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IL-1α released from damaged epithelial cells is sufficient and essential to trigger inflammatory responses in human lung fibroblasts

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Activation of the innate immune system plays a key role in exacerbations of chronic lung disease, yet the potential role of lung fibroblasts in innate immunity and the identity of epithelial danger signals (alarmins) that may contribute to this process are unclear. The objective of the study was to identify lung epithelial-derived alarmins released during endoplasmic reticulum stress (ER stress) and oxidative stress and evaluate their potential to induce innate immune responses in lung fibroblasts. We found that treatment of primary human lung fibroblasts (PHLFs) with conditioned media from damaged lung epithelial cells significantly upregulated interleukin IL-6, IL-8, monocyte chemotactic protein-1, and granulocyte macrophage colony-stimulating factor expression ($P < 0.05$). This effect was reduced with anti-IL-1α or IL-1Ra but not anti-IL-1β antibody. Costimulation with a Toll-like receptor 3 ligand, polyinosinic–polycytidylic acid (poly I:C), significantly accentuated the IL-1α-induced inflammatory phenotype in PHLFs, and this effect was blocked with inhibitor of nuclear factor kappa-B kinase subunit beta and TGFβ-activated kinase-1 inhibitors. Finally, *Il1r1* −/− and *Il1a* −/− mice exhibit reduced bronchoalveolar lavage (BAL) neutrophilia and collagen deposition in response to bleomycin treatment. We conclude that IL-1α plays a pivotal role in triggering proinflammatory responses in fibroblasts and this process is accentuated in the presence of double-stranded RNA. This mechanism may be important in the repeated cycles of injury and exacerbation in chronic lung disease.

INTRODUCTION

Clearance of invading pathogens and effective tissue repair is managed in the respiratory tract by tightly controlled immune responses involving the regulated recruitment, activation, and subsequent resolution of inflammatory cells.[1] In chronic lung disease where there is dysregulation of repair processes resulting in failure to fully resolve inflammation, further bystander tissue damage can result from the cytotoxic properties of soluble proteases and reactive oxygen species released from activated neutrophils and macrophages.[2–5] This chronic response can lead to the development of fibrosis, characterized by intensive fibroproliferation and activation of airway or parenchymal fibroblasts.

Fibroblasts constitute up to 30% of cells in the normal lung interstitum, and in the diseased lung can account for 50% of cells.[6] The role of activated fibroblasts as the major fibrogenic cell of the lung is well characterized, but less is known about the potential for these cells to contribute to innate immune and inflammatory responses.

A common pathological feature of chronic inflammatory lung diseases is epithelial damage that may lead to release of alarmins (damage-associated molecular patterns) that trigger further inflammation and mediate recruitment of immunocytes to the site of injury. Epithelial damage may result from a variety of causes including microbial attack, proteolytic damage, the effects of bystander reactive oxygen species,[7,8] and endoplasmic reticulum (ER) stress.[9,10] Two factors that may contribute to ER stress in chronic inflammatory and fibrotic lung diseases are oxidative stress induced by cigarette smoke[11] and viral infections that trigger ER stress and unfolded

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protein response in an inositol-requiring enzyme 1-dependant manner.[12] On the cellular level, ER stress is caused by accumulation of unfolded or misfolded proteins that may be a consequence of a mutation or intensified translation of proteins that cannot be folded in an efficient manner.[13] In addition, recent studies revealed that oxidative stress may also trigger the unfolded protein response by upregulating glucose-regulated protein 78 (GRP78) and protein ubiquitination.[14] ER stress activates unfolded protein response that may trigger prosurvival mechanisms such as ER stress-associated protein degradation or may promote apoptotic mechanisms, including caspase-1 activation.[14] Although the activation of unfolded protein response has been demonstrated in several chronic inflammatory diseases including chronic obstructive pulmonary disease (COPD),[9] it is unclear as to how ER stress may contribute to inflammatory responses.

Here we show that conditioned media from primary human bronchial epithelial cells (PBECs) damaged with ER stress or oxidative stress are able to trigger a proinflammatory phenotype in primary human lung fibroblasts (PHLFs). We demonstrate that interleukin (IL)-1α (and not IL-1β) is the critical innate immune trigger released by stressed lung epithelial cells, and we show that PHLFs are characterized by responsiveness to selected pathogens and damage-associated molecular patterns that is distinct from that of macrophages. We reveal the potential for synergistic proinflammatory actions of Toll-like receptor 3 agonists and IL-1α on fibroblasts that are likely to be highly relevant in viral-driven exacerbations of chronic lung disease. Finally, we demonstrate that *Il1a* −/− and *Il1r1* −/− mice exhibit reduced bronchoalveolar lavage (BAL) neutrophilia and collagen deposition in response to bleomycin treatment.

RESULTS

Conditioned media from epithelial cells damaged with ER stress and oxidative stress trigger a proinflammatory phenotype in lung fibroblasts

