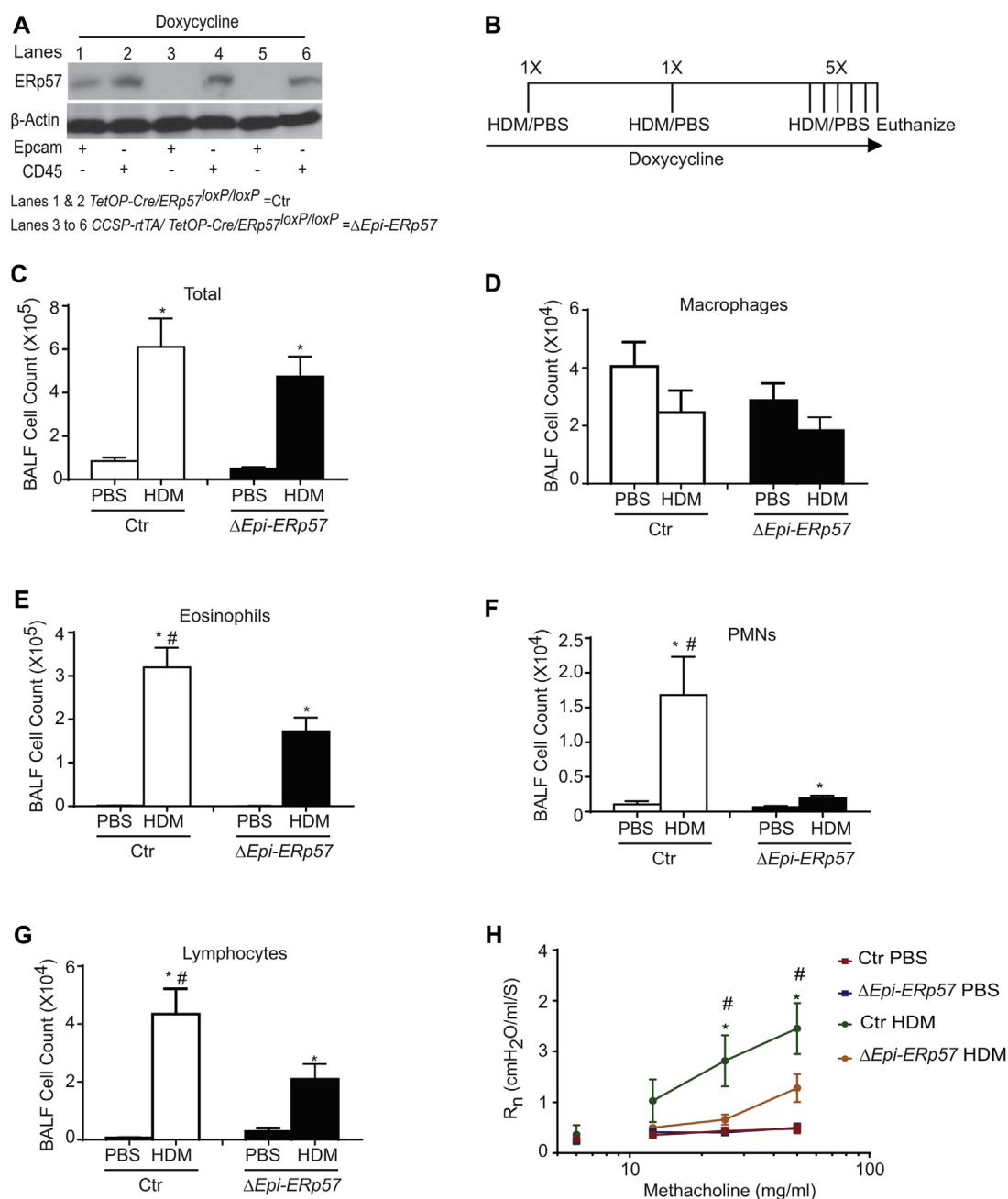


FIG 2. HDM-induced experimental asthma is attenuated in ERp57-ablated mice. **A**, Ablation of ERp57 from EpCAM⁺ epithelial cells in mice containing the *CCSP-rtTA/TetO-Cre/ERp57^{loxP/loxP}* allele. β -Actin was used as a loading control. **B**, HDM or PBS instillation regimen. **C-G**, Analysis of inflammatory and immune cells in the bronchoalveolar lavage fluid (BALF). **H**, Analysis of methacholine-induced AHR in mice. * $P < .05$, significant differences compared with PBS groups. # $P < .05$, significant differences compared with the HDM groups.

those challenged with PBS (Fig 1, B). To examine whether ERp57 levels were increased in specific cell types of the lung, as observed in human subjects, we stained for ERp57 from both PBS- and HDM-challenged lungs. Immunohistochemistry of the lungs showed that there was a dramatic increase in ERp57 levels in the epithelium of the HDM-treated lungs compared with the PBS-treated lungs (Fig 1, C). These results show that both human subjects with asthma and mice sensitized and challenged with HDM show increases in ER stress associated with an increase in ERp57 levels predominantly in the lung epithelium.

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

To determine whether increased ERp57 levels in AECs might contribute to allergic airways disease in mice, we investigated whether specific deletion of ERp57 in AECs attenuated pathophysiology associated with HDM challenge. To answer this question, we generated a doxycycline-inducible triple-transgenic *CCSP-rtTA/TetO-Cre/ERp57^{loxP/loxP}* (Δ Epi-ERp57) mouse to delete ERp57 specifically in lung epithelial cells. The mice carrying *CCSP-rtTA/ERp57^{loxP/loxP}* or *CCSP-rtTA/TetO-Cre*

