TLR4 Protein Contributes to Cigarette Smoke-induced Matrix Metalloproteinase-1 (MMP-1) Expression in Chronic Obstructive Pulmonary Disease

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Cigarette smoke is the major risk factor associated with the development of chronic obstructive pulmonary disease and alters expression of proteolytic enzymes that contribute to disease pathology. Previously, we reported that smoke exposure leads to the induction of matrix metalloproteinase-1 (MMP-1) through the activation of ERK1/2, which is critical to the development of emphysema. To date, the upstream signaling pathway by which cigarette smoke induces MMP-1 expression has been undefined. This study demonstrates that cigarette smoke mediates MMP-1 expression via activation of the TLR4 signaling cascade. In vitro cell culture studies demonstrated that cigarette smoke-induced MMP-1 was regulated by TLR4 via MyD88/IRAK1. Blockade of TLR4 or inhibition of IRAK1 prevented cigarette smoke induction of MMP-1. Mice exposed to acute levels of cigarette smoke exhibited increased TLR4 expression. To further confirm the in vivo relevance of this signaling pathway, rabbits exposed to acute cigarette smoke were found to have elevated TLR4 signaling and subsequent MMP-1 expression. Additionally, lungs from smokers exhibited elevated TLR4 and MMP-1 levels. Therefore, our data indicate that TLR4 signaling, through MyD88 and IRAK1, plays a predominant role in MMP-1 induction by cigarette smoke. The identification of the TLR4 pathway as a regulator of smoke-induced protease production presents a series of novel targets for future therapy in chronic obstructive pulmonary disease.

Expression of the interstitial collagenase matrix metalloproteinase-1 (MMP-1) is tightly regulated during embryonic development and disease (1). Elevated expression of MMP-1 is observed in several pulmonary diseases associated with cigarette smoke exposure, including emphysema (2) and lung cancer (3, 4). Studies in our laboratory demonstrated that cigarette smoke up-regulates MMP-1 expression in lung epithelial cells through a MAPK-driven pathway (2). Increased expression of MMP-1 is seen in patients with emphysema (5, 6), and transgenic expression of human MMP-1 in the lung leads to lung destruction and emphysema in mice (7, 8). The MMP-1 promoter is a direct target of cigarette smoke in lung epithelial cells (9), and a cigarette smoke response element is observed in the MMP-1 promoter (10). To block this pathway, it is important to identify the receptor involved in cigarette smoke exposure recognition and subsequent MMP-1 expression.

Major regulatory receptors upstream of MAPK are the Toll-like receptors (TLRs), which are key pattern recognition receptors in innate immunity (11). Classically, TLRs recognize a variety of pathogen-associated microbial patterns. However, recent evidence suggests that TLR signaling is not restricted to microorganism particles but that TLRs recognize a wide variety of signals such as heat shock proteins (12), hyaluronan fragments (13), oxidative stress (14), neutrophil elastase (15), and fibronectin fragments (16). In this non-classical signaling, TLRs have also been associated with disease pathology in several clinical conditions such as atherosclerosis (17), osteoarthritis (16), idiopathic pulmonary fibrosis (18), cancer (19, 20), and emphysema (21, 22). TLRs initiate signaling pathways similar to IL-1, involving molecules such as MyD88 (myeloid differentiation factor 88), IL-1 receptor-associated kinases (IRAKs), and NF-κB activation. All TLRs (except TLR3 and TLR4) utilize the MyD88-dependent pathway. TLR4 can signal in an MyD88-dependent or MyD88-independent pathway (23). TLRs play a role in lung disease, but the direct functional role of TLRs in chronic obstructive pulmonary disease has yet to be elucidated. TLRs also represent a therapeutic target in other lung diseases such as cystic fibrosis (24) and idiopathic pulmonary fibrosis (18). Recently, cigarette smoke was demonstrated to induce inflammation in lungs via TLR4/MyD88 and IL-1 receptor-1/MyD88 signaling (25). However, the direct impact on protease production remains unexplained.