To determine the potential for damaged epithelial cells to influence fibroblast behavior, human bronchial epithelial cells (16HBE14o−) were damaged by pulsing for 2 h with thapsigargin (TG) to induce ER stress or H_2O_2 to induce oxidative stress. After 2 h, the culture medium was replaced, and following 24 h of incubation it was collected and used to treat human lung fibroblasts (MRC5) for 5 h. Both TG (**Figure 1c**) and H_2O_2 (**Supplementary Figure S1A** online) treatment significantly reduced epithelial cell viability. The induction of ER stress following TG treatment was confirmed by XBP-1 (X-box binding protein 1) splicing (**Figure 1a**) and an increase in GRP78 gene expression (**Figure 1b**). Control media from 16HBE14o− had a modest influence on the expression of transcripts for IL-6 and IL-8 in MRC5 cells. In contrast, media from TG (**Figure 1d,e**) or H_2O_2 (**Supplementary Figure S1B,C**)-damaged 16HBE14o− strongly induced transcripts for each cytokine. To explore signaling pathways through which these effects may be mediated, we determined pattern recognition receptor (PRR) gene expression in MRC5 cells and PHLFs. Quantitative real time polymerase chain reaction revealed high expression of IL-1R in PHLFs that was confirmed at protein level by western blot and immunofluorescence (**Supplementary Figures S2A–C**), whereas Toll-like receptors were expressed at considerably lower or undetectable levels. An analogous pattern of PRR expression was found with MRC5 cells (**Supplementary Figure S2D**), confirming that this cell line can be employed as a model for PHLF innate immune signaling. However, a different pattern of PRR expression was found for THP1 macrophages (**Supplementary Figure S2E**), indicating that PHLFs and MRC5 cells have a PRR distinct expression profile compared with classical immunoregulatory cells.

ER stress- and oxidative stress-induced epithelial damage leads to release of damage-associated molecular patterns

From the data above we hypothesized that IL-1R signaling could be required for the stimulation of an inflammatory phenotype in fibroblasts induced by damaged epithelia. IL-1α and IL-1β are two major cytokines able to activate IL-1R, and both of them may be released by stressed cells. Therefore, to establish whether ER stress and oxidative stress may trigger release of these cytokines, PBECs were exposed to varying doses of TG (**Figure 2**) or H_2O_2 (**Supplementary Figure S3**). Induction of ER stress in PBECs was confirmed by XBP-1 splicing (**Figure 2a**) and an increase in GRP78 gene expression (**Figure 2b**). TG induced a dose-dependent decrease in PBEC cell viability (**Figure 2c**) and led to release of IL-1α (**Figure 2d**) and high-mobility group protein B1 (HMGB1), a classical marker of cellular damage (**Figure 2e**). IL-1β release was also detected in conditioned media from TG-damaged PBECs, but its concentration was significantly lower compared with IL-1α levels (**Figure 2d**). HMGB1 release was strictly associated with cell death, whereas elevated levels of IL-1α could be detected even after treatment with TG doses (7.5–10 μM) that did not cause detectable decrease in PBEC cell viability. An analogous pattern of PBEC cell death and alarmin release was observed in response to H_2O_2-induced oxidative stress (**Supplementary Figure S3**). Pretreatment of PBECs with *N*-acetyl-L-cysteine, a potent antioxidant, inhibited H_2O_2-induced IL-1α release and cell death (**Supplementary Figure S4**). Furthermore, IL-1α was localized by immunohistochemistry to the human bronchial and alveolar epithelium in tissue sections, confirming its presence in the epithelium of the human lung (**Supplementary Figure S5**).

IL-1α is the predominant epithelial damage-associated molecular pattern triggering a proinflammatory phenotype in lung fibroblasts

From the data above we hypothesized that IL-1α/IL-1R signaling could be required for stimulation of an inflammatory phenotype in fibroblasts by damaged epithelia. To test this hypothesis, PHLFs were treated with conditioned media from ER stress-damaged PBECs that had been preincubated with neutralizing antibody (NAb) for IL-1α or IL-1β. To establish a