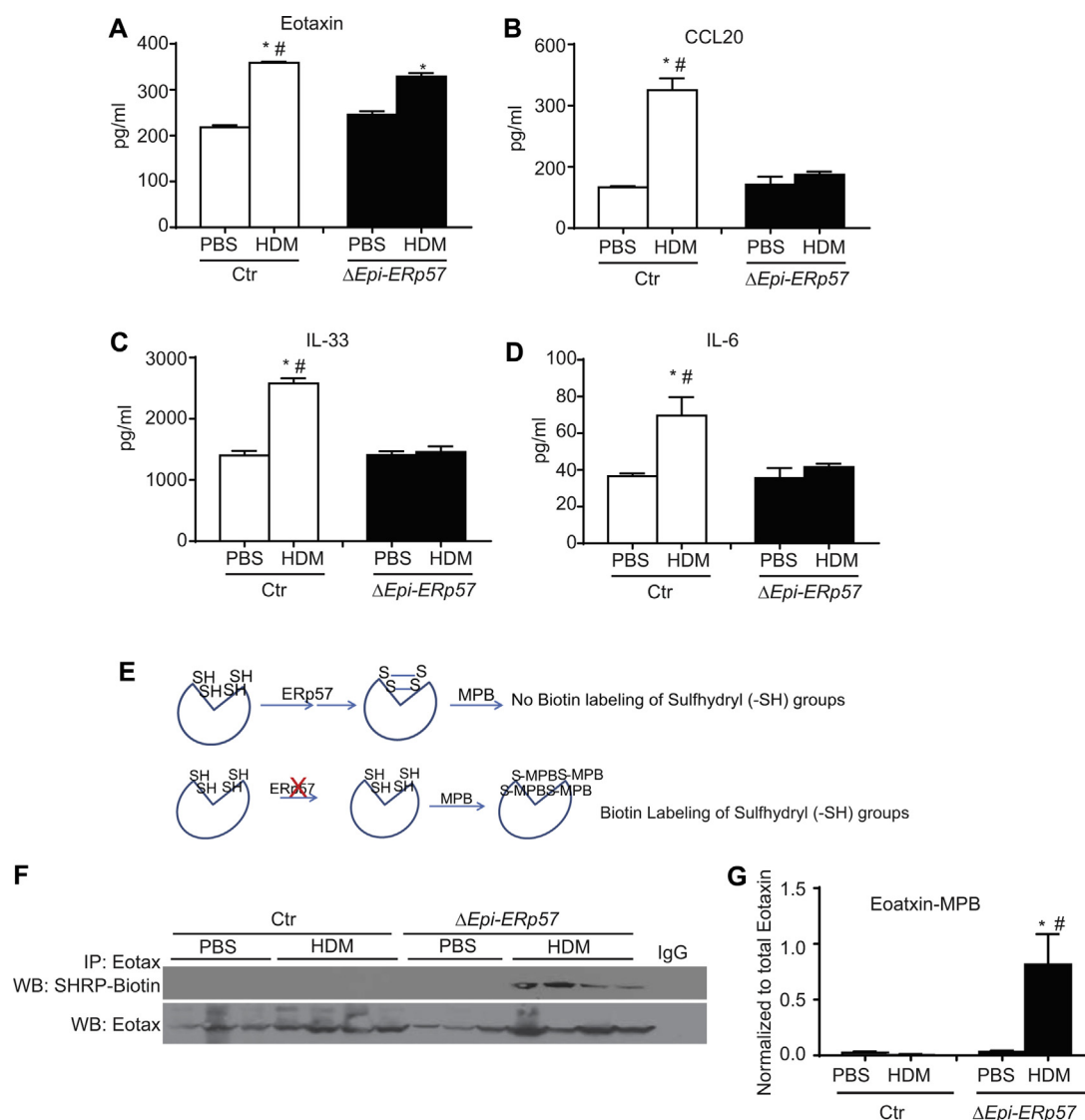


FIG 3. Deletion of ERp57 in AECs decreases levels of various cytokines and chemokines secreted from epithelial cells. **A-D**, ELISA for cytokines and chemokines. **E**, Biotin labeling of free sulfhydryl (-SH) groups with MPB. **F**, Western blot (WB) analysis of sulfhydryl (-SH) content of eotaxin by using MPB labeling and immunoprecipitation (IP). **G**, Densitometry of eotaxin-MPB in Fig 3, F. * $P < .05$, significant differences compared with PBS groups. # $P < .05$, significant differences compared with HDM groups.

were used as controls (Ctrl mice). Our analysis showed that there was a clear decrease in ERp57 levels in the epithelial cell adhesion molecule (EpCAM)⁺ cells of lungs of the doxycycline-treated $\Delta Epi-ERp57$ mice compared with those of Ctrl mice (Fig 2, A). For the experiments with allergen challenge, all mice were maintained on doxycycline for the length of the experiment beginning 5 days before initial sensitization (Fig 2, B). Analysis of total cells in bronchoalveolar lavage fluid showed that there was an increased influx in cells in both HDM-treated groups Ctrl and $\Delta Epi-ERp57$ mice (Fig 2, C). Analysis of specific inflammatory and immune cell types indicated a significant attenuation of eosinophils, neutrophils, and lymphocytes in $\Delta Epi-ERp57$ mice challenged with HDM compared with Ctrl 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 epithelium-specific ablation of ERp57 on AHR to increasing doses of inhaled methacholine (12.5, 25, and 50 mg/mL). These measurements revealed a significant decrease in AHR, as measured based on changes in central airways resistance (R_n) in $\Delta Epi-ERp57$ mice challenged with HDM compared with Ctrl 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 eosinophil, neutrophil, and lymphocyte counts and AHR. Therefore we investigated whether

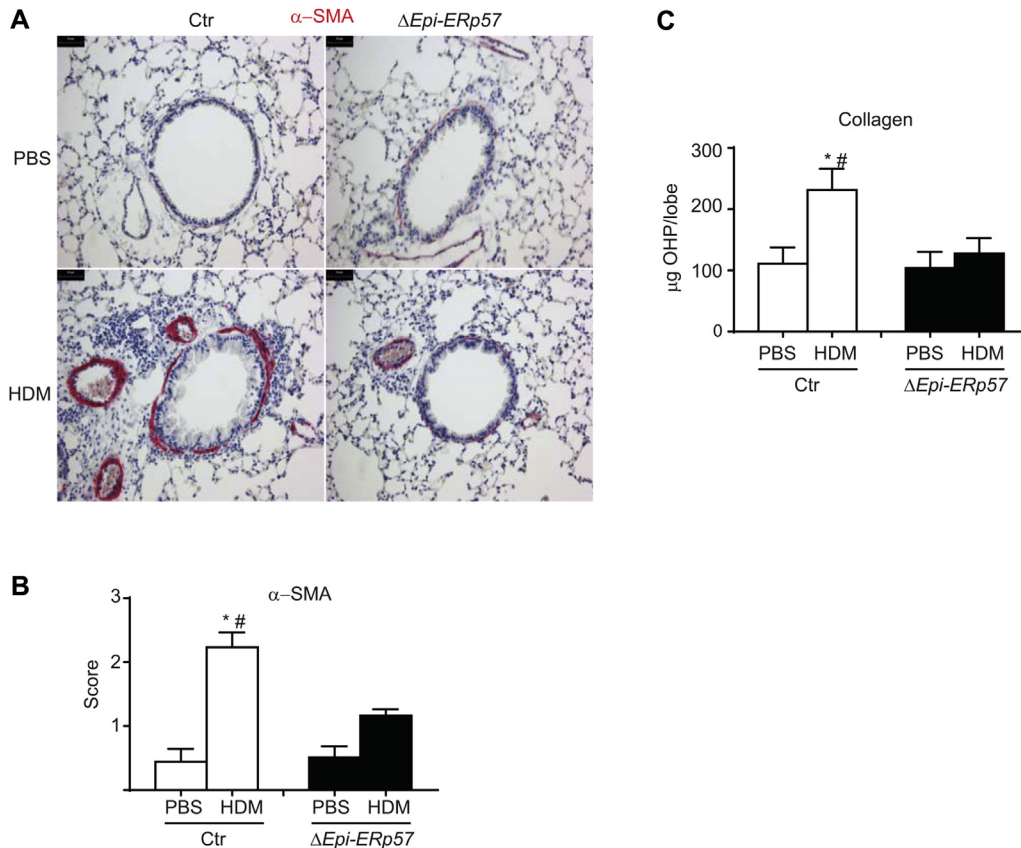


FIG 4. Ablation of ERp57 in lung epithelial cells decreases smooth muscle hypertrophy and collagen deposition. **A**, IHC staining for α -SMA in PBS- and HDM-challenged lungs from Ctr and Δ ERp57 mice. **B**, Histologic scores for α -SMA. **C**, Analysis of collagen deposition. * $P < .05$, significant differences compared with PBS groups. # $P < .05$, significant differences compared with HDM groups. Scale bars = 50 μ m.