TLR4 deficiency causes pulmonary emphysema in mice that is not associated with an inflammatory response in bronchoalveolar lavage or lung tissue de nova (26); however, subacute cigarette smoke has been demonstrated to induce TLR4-dependent inflammation (25, 27). Therefore, the role of TLR4 in cigarette smoke-induced emphysema remains unclear. Because cigarette smoke-induced MMP-1 expression contributes to the development of chronic obstructive pulmonary disease (5, 7–10, 28), we used human primary small airway epithelial (SAE) cells to elucidate the relationship between TLRs and MMP-1 signaling under cigarette smoke conditions. Additionally, mice and rabbits were exposed to cigarette smoke, and subsequent TLR4 expression was determined. Our approach...
examines the TLR4 response following cigarette smoke exposure and the subsequent effect of TLR4 blockade on MMP-1 expression in SAE cells. Our data suggest that TLRs play a role in the expression profile of MMPs that can directly contribute to disease progression.

**EXPERIMENTAL PROCEDURES**

**Primary Human Cell Culture and Reagents Used**—Human SAE cells were cultured according to the instructions of the supplier (Lonza, San Diego, CA). Cells were serum-starved 6 h prior to stimuli and remained in serum-free conditions during stimulation. Unless specified, all reagents were purchased from Sigma. When examining IRAK1 and ERK inhibition, the IRAK1/4 (Calbiochem 407601) and ERK (Calbiochem 513000; PD98059) inhibitors were added to the culture medium at 50 and 2 μM, respectively, 1 h prior to cigarette smoke extract (CSE) stimulation. Cells were treated with 1.5 μM C6-ceramide (Cayman Chemicals, Ann Arbor, MI) or 1 μg/ml monophosphoryl lipid A (MPL; InvivoGen, San Diego, CA) as positive inducers of TLR4 signaling. All other TLR ligands were obtained from a human TLR1–9 agonist kit (InvivoGen trlkit1h). SAE cells were treated with 5 mM N-acetyl-L-cysteine 1 h prior to CSE stimulation. Cells were pretreated with 20 μg/ml anti-human TLR4 antibody (eBioscience 14-9917) for 1 h prior to CSE treatment (29). IL-8 and MMP-1 levels were determined by ELISA using a human CXCL8/IL-8 Quantikine ELISA kit (R&D Systems D8000C) and the MMP-1 human Bio- trak assay (GE Healthcare RPN2610), respectively. Tissue inhibitor of metalloproteinase-1 (TIMP-1) and active MMP-1 levels were determined using a human TIMP-1 Quantikine ELISA kit (R&D Systems D8000C) and the MMP-1 human Bio- trak assay (GE Healthcare RPN2610), respectively. Tissue inhibitor of metalloproteinase-1 (TIMP-1) and active MMP-1 levels were determined using a human TIMP-1 Quantikine ELISA kit (DTM100) and a human active MMP-1 Fluorokine E kit (FM100), respectively (R&D Systems).

**Animals**—To determine the in vivo effects of smoke exposure, 8-week-old C57BL/6J mice were exposed to cigarette smoke in a specially designed smoking chamber (Teague Enterprises, Davis, CA). Mice were exposed daily to 5 h of mainstream (active) and sidestream (passive) smoke from 3R4F research-grade cigarettes (University of Kentucky, Lexington, KY) for 5 days/week for 8 weeks. Levels of carboxyhemoglobin in the blood did not exceed 10%. Control mice were exposed to room air. Animals were provided food and water ad libitum. Mice were killed by an overdose of isoflurane. Following smoke exposure, lungs were collected for protein and mRNA analysis. New Zealand White rabbits (Covance) were also exposed to cigarette smoke for 16 weeks under the same conditions described above (n = 10). Following smoke exposure, lungs were collected for protein analysis. All experiments were approved by the Institute for Animal Care and Use Committee at Columbia University.

**Human Lung Samples**—Human lung tissues were collected from patients at the New York Columbia Presbyterian Medical Center (New York, NY) under institutional guidelines as described previously (6). Samples were from age-matched patients that were nonsmokers without chronic obstructive pulmonary disease (n = 4) and smokers with chronic obstructive pulmonary disease (n = 10). All of the emphysema subjects had advanced disease (Gold 3 or greater) and had not smoked for at least 4 months prior to surgery (lung volume reduction surgery or transplant). The control samples were obtained from age-matched individuals who underwent lung resection for benign nodules. The Institutional Review Board at Columbia University approved use of these samples.