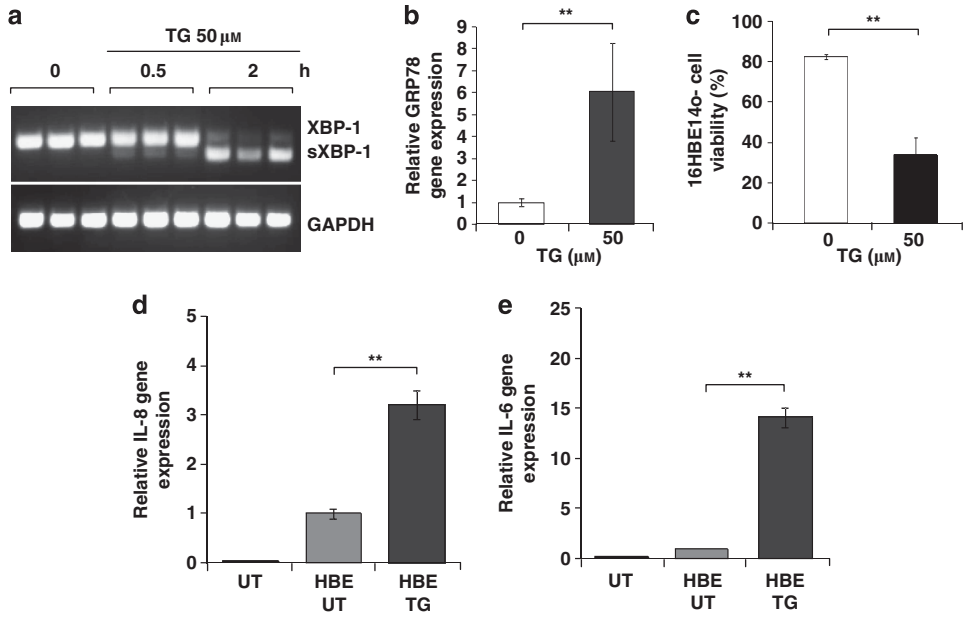

Figure 1 Conditioned media from 16HBE14o − cells damaged with thapsigargin (TG)-induced endoplasmic reticulum (ER) stress stimulates expression of proinflammatory cytokines in normal human lung fibroblasts (MRC5). (**a**) The 16HBE14o − (HBE) cells were treated with 50 μM TG for 0.5 or 2 h after which the induction of ER stress was confirmed using XBP-1 (X-box binding protein 1) splicing assay. (**b**) In addition, 2 h of treatment with 50 μM TG followed by 4 h of incubation at 37 °C induced upregulation of glucose-regulated protein 78 (GRP78) gene expression. To investigate the influence of TG on cell viability, the cells were pulsed with 50 μM TG for 2 h, after which the media were replaced and the cells were reincubated for a further 24 h. (**c**) Propidium iodide (PI) staining revealed that TG treatment significantly reduced cell viability. MRC5 cells treated for 5 h with conditioned media from damaged 16HBE14o − epithelial cells showed increased gene expression of (**d**) interleukin-8 (IL-8) and (**e**) interleukin-6 (IL-6) compared with fibroblasts treated with media from undamaged cells. Data represented by mean ± s.e.m.; $n = 6$ per group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UT, untreated cells. $^{**}P < 0.01$.

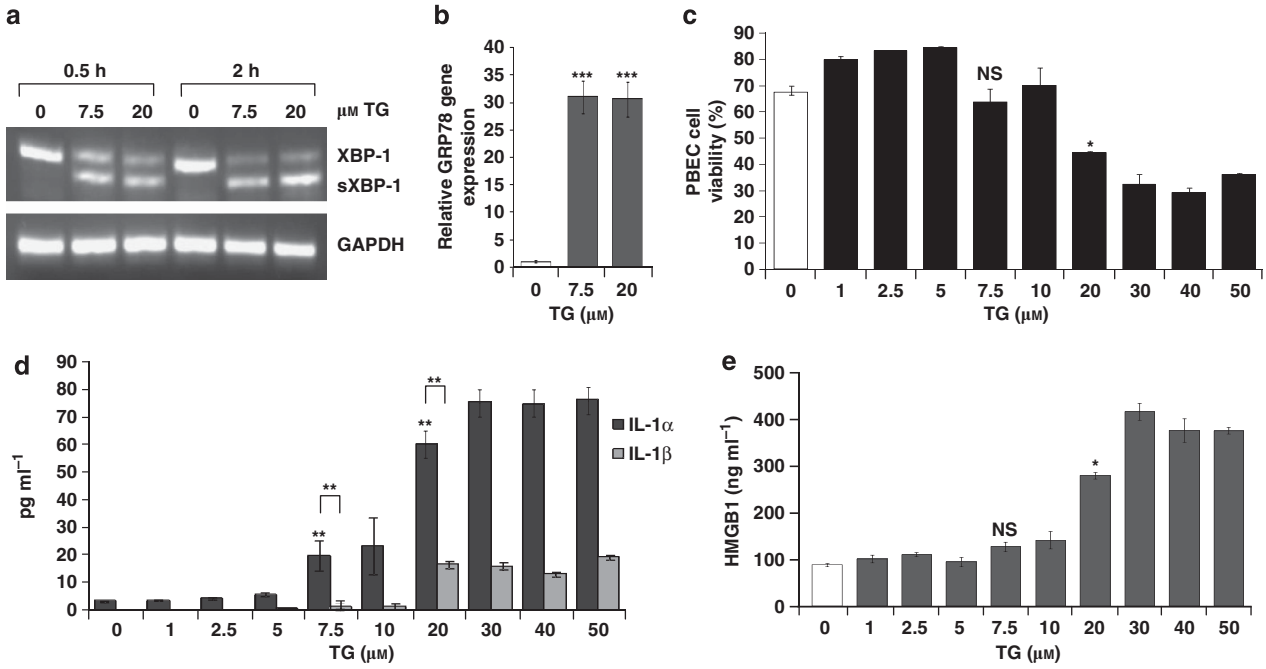

Figure 2 Endoplasmic reticulum (ER) stress induces primary human bronchial epithelial cell (PBEC) damage and alarmin release. (**a**) PBECs were treated with 7.5 or 20 μM TG for 0.5 or 2 h after which the induction of ER stress was confirmed using XBP-1 (X-box binding protein 1) splicing assay. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (**b**) Treatment with thapsigargin (TG) for 2 h, followed by 4 h of incubation at 37 °C induced upregulation of glucose-regulated protein 78 (GRP78) gene expression in PBECs. To investigate the influence of TG on PBEC viability and alarmin release, the cells were pulsed with the indicated doses of TG for 2 h, after which the media were replaced and the cells were reincubated for a further 24 h. (**c**) TG treatment significantly reduced PBEC culture viability and upregulated release of alarmins: (**d**) interleukin (IL)-1α, IL-1β, and (**e**) high-mobility group protein B1 (HMGB1). Asterisks correspond to the statistical significance between untreated controls and cells treated with 7.5 or 20 μM TG (conditioned media from PBECs treated with these doses of TG were used to treat primary human lung fibroblasts (PHLFs) in the subsequent experiments). NS, nonsignificant. Data represented by mean ± s.e.m. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