these decreases could be explained by decreases in epithelium-derived cytokine and chemokine levels. Our analysis from whole-lung tissue lysates from Ctr and Δ Epi-ERp57 mice showed a slight but significant decrease in eotaxin levels and highly significant decreases in CCL20, IL-33, and IL-6 levels in HDM-challenged Δ Epi-ERp57 mice compared with Ctr HDM-challenged mice (Fig 3, A-D). Although there were decreased neutrophil counts in the Δ Epi-ERp57 mice, we did not find any significant alterations in production of the epithelium-derived neutrophil chemoattractant granulocyte colony-stimulating factor in PBS- or HDM-challenged mice on both genetic backgrounds (data not shown).

ERp57 is a PDI that specifically facilitates disulfide (-S-S-) bond formation on glycoproteins being processed and secreted by the ER.²¹ We next determined whether deletion of ERp57 in the airways epithelium had any effect on cytokines, such as eotaxin, a glycoprotein containing 2 disulfide bonds (<http://www.uniprot.org/uniprot/P48298>). Our analysis using labeling of cysteine sulfhydryl (-SH) groups (Fig 3, E) of eotaxin and subsequent immunoprecipitation revealed considerably fewer disulfide bonds in eotaxin in the lungs of Δ Epi-ERp57 mice compared with Ctr mice (Fig 3, F and G) challenged with HDM. Collectively, these results suggest that ERp57 deletion attenuates HDM-induced cytokine production and might also affect function because of the lack of disulfide bonds.

Epithelium-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 total lung lysates. Our results show that mRNA expression of MUC5AC or Gob5 was not decreased in Δ Epi-ERp57 mice challenged with HDM compared with that seen in Ctr mice challenged with HDM (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). Staining for mucus in the airways by using periodic acid-Schiff (PAS) also showed similar mucus production in AECs of Δ Epi-ERp57 and Ctr mice challenged with HDM (see Fig E2, C and D). These results indicate that AEC-specific deletion of ERp57 has no effect on HDM-induced mucus production.

Lung epithelium-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.^{11,15,22} Therefore we next determined whether ERp57 deletion in mice decreases HDM-induced disulfide (-S-S-)-mediated oligomerization of

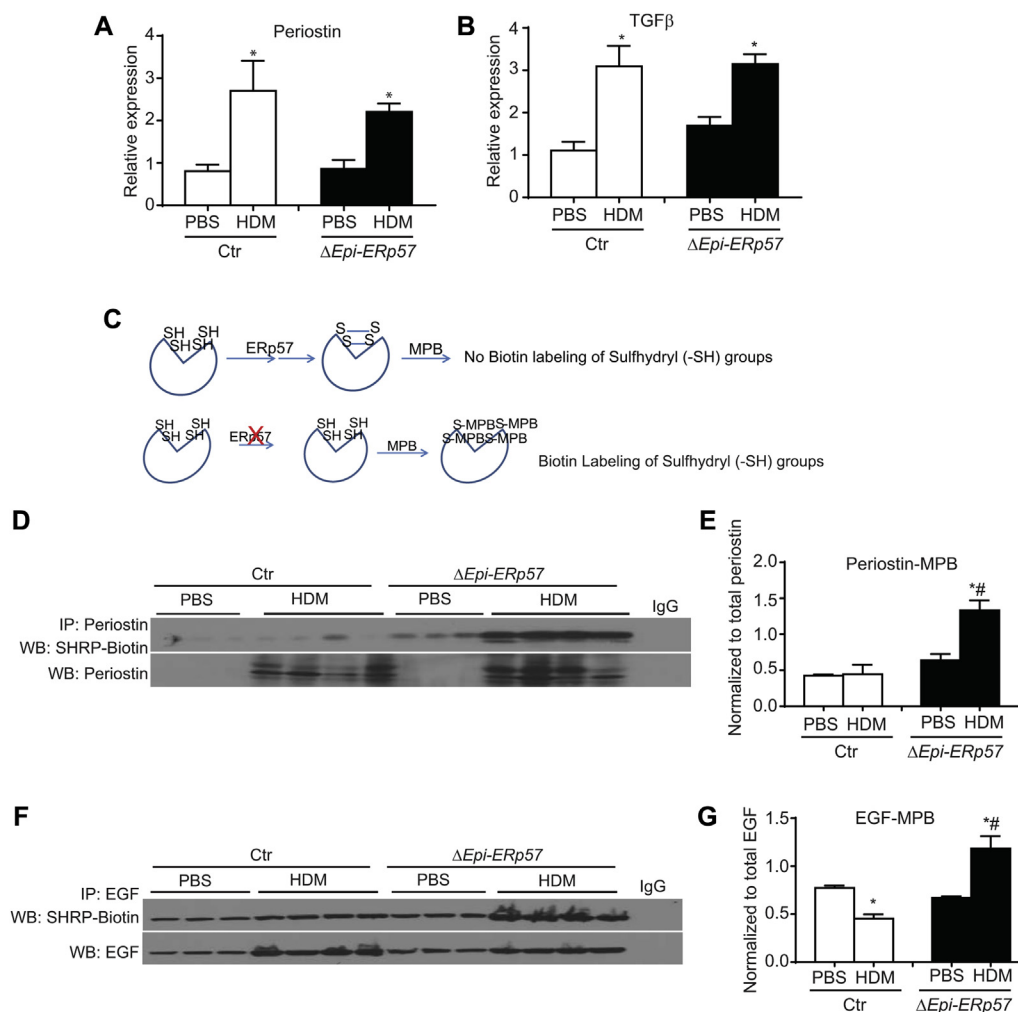


FIG 5. Ablation of ERp57 in lung epithelial cells decreases disulfide bonds (-S-S-) in profibrotic growth factors. **A** and **B**, Analysis of mRNA for epithelium-derived growth factors. **C**, Biotin labeling of free sulfhydryl (-SH) by MPB. **D**, Western blot (WB) analysis of sulfhydryl (-SH) content of periostin. **E**, Densitometry of periostin-MPB in Fig 5, **D**. **F**, Western blot analysis of sulfhydryl (-SH) content of EGF. **G**, Densitometry of the EGF-MPB in Fig 5, **F**. * $P < .05$, significant differences compared with PBS groups. # $P < .05$, significant differences compared with HDM groups. IP, Immunoprecipitation.