**Preparation of CSE and Cell Treatment**—CSE was prepared using a modified protocol (30). Briefly, a Barnant vacuum pump operating at constant airflow was used to draw the smoke of one 3R4F research-grade cigarette through 25 ml of Dulbecco’s phosphate-buffered saline. This solution (100% CSE) was adjusted to pH 7.4, treated twice with an endotoxin removal reagent (MO BIO 12625-25) as described by manufacturer, sterile-filtered (0.22 μm), and added to growth medium to a final concentration of 5% within 60 min of preparation. Endotoxin levels in CSE were examined using a ToxinSensor chromogenic Limulus amebocyte lysate endotoxin assay kit (Gen- Script, Piscataway, NJ) as described by manufacturer.

**Quantitative PCR (qPCR) Analysis**—Total RNA was isolated from cells and lung tissue using an RNeasy minikit (Qiagen, Valencia, CA) as described by the manufacturer. The gene transcript levels of human MMP-1 (Hs00899658_m1) and two housekeeping genes (ACTB (Hs99999903_m1) and GAPDH (Hs02758991_g1)) were quantified by real-time PCR with the use of an ABI PRISM® 7900HT sequence detection system (Applied Biosystems). TaqMan® gene expression assays were purchased from Applied Biosystems.

**Immunoblotting**—Cell monolayers were collected in cold phosphate-buffered saline and resuspended in 100 μl of protein lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% glycerol, 1 mM EDTA, and 0.05% Tween 20 (pH 9.0)). Tissue samples were homogenized using a modified protocol (30). Briefly, a Barnant vacuum pump operating at constant airflow was used to draw the smoke of one 3R4F research-grade cigarette through 25 ml of Dulbecco’s phosphate-buffered saline. This solution (100% CSE) was adjusted to pH 7.4, treated twice with an endotoxin removal reagent (MO BIO 12625-25) as described by manufacturer, sterile-filtered (0.22 μm), and added to growth medium to a final concentration of 5% within 60 min of preparation. Endotoxin levels in CSE were examined using a ToxinSensor chromogenic Limulus amebocyte lysate endotoxin assay kit (Gen-Script, Piscataway, NJ) as described by manufacturer.

**Immunohistochemistry**—SAE cells were cultured and stimulated overnight. Cells were fixed with 4% paraformaldehyde, washed with PBS, permeabilized with 0.2% Triton X-100, and blocked with 2% BSA. Cells were stained with anti-MMP-1 antibody. Detection was performed with Alexa Fluor-labeled anti-goat fluorescent antibody (Invitrogen, Carlsbad, CA). Samples were counterstained with DAPI. Isotype control goat IgG was used as a negative control in each assay. Paraffin-embedded human samples were sectioned at 6 μm. Non-enzymatic unmasking was accomplished by boiling the slides in a microwave oven in Tris/EDTA buffer (10 mM Tris base, 1 mM EDTA, and 0.05% Tween 20 (pH 9.0)). Endogenous peroxidase was quenched by a 10-min incubation in 3% H₂O₂. Sections were stained with anti-TLR4 polyclonal antibody. Detection was performed with Alexa Fluor-labeled anti-rabbit fluorescent antibody (Invitrogen), and samples were counterstained with...
RESULTS

Cigarette Smoke-induced MMP-1 Is Independent of Microbial Toxins—The chronic inflammation and protease/anti-protease imbalance caused by cigarette smoke have been associated with the tar and gas phases of smoke, as well as the microbial toxins in the tobacco (31, 32). We assessed the endotoxin levels in CSE and observed high levels of endotoxin in CSE (Fig. 1A), similar to levels observed previously (33). However, the endotoxin present in CSE could be removed, resulting in an endotoxin-free CSE preparation. CSE were examined for MMP-1 induction pre- and post-endotoxin removal. Previously, LPS was shown to induce MMP-1 via TLR4 and AP-1 activation (34, 35). CSE induced MMP-1 independent of the presence of endotoxin in SAE cells (Fig. 1B). Therefore, tobacco-associated endotoxin is not the principal source for MMP-1 expression in SAE cells. Additionally, CSE-induced IL-8 in SAE cells was also independent of the presence of endotoxin (Fig. 1C).