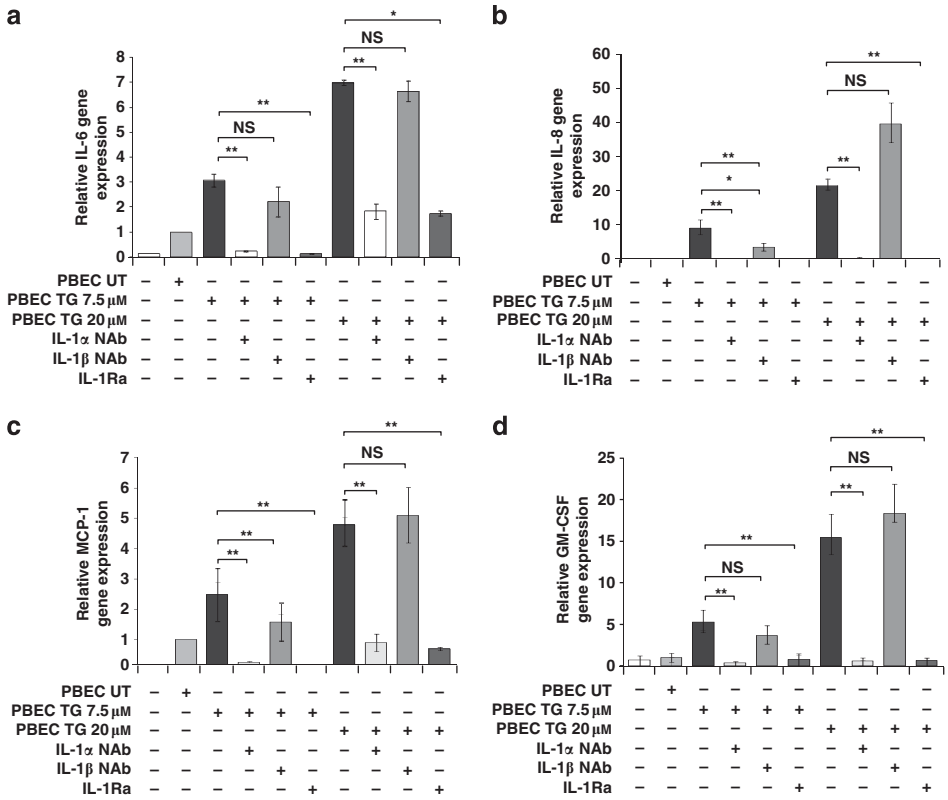

Figure 3 Interleukin-1α (IL-1α) is the key alarmin released from endoplasmic reticulum (ER) stress-damaged epithelial cells that activates expression of proinflammatory cytokines in primary human lung fibroblasts (PHLFs). PHLF's were treated for 5 h with conditioned media from untreated primary human bronchial epithelial cells (UT PBECs) or PBECs pulsed for 2 h with 7.5 or 20 μM thapsigargin (PBEC TG) and collected after a further 24 h. Before adding to the PHLFs, the conditioned media were preincubated for 1 h at 37 °C in the presence or absence of IL-1α (4 μg ml^{-1}) or IL-1β (4 μg ml^{-1}) neutralizing antibody (NAb). To inhibit IL-1R signaling, PHLFs were pretreated for 1 h with IL-1Ra (500 ng ml^{-1}) after which the culture media were replaced with conditioned media from damaged epithelial cells containing the same concentration of IL-1Ra. Following 5 h of treatment, samples were collected for RNA. IL-1α NAb and IL-1Ra significantly reduced gene expression of (**a**) IL-6, (**b**) IL-8, (**c**) monocyte chemotactic protein-1 (MCP-1), and (**d**) granulocyte macrophage colony-stimulating factor (GM-CSF) induced following treatment with conditioned media from PBECs damaged with 20 μM TG. NS, nonsignificant. Data represented by mean ± s.e.m. from three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

requirement for IL-1R, similar experiments were carried out in PHLFs pretreated with IL-1R antagonist (IL-1Ra). IL-1α NAb and IL-1Ra significantly reduced induction of IL-6, IL-8, monocyte chemotactic protein-1, and granulocyte macrophage colony-stimulating factor gene expression in PHLFs treated with conditioned media from PBECs damaged with TG (**Figure 3**). In contrast, IL-1β NAb had mild influence or was without significant effects. In all cases, the treatment with IL-1α NAb resulted in reduction of the measured cytokine expression close to control level (untreated fibroblasts), suggesting IL-1α is the predominant epithelial cell-derived factor responsible for inducing an inflammatory phenotype in fibroblasts. In addition, IL-1α NAb and IL-1Ra blocked IL-6, IL-8, monocyte chemotactic protein-1, and granulocyte macrophage colony-stimulating factor gene expression in fibroblasts treated with conditioned media from oxidative stress-damaged PBECs (**Supplementary Figure S6**). The efficacy of IL-1α NAb, IL-1β NAb, and IL-1Ra was verified with a functional assay using IL-6 gene expression as a surrogate of IL-1R activation (**Supplementary Figure S7**).

PHLFs show unique responsiveness to danger signals

To confirm the observations described above, PHLFs were treated for 24 h with increasing doses of IL-1α, after which conditioned media from the cells were collected and subjected to enzyme-linked immunosorbent assay (ELISA) for IL-8. The analysis revealed that IL-1α treatment significantly increased the amount of released IL-8 protein from PHLFs (**Figure 4a**). Interestingly, neither IL-1α nor IL-1β treatment resulted in increased secretion of IL-8 from primary human alveolar macrophages (**Figure 4b**).

Bacterial and viral infections have been recognized as important risk factors in exacerbations of chronic fibrotic lung diseases. Therefore, we investigated the responsiveness of PHLFs to bacterial lipopolysaccharide and polyinosinic–polycytidylic acid (poly I:C)—an analog of viral RNA. PHLFs moderately responded to poly I:C, as demonstrated by a significant upregulation of IL-6 gene expression, but showed only a weak responsiveness to bacterial lipopolysaccharide and no response to another Toll-like receptor 4 ligand, HMGB1 (**Figure 4c–f**).