Bak and activation of caspase-3 (apoptotic marker). We examined the redox modification of Bak using nonreducing (-DTT) denaturing (+SDS) polyacrylamide gel electrophoresis. Our results in Fig E3 in this article's Online Repository at www.jacionline.org demonstrate that ER stress-mediated increases in ERp57 levels were associated with the promotion of disulfide (-S-S-) mediated oligomers of Bak, as evidenced by dithiothreitol (DTT)-mediated decomposition of oligomers in HDM-challenged lungs of Ctr mice compared with that in Δ Epi-ERp57 mice (see Fig E3, A). As a consequence, HDM-induced caspase-3 activity was also attenuated in ERp57-deleted Δ Epi-ERp57 mice compared with HDM-challenged Ctr mice (see Fig E3, B). Furthermore, staining for both ERp57 and active caspase-3 in serial sections (5 μ m apart) also revealed a dramatic decrease in active caspase-3 levels in the same regions of the airway epithelium that correspond to decreases in ERp57 in Δ Epi-ERp57 mice compared with HDM-challenged Ctr mice (see Fig E3, C). These results indicate that ablation of ERp57 in lung epithelial cells results in decreased

disulfide (-S-S-) mediated oligomerization of Bak and decreased allergen-induced apoptosis in the lung compared with that seen in nonablated (Ctr) mice.

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

To examine the role of ERp57 in structural airway remodeling, we assessed airways smooth muscle and collagen content after HDM exposure in Ctr and Δ Epi-ERp57 mice. HDM challenge led to increases in α smooth muscle actin (α -SMA) levels in the peribronchiolar region of HDM-challenged Ctr mice. Semiquantitative scoring by 3 independent scientists blind to the identity of the samples revealed significant decreases in α -SMA staining in HDM-challenged Δ Epi-ERp57 mice compared with Ctr mice (Fig 4, A and B). Similarly, biochemical analysis of collagen deposition showed a reduction in collagen levels after HDM challenge in Δ Epi-ERp57 compared with Ctr mice (Fig 4, C).

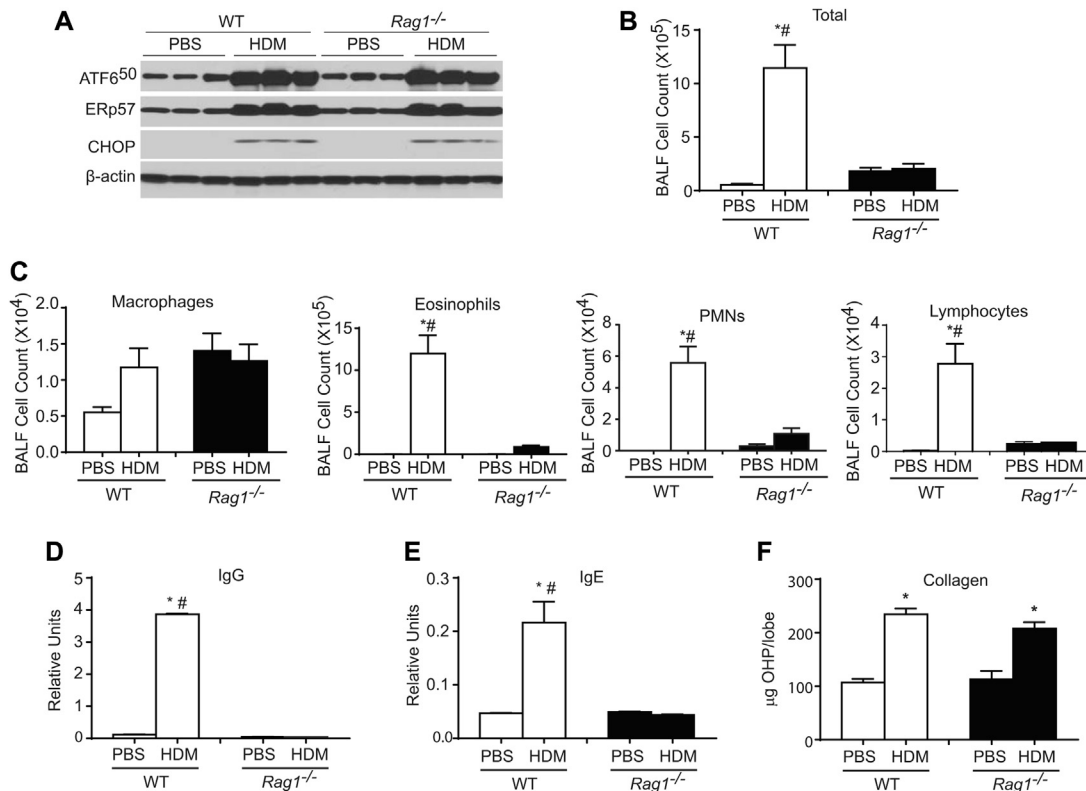


FIG 6. T or B cells are not required for HDM-induced ER stress activation and collagen deposition. **A**, Western blot analysis for various ER stress markers and ERp57 in WT and *Rag1*^{-/-} mice. β-Actin was used as a loading control. **B** and **C**, Analysis of inflammatory and immune cells in bronchoalveolar lavage fluid (BALF). **D** and **E**, Assay for production of IgG and IgE. **F**, Hydroxyproline assay. **P* < .05, significant differences compared with PBS groups. #*P* < .05, significant differences compared with HDM groups.

Analysis of the profibrotic growth factors TGF-β and periostin produced by epithelial cells during injury^{23,24} did not show significant differences in expression (Fig 5, A and B). Glycoproteins, such as periostin and epidermal growth factor (EGF; also produced by AECs),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 *ΔEpi-ERp57* mice compared with Ctr mice challenged with HDM (Fig 5, D-G). Collectively, these results indicate that ER stress mediators, specifically ERp57, control allergen-induced airways fibrosis in the lung.

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 levels were induced as a consequence of the strong adaptive immune response, we compared the response to HDM challenge in WT mice and mice deficient in *Rag1*. *Rag1* encodes an enzyme involved in recombination of the immunoglobulin and T-cell receptor genes and is essential for the generation of mature B and T lymphocytes,²⁵ 2 essential components of the adaptive immune system. The results (Fig 6) demonstrate that although HDM increased ER stress markers, such as ATF6⁵⁰ and CHOP,

in both WT and *Rag1*^{-/-} mice, there was no difference in upregulation of ER stress markers between the groups. Furthermore, we also observed similar increases in ERp57 levels in HDM-challenged WT and *Rag1*^{-/-} mice (Fig 6, A). As expected, *Rag1*^{-/-} mice exhibited dramatic decreases in total bronchoalveolar lavage fluid cells, eosinophil, PMN, and lymphocyte counts and immunoglobulin production (IgG and IgE; Fig 6, B-E). Interestingly, we did not see any significant alterations in collagen content in HDM-challenged *Rag1*^{-/-} mice compared with WT mice (Fig 6, F). These results suggest 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 asthmatic patients exhibit a marked increase in ERp57 levels compared with those in nonasthmatic subjects, predominantly in the lung epithelium. Using a murine model of allergic asthma, we demonstrated that epithelium-specific upregulation of PDI-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, upregulation of ERp57, and airways fibrosis.