Activation of TLR4 Leads to Increased MMP-1 Expression in SAE Cells—Previous studies have shown that cigarette smoke regulates TLR4 (36) and IL-8 (37) expression in human macrophages. Therefore, we assessed the MMP-1 response to ligands of various TLRs by treating SAE cells with several ligands specific to TLR activation. We observed that only TLR3 (poly(I:C)) and TLR4 (LPS, MPL, and ceramide) ligands resulted in increased MMP-1 expression in SAE cells (Fig. 2A). Several TLR ligands (TLR2, heat-killed preparation of Listeria monocytogenes; TLR3, poly(I:C); TLR4, LPS, MPL, and ceramide; TLR5, flagellin; and TLR8, ssRNA40) resulted in increased levels of secreted MMP-1 in the SAE cell medium (Fig. 2B). Therefore, TLR2, TLR5, and TLR8 activation leads to MMP-1 secretion without altered gene expression. Others have also demonstrated that poly(I:C) regulates TLR3 and influences expression of several MMPs and adaptor proteins in primary lung epithelial cells, which could also influence lung morphology in patients exposed to smoke and viral infections (38). Additionally, the prominent anti-protease that regulates MMP-1 activity, TIMP-1, was assessed in SAE cells following exposure to TLR ligands. Interestingly, TIMP-1 gene expression levels were altered by all TLR ligands except TLR3 and TLR4 ligands (supplemental Fig. S1A). However, TIMP-1 protein levels were unaltered in the culture medium 24 h after stimulation (supplemental Fig. S1B).
CSE-induced IRAK1 Phosphorylation and Blockade of IRAK1/4 Prevent Cigarette Smoke-induced MMP-1—Utilizing SAE cells, blocking TLR4 signaling using a TLR4 inhibitory antibody resulted in a significant reduction in CSE-mediated MMP-1 induction (Fig. 3A). It is well established that MyD88 recruitment leads to IRAK1 phosphorylation, which ultimately leads to the subsequent expression of numerous genes (39). A short-term exposure (30 min) of CSE was sufficient to induce IRAK1 phosphorylation and loss of total IRAK1 (Fig. 3B) at similar levels to ceramide and MPL stimulation. Additionally, blockade of TLR4 using a specific TLR4 inhibitory antibody led to the inhibition of CSE-induced IRAK1 phosphorylation (Fig. 3C). Ceramide induces MMP-1 expression by similar means to CSE via MAPKs (40), and ceramide has been shown to activate TLR4 signaling (41). Interestingly, we observed that CSE induced MMP-1 to the same levels as ceramide and to greater levels compared with MPL (Figs. 2A and 3D). To determine the importance of IRAK1 activity in CSE-induced MMP-1, we blocked CSE-induced IRAK1 activity and examined the subsequent MMP-1 levels. We utilized an IRAK1/4 inhibitor that is a cell-permeable benzimidazole compound, which acts as a potent and selective inhibitor of IRAKs and has little activity against other kinases (42). Blockade of IRAK1 activity using the IRAK1/4 inhibitor led to inhibition of CSE-induced MMP-1, as confirmed by qPCR (Fig. 3D, upper panel), ELISA (Fig. 3D, lower panel), and immunohistochemistry (Fig. 3E). Utilizing siRNA technology, IRAK1 expression was disrupted (Fig. 4A) and resulted in a significant reduction in CSE-induced MMP-1 expression and secretion (Fig. 4, B and C), whereas control siRNA had no effect on the CSE induction of MMP-1 expression. Additionally, the silencing study resulted in reduced levels of active MMP-1 in the cell medium without altering TIMP-1 levels (Fig. 4, D and E).

CSE-induced ERK Phosphorylation Is IRAK1-dependent—Because CSE stimulation leads to phosphorylation of ERK in SAE cells (2), we examined the phosphorylation of ERK in the presence and absence of IRAK1/4 inhibition. Consistent with TLR4 upstream regulation, inhibition of IRAK1/4 activity reduced CSE-induced phospho-ERK levels (Fig. 5A). The blockade of ERK activity resulted in reduced CSE-, LPS-, and ceramide-induced MMP-1 expression (Fig. 5B) and MMP-1 activity (Fig. 5C) without altering TIMP-1 levels (supplemental Fig. S2).