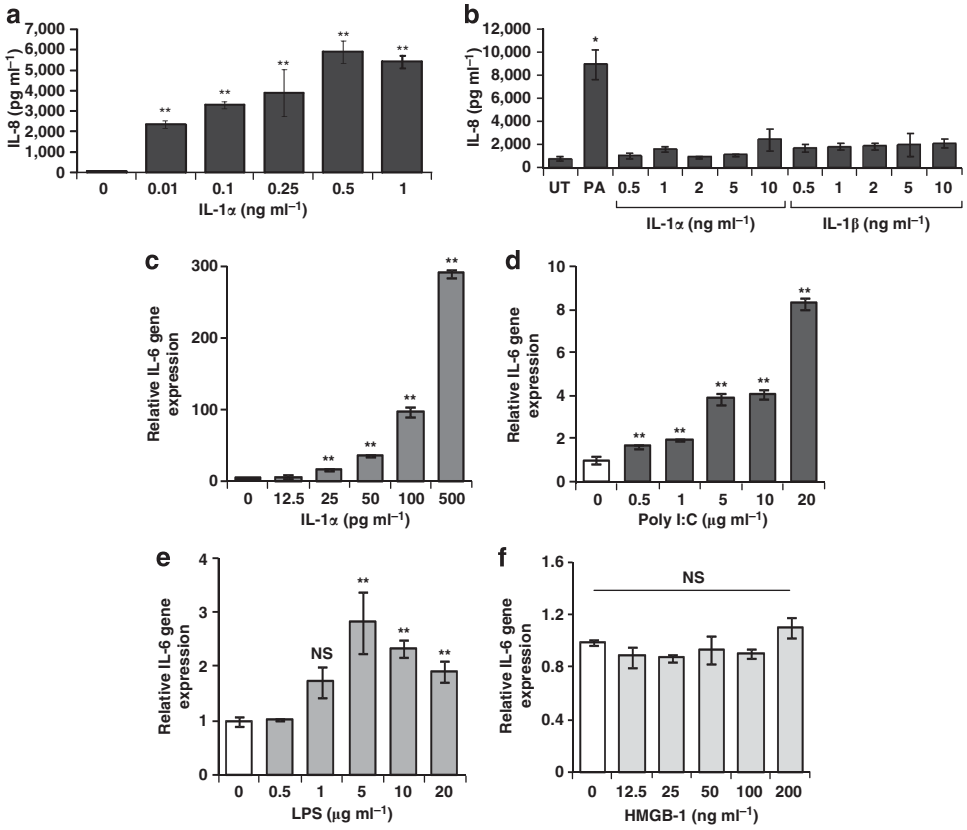

Figure 4 Primary human lung fibroblasts (PHLFs) show unique responsiveness to pattern recognition receptor (PRR) ligands. (**a**) PHLFs ($n=6$) and (**b**) primary human alveolar macrophages ($n=4$) were treated as indicated with interleukin (IL)-1α, IL-1β, or *Pseudomonas aeruginosa* (PA) for 24 h, after which conditioned media were collected and subjected to enzyme-linked immunosorbent assay (ELISA) for IL-8. IL-1α treatment resulted in increased secretion of IL-8 from (**a**) PHLFs but not from (**b**) macrophages. In addition, 5 h of challenge with selected PRR ligands including (**c**) IL-1α, (**d**) polyinosinic–polycytidylic acid (poly I:C), (**e**) lipopolysaccharide (LPS), and (**f**) high-mobility group protein B1 (HMGB1) revealed that PHLFs strongly respond to IL-1α and poly I:C but show moderate response to LPS and no response to HMGB1. $^{*}P<0.05$, $^{**}P<0.01$.

Poly I:C synergizes with IL-1α to stimulate fibroblast cytokine expression via TGFβ-activated kinase-1/inhibitor of nuclear factor kappa-B kinase subunit beta signaling

As primary human lung fibroblasts strongly respond to IL-1α and poly I:C, we investigated the potential for an additive or synergistic effect of these two factors. PHLFs were treated with poly I:C or IL-1α alone or in combination for 24 h before quantification of IL-6 and IL-8 expression. The immunocytofluorescent analysis showed that combined IL-1α and poly I:C treatment markedly stimulated accumulation of IL-6 within intracellular granules of fibroblasts (**Figure 5a**), as well as increased IL-6 and IL-8 gene expression (**Figure 5b–e**) and protein secretion (**Supplementary Figure S8**). Inhibitors of TAK1 and IKK2 kinases blocked the individual and combined effects of IL-1α and poly I:C on both IL-6 and IL-8 gene expression (**Figure 5b–e**) and protein release (**Supplementary Figure S8**). From these data we conclude that a TGFβ-activated kinase-1/inhibitor of nuclear factor kappa-B kinase subunit beta signaling pathway mediates IL-1α and poly I:C stimulation of a proinflammatory phenotype in lung fibroblasts.

Il1r1−/− *and Il1a*−/− mice exhibit reduced BAL neutrophilia and collagen deposition in response to bleomycin treatment

C57Bl/6 wild-type, *Il1r1*−/−, and *Il1a*−/− mice were intratracheally instilled with bleomycin sulfate (0.15 U) and killed on day 7 or day 21. Saline-treated C57Bl/6 mice were used as a control. Bleomycin has previously been shown to induce significant levels of oxidative stress in lung epithelial cells,[15] and treatment with *N*-acetyl cysteine, a potent antioxidant, has been shown to reduce bleomycin-induced fibrosis.[16] Bleomycin treatment of C57Bl/6 mice increased the levels of peroxiredoxin-SO3, a marker of induction of oxidative stress, in lung epithelial cells (**Figure 6a**) and induced a marked influx in both the frequency (**Figure 6b,c**) and number (data not shown) of Ly6G+ neutrophils into the BAL 7 days after treatment. This influx is significantly reduced in both *Il1r1*−/− and *Il1a*−/− mice. In addition, bleomycin treatment of C57Bl/6 mice induced dramatic tissue remodeling and collagen deposition by day 21 (**Figure 6d,e**). Again, both tissue remodeling and collagen deposition were significantly reduced in both *Il1r1*−/− and *Il1a*−/− mice.