Perturbations in ER homeostasis can cause UPR, leading to inflammation and, when unresolved, cell death.²⁶ Recent reports suggest that UPR-mediated activation of the transcription factor

X-box binding protein 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 peribronchiolar fibrosis. In our earlier work we demonstrated that *in vivo* a complex allergen, HDM, induces higher levels of UPR markers and upregulation of ERp57 compared with murine models of LPS or ovalbumin plus LPS challenge.¹¹ Additionally, in contrast to recent reports,^{16,17} our studies with human epithelial cells (*in vitro*) demonstrated activation of ATF6 α and upregulation of ERp57.¹¹ We believe that differences in the activation of specific UPR transducers might be due to the complex signaling pathways activated by multiple pattern recognition receptor agonists, uric acid, and proteases in the epithelium by HDM compared with the simple antigen ovalbumin or the Toll-like receptor 4 agonist LPS.^{7,9} Thus our results indicate a multifaceted mechanism of allergen-specific activation of ER stress mediators in mouse and human AECs.

Interestingly, ORMDL sphingolipid biosynthesis regulator 3 (ORMDL3), a protein linked to asthma susceptibility, contributes to alterations in ceramide and calcium homeostasis²⁷⁻²⁹ and can induce ATF6 α activation in epithelial cells.²⁹ How ATF6 α activation contributes to asthma pathophysiology remains to be determined. Previous work performed by our laboratory demonstrated that ATF6 α knockdown in human bronchiolar epithelial cells decreased HDM-induced upregulation of ERp57 and apoptosis.¹¹ However, these studies did not address the role of ERp57 as a regulator of HDM-induced proinflammatory/profibrotic response from epithelial cells *in vivo*. Our experiments here demonstrate that lung epithelium-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 epithelium-derived cytokine and chemokine production.^{1,30} 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 after HDM challenge in ERp57-deleted mice. These results indicate that deletion of ERp57 in epithelial cells could directly affect production of the above epithelium-derived, innate lymphoid cell, T-cell, and dendritic cell activators¹ that are involved in innate and adaptive immune responses after HDM exposure.

We hypothesized that by being a PDI with specificity toward glycoproteins (eg, cytokines and growth factors), ERp57 could be involved in formation of disulfide bonds (-S-S-) in specific cytokines. Furthermore, ERp57 ablation would likely both decrease the secretion of cytokines and also affect their function. We tested this hypothesis on eotaxin because 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 with Ctr mice challenged with HDM. On the basis of our results, we speculate that although eotaxin is produced in copious amounts, deficiency in disulfides might 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-1.³¹ At this juncture, we can only speculate that the decrease in production of cytokines, such as IL-33, IL-6, and the chemokine CCL20, could be due to the

inactivity of Ref-1 in ERp57-deleted AECs. However, additional targeted experiments are required to substantiate the role of ERp57 in oxidative folding of eotaxin and Ref-1 regulation in the setting of HDM-induced allergic airways disease.

Mucins are large glycosylated proteins encompassing multiple disulfide bonds.³² However, it is known that mucins are folded and secreted by epithelial cells through PDI-AGR2^{16,32,33} and not ERp57. Therefore there was no significant decrease in mucin production in HDM-challenged $\Delta Epi-ERp57$ mice compared with Ctr mice.

Allergen-induced structural remodeling of the lung was thought to be a consequence of damage to the airways epithelium and subsequent cross-talk between AECs and mesenchymal and immune cells.^{34,35} 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.^{11,15,22,36} Based on the results presented herein indicating that ERp57-deleted mice showed decreased oligomerization of Bak and the apoptosis marker active caspase-3, it is reasonable to speculate that ERp57 could be regulating apoptosis of epithelial cells during HDM challenge.

Growth factors, such as EGF, TGF- β , and periostin, released by injured airway epithelium are thought to be the potential profibrotic growth factors involved in airways structural remodeling in asthmatic patients.^{1,37} The allergen-dependent chronic activation of ER stress and apoptosis can cause repeated injury to the airway epithelium. In fact, injured epithelium in asthmatic patients, as well as in mouse models, upregulate profibrotic growth factors, stimulating proliferation of the underlying smooth muscle cells and subsequently leading to collagen deposition.³⁷ Measurement of profibrotic growth factor mRNAs in our study did not yield significant differences between the 2 genotypes challenged with HDM. This suggests that ERp57 deficiency in 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)^{23,38-40} revealed more sulfhydryl groups exposed in ERp57-deleted HDM-challenged mice compared with Ctr mice challenged with HDM. These results suggest that ERp57 might 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 might not affect production but rather inhibits function because of the lack of disulfide bonds, which are required for tertiary structure, and of 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.^{41,42} Recent studies have suggested that asthmatic patients and HDM-based mouse models of asthma have subepithelial thickening marked by α -SMA (smooth muscle hyperplasia) and increased collagen deposition,⁴³⁻⁴⁵ resulting in peribronchiolar fibrosis. Accordingly, results presented here show that HDM induces severe ER stress, leading to apoptosis of AECs and subsequent fibrosis.

Deletion of ERp57 in AECs also resulted in a significant decrease in HDM-induced central airway resistance (R_n). We did not observe statistically significant differences in tissue resistance and tissue stiffness in HDM-challenged Ctr mice compared with

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 anatomic 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, and 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 caused by increased airway closure, which is likely due to mucus metaplasia.⁴⁶ 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,^{47,48} resulting in increased central airway narrowing. However, the mechanisms by which ERp57 increase AHR of the central but not peripheral airways are yet to be determined.

Collectively, our work illuminates a previously unexplored mechanism of HDM-induced UPR and epithelial ERp57. We demonstrate that ERp57 upregulation in epithelial cells plays a regulatory role in airway inflammation, peribronchiolar fibrosis, and AHR. On the basis of our results, we believe that strategies to inhibit ERp57 in AECs might offer beneficial effects in treating allergic asthma.

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Key messages

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

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METHODS

Labeling of free sulfhydryls with *n*-ethyl maleimide

Lung tissues ($n = 3$ –4 mice per group) were lysed in Tris buffer (pH 7.4) containing 125 mmol/L NaCl and 1% NP-40 with 500 μ mol/L biotinylated *n*-ethyl maleimide (MPB) for 1 hour at ambient temperature. Lysates were then frozen at -20°C overnight. Thawed lysates were centrifuged at 14,000 rpm for 10 minutes and passed through a Micro Bio-Spin (Bio-Rad Laboratories, Hercules, Calif) column to separate free MPB. The eluent whole-lung lysate was immunoprecipitated with anti-eotaxin (R&D Systems, Minneapolis, Minn), periostin (Abcam, Cambridge, United Kingdom), or EGF (Santa Cruz Biotechnology, Dallas, Tex) antibodies and sequentially probed, initially with streptavidin–horseradish peroxidase, to detect biotinylated free sulfhydryl groups and then with anti-eotaxin, periostin, or EGF protein-specific antibodies.

Nonreducing gel electrophoresis

Lung homogenates were suspended in loading buffer without the reducing agent DTT. A separate set of samples was resuspended in loading buffer with DTT to reduce the disulfide bonds. The samples were resolved by means of SDS-PAGE and subjected to Western blot analysis.