Cigarette Smoke-induced MMP-1 Expression Is Redox-regulated—Oxidative stress is a crucial contributor in lung inflammation and could play a role in CSE-induced TLR4, as TLR4 signaling in macrophages is redox-sensitive (37). First, MMP-1 expression was examined in the presence of an oxidant, H$_2$O$_2$. H$_2$O$_2$ exposure induced MMP-1 gene expression in SAE cells that was blocked by IRAK1/4 inhibition (Fig. 6A). We next examined MMP-1 levels in SAE cells following CSE exposure in the presence of an antioxidant, N-acetyl-l-cysteine. N-Acetyl-l-cysteine incubation prior to CSE stimulation suppressed the up-regulation of MMP-1 gene expression (Fig. 6B) by CSE and H$_2$O$_2$. Additionally, blockade of ERK activity resulted in reduced H$_2$O$_2$-induced MMP-1 activity (Fig. 6C) without altering TIMP-1 levels (supplemental Fig. S2). Therefore, TLR4 activation by cigarette smoke appears to act in a redox-dependent manner.

Cigarette Smoke Exposure Induces TLR4 Expression in Vivo—Acute cigarette smoke exposure results in LPS-independent TLR4 activation in mice (25), and smoke-exposed mice are more susceptible to viral infections via TLR3 signaling (43).
Following our *in vitro* analysis, we examine TLR3 and TLR4 levels in mice exposed to 8 weeks of cigarette smoke. Mice had slightly lower levels of TLR3 following smoke exposure but significantly increased TLR4 expression (Fig. 7A). Additionally, increased MyD88 levels were observed in the lungs of mice exposed to cigarette smoke (Fig. 7B). Because mice do not express MMP-1, we examined TLR4 and MMP-1 levels in rabbits following 16 weeks of cigarette smoke exposure. Similar to our murine smoking model, rabbit lungs had increased levels of TLR4 following smoke exposure (Fig. 7D). Because mice do not express MMP-1, we examined TLR4 and MMP-1 levels in rabbits following 16 weeks of cigarette smoke exposure. Similar to our murine smoking model, rabbit lungs had increased levels of TLR4 following smoke exposure (Fig. 7D). Because mice do not express MMP-1, we examined TLR4 and MMP-1 levels in rabbits following 16 weeks of cigarette smoke exposure. Similar to our murine smoking model, rabbit lungs had increased levels of TLR4 following smoke exposure (Fig. 7D).

**DISCUSSION**

In this study, we observed that cigarette smoke-induced TLR4 activation leads to MMP-1 expression in SAE cells. MMP-1 induction is sensitive to TLR4 blockade, IRAK1 inhibition, or gene silencing and also to antioxidant treatment. Additionally, we observed increased TLR4 activation in mouse and rabbit lungs exposed to cigarette smoke. Others have observed that chronic cigarette smoke exposure-induced emphysema is TLR4-independent in mice (27), but MMP-1 is not present in mice and thus may give a very different emphysema profile than that seen in humans. Additionally, expression of MMP-1 may be necessary in emphysema initiation upon acute cigarette smoke exposure. Early induction of TLR4 and subsequent MMP-1 expression may have an impact on lung destruction and remodeling in humans.

Cigarette smoke contains many components, including LPS. Following endotoxin removal from CSE, both LPS and endotoxin-free cigarette smoke still activate some of the same intracellular signaling pathways and genes such as MAPKs, IL-8, and MMP-1. However, LPS and cigarette smoke also exhibit differences in signaling in many cells, including the SAE cells in this study. Independent of smoke, endotoxin can induce bronchoconstriction, TNF, IL-12 p40, keratinocyte-derived cytokine expression, and neutrophil chemotraction, which are TIRAP- and MyD88-dependent in mice (44). Also, LPS can regulate MMP-1 expression in monocytes through a p38/prostaglandin E2-dependent pathway and MMP-9 expression through the ERK1/2 pathway (45). In our primary cell model, MMP-9 levels were unaffected by CSE, but MMP-9 was regulated by several TLR ligands, including LPS (supplemental Fig. S3). Therefore, it appears that TLR4 regulation by CSE is independent of LPS and that a non-classical TLR4 recognition occurs in the lung under...
smoking conditions. Additionally, the secretion of MMP-1 (without gene expression) following stimulation with several TLR ligands (TLR2, TLR5, and TLR8) suggests other possible means of altering collagenase activity, possibly through PKC, PKA, or protein-tyrosine kinases (46). PKC has been demonstrated to act as a novel signal transducer in TLR4-mediated NF-B activation via TRAF6 signaling (47). The involvement of protein kinases in MMP-1 secretion is an area of investigation in our future studies. Interestingly, TLR signaling also plays a key role in free cholesterol accumulation and inflammatory cell infiltration in atherosclerotic lesion mouse models (48). MyD88-dependent TLR2 signaling mediates inflammation and MMP expression (including MMP-1) in atheroma cell cultures (17). Therefore, TLRs play several key roles in disease progression independent of microbial stimuli and may be cell- or tissue-specific.