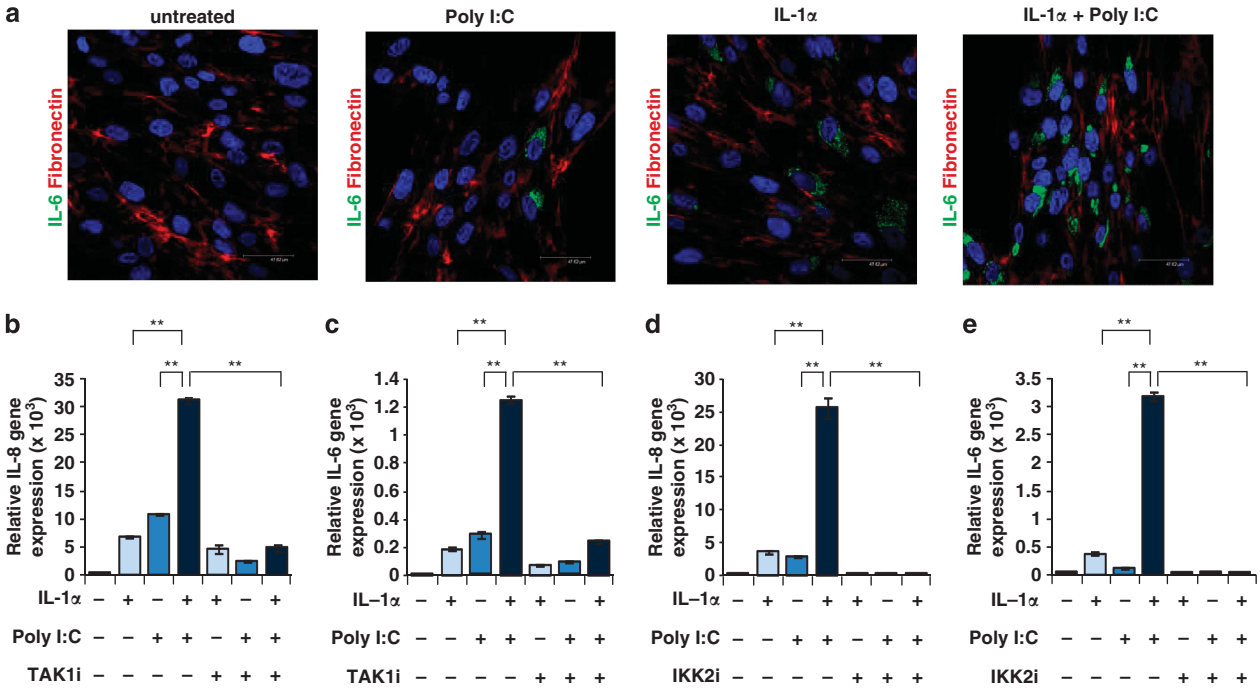

Figure 5 Interleukin (IL)-1α and polyinosinic–polycytidylic acid (poly I:C) synergize in the induction of IL-6 and IL-8 expression in primary human lung fibroblasts (PHLFs). Cotreatment of PHLFs for 24 h with IL-1α (500 pg ml^{-1}) and poly I:C (10 μg ml^{-1}) markedly enhanced accumulation of (**a**) intracellular IL-6 protein and (**b**–**e**) gene expression of IL-8 and IL-6 in comparison with treatment with IL-1α or poly I:C alone. Pretreatment for 1 h with (**b**, **c**) TAK1 (1 μM) or (**d**, **e**) IKK2 (1 μM) inhibitors significantly reduced IL-6 and IL-8 gene expression by PHLFs in response to treatment with IL-1α and poly I:C individually or in combination. Relative gene expression was measured by qRT-PCR using *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a housekeeping gene. Intracellular IL-6 protein was visualized by immunocytofluorescence. IKK2, inhibitor of nuclear factor kappa-B kinase subunit beta; TAK-1, TGFβ-activated kinase-1; qRT-PCR, quantitative real time polymerase chain reaction; UT, untreated cells. Data represented by mean ± s.e.m.; $n = 6$ per group. $^{**}P < 0.01$.

DISCUSSION

Repetitive damage to the airway or alveolar epithelium, associated with inflammation and tissue remodeling, are common features of many chronic respiratory diseases including idiopathic pulmonary fibrosis,[17] COPD,[7] asthma,[14] and bronchiolitis obliterans syndrome (BOS).[18] Chronic inflammation, characterized by intensive protein production, may also cause ER stress and an unfolded protein response that may lead to programmed cell death.[19] The lung epithelium is the first point of contact for inhaled oxidants/free radicals and these species are normally neutralized by antioxidants present in epithelial lining fluid.[7,20] Defining how oxidant and ER stress-induced epithelial cell damage stimulates inflammation and identifying the molecular mediators that cross-talk between damaged and dying epithelial cells and the immune system are important goals necessary to improve our knowledge of the pathophysiological events that underlie chronic pulmonary disease.

Epithelial injury is a well-recognized histological feature in patients with chronic inflammatory lung diseases including COPD and BOS, and both alloimmune and nonalloimmune insults to the airway epithelium after lung transplantation have been associated with an increased incidence of BOS.[21] Our work described here makes the case for a new paradigm, specifically that bronchial epithelium damaged with ER or oxidative stress can induce sterile innate immune signaling in lung fibroblasts, resulting in a phenotypic switch toward a highly proinflammatory state. We have shown that the proinflammatory lung fibroblasts secrete vast quantities of the neutrophil chemokine IL-8 and the pleiotropic cytokine IL-6 that can induce T-cell[22] and monocyte[23] differentiation.