HDM model of allergic airways disease

For all experiments, 8- to 12-week-old mice were used, as approved by the Institutional Animal Care and Use Committee. Mice ($n = 10$ per group) were anesthetized with isoflurane and exposed to 50 μ g of HDM (containing 35 endotoxin units/mg; GREE, Lenoir, NC) extract suspended in PBS through intranasal administration on day 0 and boosted again on day 7. Mice were then administered 50 μ g of HDM consecutively on days 14 to 18 and killed 24 hours after final exposure. The control group was given 50 μ L of sterile PBS alone at all time points.

Deletion of ERp57

Confirmation of deletion of ERp57 from epithelial cells in mice containing *CCSP-rtTA/TetOP-Cre/Erp57^{loxP/loxP}* was done after administration of doxycycline-containing food for 5 days. Single-cell lung suspensions were prepared, and cells were sorted by using flow cytometry. The EpCAM⁺CD45^{low} Sca1^{low} fraction of lung cells was isolated for assessment of ERp57 expression by means of Western blot analysis. β -Actin was used as a loading control. CD45⁺ cells were selected as nonepithelial controls.

Assessment of AHR

Mice ($n = 8$ –10 per group) were anesthetized with an intraperitoneal injection of pentobarbital sodium (90 mg/kg), tracheotomized with an 18-gauge cannula, and mechanically ventilated at 200 breaths/min with a flexiVent computer-controlled small-animal ventilator (SCIREQ, Montreal, Quebec, Canada). While on the ventilator, mice also received the paralytic pancuronium bromide. Newtonian resistance (R_n) was calculated, as previously described.^{E1,E2} Airway responsiveness is represented as the average of the 3 peak measurements for each animal obtained at incremental methacholine doses.

Bronchoalveolar lavage fluid processing

Bronchoalveolar lavage fluid from mice ($n = 8$ –10 per group) was collected. Total and differential cell counts were performed, as previously described.^{E3} Briefly, cells were isolated by means of centrifugation, and total cell counts were enumerated with the Advia 120 Automated Hematology Analyzer System (Siemens Healthcare, Malvern, Pa). Differential cell counts were obtained through cytopspins with Hema3 stain reagents (Fisher Scientific, Waltham, Mass). Differentials were performed on a minimum of 300 cells per animal.

Western blot analysis

After dissection, right lung lobes were flash-frozen for protein analysis. Lungs were pulverized and lysed in buffer containing 137 mmol/L Tris/HCl (pH 8.0), 130 mmol/L NaCl, and 1% NP-40. Proteins from cell lysates were prepared in the same buffer. Insoluble proteins were pelleted by means of centrifugation, and after protein quantitation of the supernatant, samples were suspended in loading buffer with DTT and resolved by using SDS-PAGE. Proteins were transferred to polyvinylidene difluoride, and membranes were probed by using a standard immunoblotting protocol with the following primary antibodies: glucose-regulated protein 78, ATF6⁵⁰ and CHOP (Abcam), ERp57, glucose-regulated protein 94 (Stressgen, San Diego, Calif), and β -actin (Sigma, St Louis, Mo).

Measurement of collagen and immunohistochemistry

Collagen content was measured by using the hydroxyproline assay ($n = 8$ –10 per group). Briefly, lung lobes were diced and placed in 500 μ L of 10 mg/mL pepsin in 0.5 mol/L acetic acid for 3 hours at 37°C or until lungs were completely digested. The digest was spun at 10,000g for 10 minutes at room temperature. Fifty microliters of the supernatant was mixed vigorously with 500 μ L of Sircol dye (Bicolor.co.uk) solution for 30 minutes and then spun again at 10,000g for 10 minutes. Excess dye was decanted off, and the resulting pellet was dissolved in 500 μ L of an alkaline solution, 200 μ L of which was pipetted in duplicates into a 96-well plate and measured at 540 nm. Fixed sections were prepared for immunostaining by means of deparaffinization with xylene and rehydration through a series of ethanols to evaluate regional changes in ERp57 (Stressgen), α -SMA (Sigma), and active caspase-3 (Cell Signaling, Danvers, Mass) levels. For antigen retrieval, slides were heated for 20 minutes in 95°C citrate buffer (pH 6.0) and then rinsed in distilled water. Sections were then blocked for 1 hour in blocking serum per the manufacturer's instructions (Vectastain Alkaline Phosphatase Universal; Vector Laboratories, Burlingame, Calif). Slides were then washed in TBS with 0.1% Tween-20 3 times for 5 minutes each, followed by incubation with primary antibody for α -SMA (Sigma) overnight at 4°C . Sections were washed again and incubated with a biotinylated universal secondary antibody (Vectastain Alkaline Phosphatase Universal, Vector Laboratories) for 30 minutes at room temperature. Slides were washed and incubated with the Vectastain ABC-AP reagent (prepared per manufacturer's instructions) for 30 minutes at room temperature. Sections were then incubated with Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories) for 20 minutes at room temperature, rinsed with tap water, and counterstained with Mayer hematoxylin. The ERp57 and active caspase-3 detection in Fig E3, C, was performed on serial sections that were 5 μ m apart on the same slides.

Semiquantitative scoring

Imaging for α -SMA, ERp57, and PAS was performed at $\times 20$ magnification. Histochemistry staining was assessed with a blinded scoring system by 3 independent investigators with the following scale: 0, no reactivity; 1, minimal staining; 2, moderate staining; and 3, prominent staining. Scores were averaged according to treatment group.

ELISA

Eotaxin, granulocyte colony-stimulating factor, CCL20, IL-33, and IL-6 were detected by means of ELISA in lung homogenates (normalized for protein), according to the manufacturer's instructions (R&D Systems).

Measurement of serum IgG₁ and IgE levels

After death, blood was collected by means of heart puncture and immediately spun through a microtainer. Serum was collected, and IgG₁ and IgE content was determined by using an ELISA-based method with a 96-well plate preincubated with 1 μ g/mL HDM.

Analysis of mRNA from tissue samples

Right lung lobes were flash-frozen and pulverized, and total RNA was isolated and purified with the RNeasy kit (Qiagen, Hilden, Germany). One microgram of RNA was reverse transcribed to cDNA for quantitative assessment of gene expression by using SYBR Green (Bio-Rad Laboratories) to measure periostin, TGF- β , mucin 5ac (MUC5ac), and mclca3 (Gob5) expression. Expression values were normalized to the housekeeping gene cyclophilin. The primers used in this study are as follows: MUC5ac, forward 5'-CAGTGAATTCTGGAGGCCAACAAAGGTAGAG-3' and reverse 5'-CTAAGCTTAGATCTGGTTGGGACAGCAGC-3'; GOB5, forward 5'-AC TAAGGTGGCCTACCTCAA-3' and reverse 5'-GGAGGTGACAGTCAA GGTGAGA-3'; periostin, forward 5'-CGAAGGGGACAGTATCTCCA-3' and reverse 5'-GCTTCAGAGAGGATGCCAAG-3'; and cyclophilin, forward 5'-TTCCTCCTTCACAGAATTATTCCA-3' and reverse 5'-CCAGTGCCAT

TATGG-3'; and TGF- β , forward 5'-GGCCTTGAAGCATGTAGAGG-3' and reverse 5'-GGAGAACTCGTTAGAGACGACTT-3'.