There appears to be a tight regulatory link between inflammation and protease expression in TLR4-induced MMP-1. We observed that N-acetyl-L-cysteine treatment inhibited CSE-induced TLR4 activation and MMP-1 expression. MMP-1 activity can be controlled in a redox-dependent fashion (49), but CSE-induced MMP-1 can be inhibited by an antioxidant at the transcriptional level. Hydrogen peroxide stimulus can lead to MMP-1 expression (50), which suggests that CSE-induced TLR4 activation may be mediated by oxidative stress signaling. CSE-induced TLR4 activation may also be controlled by endogenous and exogenous ceramide levels. Cigarette smoke induces neutral sphingomyelinase-2 activation, thereby increasing membrane sphingomyelin hydrolysis to ceramide (51, 52). Elevated ceramide enhances airway epithelial cell death, which causes bronchial and alveolar destruction and lung injury in pulmonary disease (52). Interestingly, ceramide induction in the lungs and subsequent signaling can be reversed by treatment with antioxidants (52, 53). Therefore, we and others (37) have demonstrated that cigarette smoke-induced TLR4 activation can be prevented by antioxidant treatment, possibly due to inhibition of cigarette smoke-mediated ceramide induction.

There are two possible means of activating TLR-regulated MMP-1 expression in SAE cells: either smoke- or LPS-induced TLR4 or virus-induced TLR3. This TLR3-regulated MMP-1 expression may be regulated by PKR signaling and strengthens the work of Kang et al. (43) showing that cigarette smoke enhances viral pulmonary immunity and lung remodeling.
Modulation of MMP expression is therefore an important therapeutic challenge. Thus far, MMP inhibitors have proven less than successful; however, by characterizing the upstream signaling pathway of MMP regulation, new targets are now viable for therapy. Several MMPs appear to have similar signaling regulation. Interestingly, MyD88, IRAK1, and TRAF6 are early mediators for IL-1-induced MMP-13 regulation through MAPKs and AP-1 activation (54). Therefore, modulation of TLR activation, inhibition of MAPKs, AP-1 inactivation, or antioxidant treatment represents an alternative means for reducing overall MMP expression. Importantly, MMP-1 can be inhibited with TAK-242 (55), an antisepis agent. LPS-induced MMP-1 expression can be suppressed in U937 mononuclear cells by simvastatin by inhibiting ERK activation (34). Additionally, because antioxidants can regulate TLR4 activation and MMP-1 expression, these compounds could be utilized to block MMP expression under disease conditions. Therefore, there are several possible means of subduing TLR4 activation and possibly cigarette smoke-induced MMP-1.

Based on this study, modulating TLRs for therapeutic treatment in lung disease represents a major approach to treating disease pathology (24, 56). MMP-1 is regulated by TLR4 under smoking conditions and also by TLR3 upon viral stimulation (poly(I:C)). Therefore, the enhancement of virus-induced emphysema by cigarette smoke observed in mice (43) could be augmented in humans. Our findings highlight the molecular signaling pathway of cigarette smoke regulation of the principal collagenase in human emphysema.

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FIGURE 8. Higher levels of TLR4 and MMP-1 in lungs of emphysema patients. A, human lungs demonstrated increased TLR4 staining (red) in emphysema lungs compared with normal lungs. Two patient samples are shown as representative of typical TLR4 staining observed in both cohorts. Nuclei were stained with Hoechst/DAPI (blue). Isotype Control represents isotype-negative staining using rabbit IgG in place of anti-TLR4 antibody. Scale bars = 100 μm. B, MyD88 and actin levels were determined in lung homogenate by Western blotting in normal and emphysema lungs. C, lung tissue MMP-1 levels were examined by qPCR. Values are means ± S.E., where n = 4 and 10 for each group, respectively. p values are shown, comparing both groups. RQ, relative quantitation.
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