Previous reports indicate that fibroblasts from chronically inflamed tissues, such as synovial fibroblasts from arthritic joints, show alterations in inflammatory gene expression and signaling cascades in comparison with fibroblasts from normal tissue, and that this modified phenotype may be maintained for many passages *in vitro*,[24] suggesting that fibroblasts may play an important role in the switch from acute to chronic inflammation. Here, we were able to show that a single alarmin, IL-1α, is both necessary and sufficient for the epithelial damage-induced phenotypic switch of lung fibroblasts to their inflammatory state. We have also demonstrated that Il1r1 −/− and Il1a −/− mice exhibit reduced BAL neutrophilia and collagen deposition in response to bleomycin treatment. Our observations are in agreement with those in a recent study by Tracy *et al.*,[25] showing that IL-1α is the major epithelial alarmin released from lung epithelial cells to induce inflammatory mediators in fibroblasts during photodynamic therapy. The implication of IL-1α in chronic lung diseases is

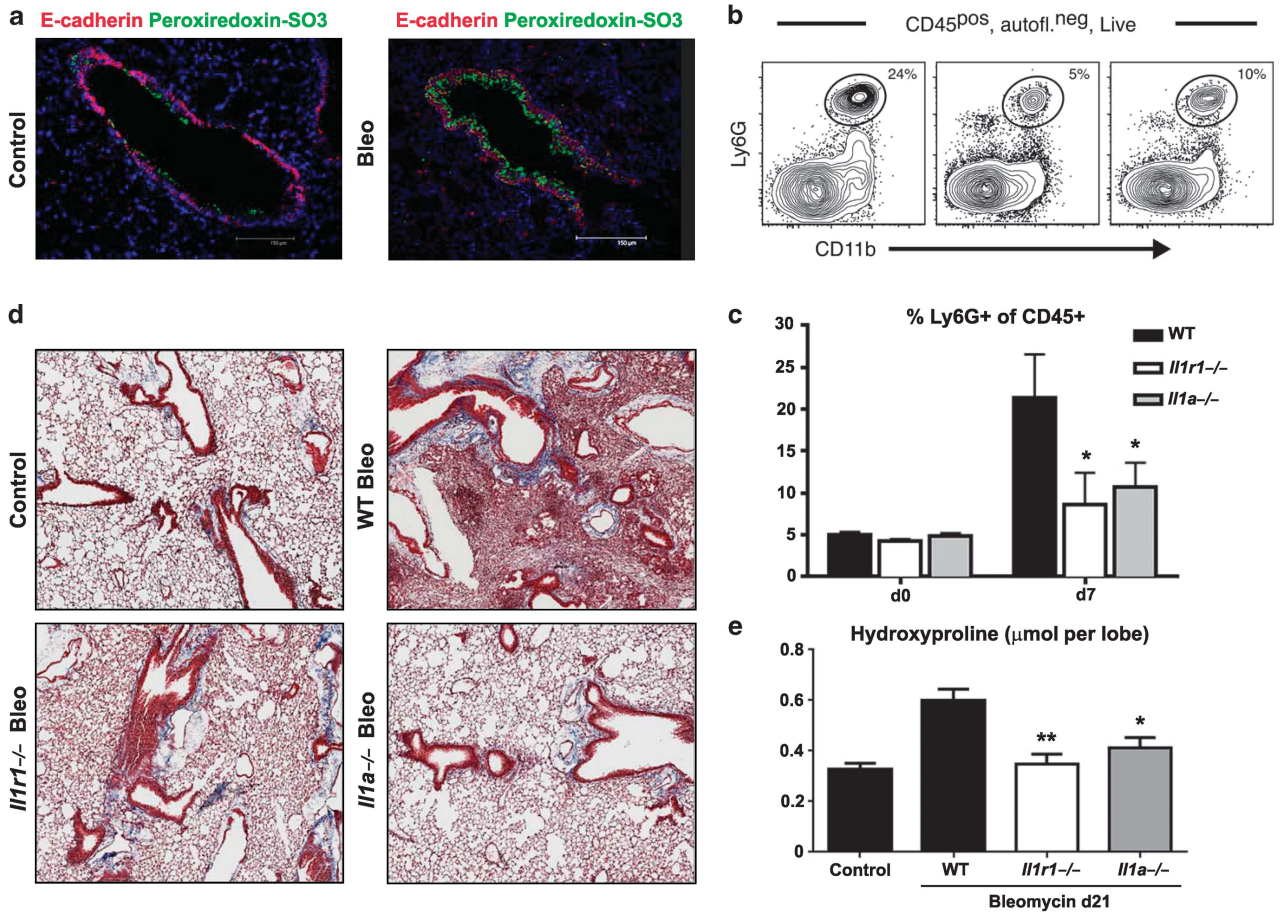

Figure 6 *Il1r1* −/− *and Il1a* −/− mice exhibit reduced bronchoalveolar lavage (BAL) neutrophilia and collagen deposition in response to bleomycin treatment. C57Bl/6 (wild-type (WT) Bleo), *Il1r1* −/− (*Il1r1* −/− Bleo), and *Il1a* −/− (*Il1a* −/− Bleo) mice were intratracheally instilled with bleomycin sulfate (0.15 U) or saline as a control and killed on day 7 or day 21. Bleomycin treatment of WT mice induced an increase in the levels of the oxidative stress marker (**a**) peroxiredoxin-SO3 and a (**b**, **c**) marked influx of Ly6G+ neutrophils into the BAL on day 7 (d7) after treatment. This influx is significantly reduced in Il1r1 −/− and Il1a −/− mice. In addition, bleomycin treatment of WT mice induced dramatic (**d**) tissue remodeling and (**e**) collagen deposition (as measured by hydroxyproline) by day 21. Both remodeling and collagen deposition were significantly reduced in Il1r1 −/− and Il1a −/− mice. Data represented by mean ± s.e.m.; $n = 7$–9 per group. $^{*}P < 0.05$, $^{**}P < 0.01$.