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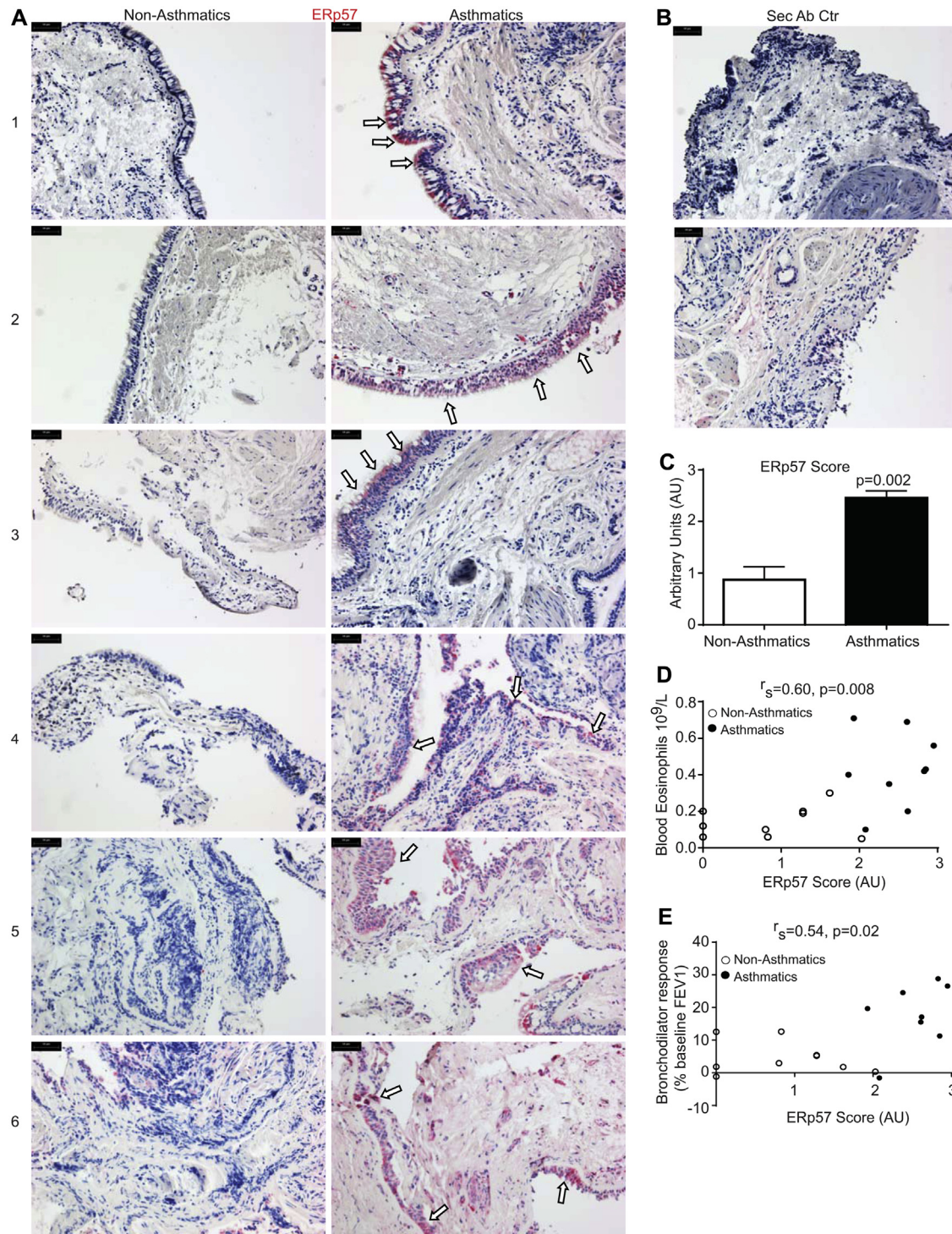


FIG E1. ERp57 levels increases are associated with increases in eosinophil counts and bronchodilator response. **A**, Representative images of paraffin-embedded human lung tissue samples obtained from asthmatic human subjects 1 to 3 were obtained from UCSF (Table E1), and those from subjects 4 to 6 were obtained from the Cleveland Clinic (Table E2). Tissues were stained for ERp57 (red). Scale bars = 50 μ m. **B**, Lung tissue samples stained with secondary antibody alone. Scale bars = 50 μ m. **C**, Semiquantitative histologic scores for ERp57. **D** and **E**, Correlations between ERp57 scores and blood eosinophil counts or bronchodilator response in nonasthmatic and asthmatic subjects.

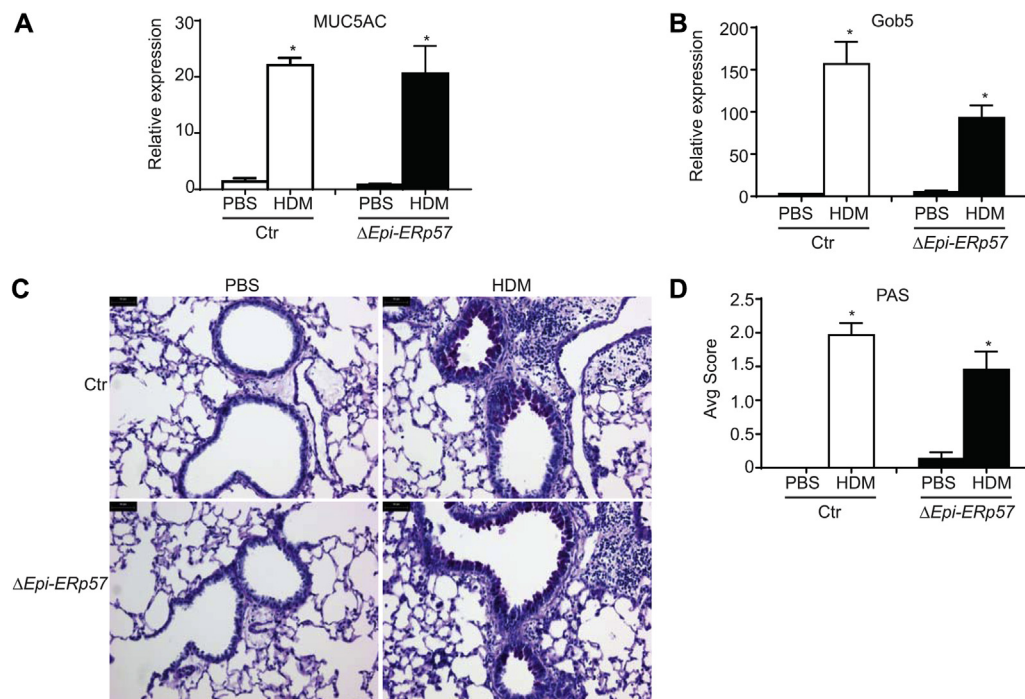


FIG E2. Deletion of ERp57 has no effect on HDM-induced mucin production. **A** and **B**, Quantitative RT-PCR analysis for MUC5AC and Gob5. **C**, Representative histopathologic images of PAS staining of WT and $\Delta ERp57$ mice. **D**, PAS scoring on Ctr and ERp57-deleted mouse lungs. * $P < .05$, ANOVA; significant differences compared with PBS groups. Scale bars = 50 μ m.

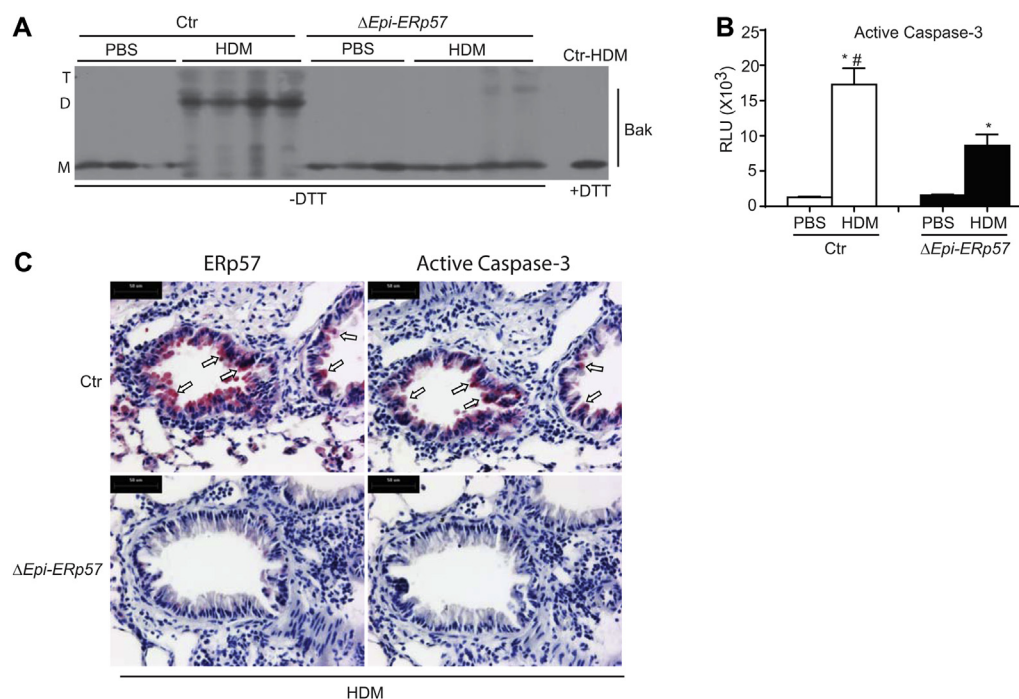


FIG E3. Deletion of ERp57 decreases intrinsic apoptosis in HDM-challenged mouse lungs. **A**, Nonreducing SDS-PAGE showing disulfide (-S-S)-mediated Bak oligomerization in response to HDM. *D*, Dimer; *M*, monomer; *T*, trimer. **B**, Caspase-3 activity, as measured in whole-lung lysates. * $P < .05$, ANOVA; significant differences compared with PBS groups. # $P < .05$, ANOVA; significant differences compared with HDM groups ($n = 7-8$ mice per group). **C**, Representative IHC images of WT and $\Delta Epi-ERp57$ mice challenged with HDM and stained with anti-ERp57 or anti-active caspase-3 antibody. Sequential lung sections 5 μ m apart were stained. Red color represents positive staining in AECs. Scale bars = 50 μ m.

TABLE E1. Subjects' baseline characteristics: UCSF

	Healthy control subjects	Asthmatic patients	<i>P</i> value
Sample size	6	6	
Age (y)	33 ± 7	34 ± 13	.89
BMI (kg/m ²)	26 ± 3	27 ± 4	.73
Sex (% female)	50	33	1
Ethnicity			
White	3	3	
African American	1	1	
Hispanic	1	1	
Asian/Pacific Islander	1	1	
FEV ₁ (% predicted)	96 ± 7	85 ± 13	.09
Δ FEV ₁ with albuterol (% of baseline)	5.2 ± 6.1	21.1 ± 6.8	.002
FEV ₁ /FVC ratio	0.80 ± 0.065	0.69 ± 0.058	.01
Methacholine PC ₂₀ (mg/mL)	>10	0.51 (0.15-1.34)	.0001
IgE (IU/mL)	14 (4-29)	301 (21-6556)	.01
Blood eosinophils (×10 ⁹ /L)	0.09 (0.05-0.30)	0.50 (0.35-0.71)	.005
FENO	15 (10-36)	69 (43-109)	.004
Positive skin prick response (no.)	1 (1-2)	4 (2-11)	.003

Values are reported as means ± SDs or medians (ranges). All data are from the baseline visit, except spirometric data, which are reported from visit 2 (immediately before initiation of inhaled corticosteroids). *P* values are based on the Welch *t* test (mean ± SD), the χ^2 test for proportions (sex), the Wilcoxon rank sum test (median [range]), or Poisson regression (skin prick tests). All subjects were nonsmokers defined as never smokers or former smokers with no smoking for at least 1 year before enrollment and total pack years of 15 or less.

FENO, Fraction of exhaled nitric oxide; FVC, forced vital capacity.

TABLE E2. Subjects' baseline characteristics: Cleveland Clinic

	Healthy control subjects	Asthmatic patients	<i>P</i> value
Sample size	3	3	
Age (y)	33 ± 7	34 ± 13	.192
BMI (kg/m ²)	26 ± 5	34 ± 2	.013*
Sex (% female)	33	33	1
Ethnicity			
White	2	1	
African American	1	2	
FEV ₁ (% predicted)	95 ± 4	68 ± 4	.007*
Δ FEV ₁ with albuterol (% of baseline)	6.1 ± 2.0	12.9 ± 3.5	.161
FEV ₁ /FVC ratio	0.76 ± 0.033	0.69 ± 0.033	.227
Methacholine PC ₂₀ (mg/ mL)	NA	1.75 (0.969-2.30)	
IgE (IU/mL)	39 (16-63)	224 (19-334)	.275
Blood eosinophils (×10 ⁹ / L)	0.17 (0.1-0.2)	0.23 (0.1-0.4)	.637
FENO	21.5 (16-26.5)	33.3 (24-43)	.248
Positive skin prick response (no.)	3 (0-6)	4 (3-5)	.512

Values are reported as means ± SDs or medians (ranges). All data are from the baseline visit, except spirometric data, which are reported from visit 2 (immediately before initiation of inhaled corticosteroids). *P* values are based on the Welch *t* test (mean ± SD), the χ^2 test for proportions (sex), the Wilcoxon rank sum test (median [range]), or Poisson regression (skin prick tests). All subjects were nonsmokers defined as never smokers or former smokers with no smoking for at least 1 year before enrollment and total pack years of 15 or less.

FENO, Fraction of exhaled nitric oxide; FVC, forced vital capacity.

*Significantly different as compared to controls.