additionally supported by the recent discovery of anti-IL-1α autoantibodies in the blood of idiopathic pulmonary fibrosis patients[17] and increased levels of IL-1Ra in lung allograft recipients who developed BOS,[18] suggesting that extracellular IL-1α is an undesirable and potentially harmful factor in fibrotic lung diseases. In addition, IL-1α is upregulated in the lungs of patients with COPD,[26] and fibroblasts isolated from the lungs of patients with COPD secrete increased levels of proinflammatory factors compared with normal fibroblasts.[27] Taken together, these observations build a case for investigating the therapeutic potential of biologics that attenuate IL-1α/IL-1R signaling in these chronic lung diseases that are associated with significant morbidity and premature mortality.

Our observation that poly I:C acts in synergy with IL-1α raises the intriguing possibility that the degree to which fibroblasts affect lung inflammation may be influenced by respiratory viral infections. Conditions such as COPD and asthma are exacerbated by community-acquired viral respiratory tract infections, and such infections also trigger immune-mediated epithelial injury in posttransplant patients associated with the development of BOS.[28] Furthermore, respiratory viruses have been implicated with acute exacerbations in patients with idiopathic pulmonary fibrosis that are associated with significant mortality in this group.[29] Extracellular double-stranded viral RNA molecules released from dying or damaged viral-infected lung epithelial cells would, as shown here with poly I:C, be able to enter lung fibroblasts and synergize with IL-1α/IL-1R signaling to exert a powerful proinflammatory effect.

In addition, our observations suggest that fibroblasts show a unique and distinct PRR expression profile and responsiveness to PRR ligands than alveolar macrophages, suggesting that these two cell types may play different roles in the recognition of danger signals and triggering innate immune responses during infection and trauma.

In summary, we have presented data revealing an IL-1α and double-stranded RNA signaling network that operates to trigger a powerful proinflammatory phenotype in lung fibroblasts in response to ER stress- and reactive oxygen species-induced epithelial cell damage. We suggest this network may facilitate the establishment of a chronic inflammatory disease state in the lung and that targeting its different components individually or in combination may be of benefit in the prevention of tissue destruction in patients with chronic lung disease.

Study approval

All participants provided informed consent before participation in this study. All aspects of the study were approved by Newcastle and North Tyneside Local Research Ethics Committee (Ref. 2001/179).

METHODS

Antibodies. Antibodies used for immunoblotting and immunofluorescence were as follows: IL-1R1 (Abcam, Cambridge, MA), IL-6 (R&D Systems, Minneapolis, MN), IL-1α (Abcam), fibronectin (Abcam), peroxiredoxin-SO3 (Abcam), and E-cadherin (BD Bioscience, San Jose, CA). Neutralizing antibodies were as follows: anti-human: IL-1β and IL-1α (R&D Systems).

Chemical inhibitors. TAK1i ((5Z)-7-Oxozeaenol) and IKK-2i IV (5-(p-Fluorophenyl)-2-ureido]thiophene-3-carboxamide) were purchased from Calbiochem (Nottingham, UK). Human recombinant IL-1Ra was obtained from R&D Systems.

Primers. The quantitative real time polymerase chain reaction primers have been listed in **Supplementary Table S1** online.

Primary cell isolation and culture. Human PBECs were obtained by bronchial brushings from stable posttransplant patients and were cultured on 0.5% Purecol (Invitrogen, Carlsbad, CA)-coated dishes in SABM medium containing 2% fetal bovine serum (FBS), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Lonza, Basel, Switzerland). PHLFs were isolated using collagenase II digestion technique. Briefly, lung tissue pieces were minced, washed on a 40 μm filter with Dulbecco's modified Eagle's medium/F12 (Sigma, St Louis, MO), and digested with 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) at room temperature for 2 h. After washing with 1 × phosphate-buffered saline (PBS), the cells were filtered through large gauze followed by 100 μm filter. The cells were collected by centrifugation at 300 *g* and plated at a density of 80,000 cells cm^{-2} in Dulbecco's modified Eagle's medium/F12 (Sigma) supplemented with 10% FBS, 1% L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. The cells were then left to attach for 1 h, after which they were washed with 1 × PBS and fresh culture medium was added.

Primary alveolar macrophages were obtained from BALs of stable lung transplant recipients. The BALs were centrifugated at 1,500 *g* for 5 min, after which the pellets were washed twice with PBS and suspended in RPMI medium containing 10% FBS (Sigma).

Cell line culture. MRC5 cells were cultured in Dulbecco's modified Eagle's medium/F12 (Sigma) containing 10% FBS, 1% L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

The 16HBE14o − cells were maintained in MEM/EBSS (Thermo Scientific, Logan, UT) supplemented with 10% FBS, 1% L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

THP-1 cells were maintained in RPMI medium (Sigma) supplemented with 10% FBS, 1% L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

All cells were maintained at 37 °C in an atmosphere of 5% CO_2.

Mice. C57Bl/6 (WT) mice were purchased from Taconic Farms (Hudson, NY). *Il1r1* − / − mice on the B6 background were obtained through a supply contract between the National Institute of Allergy and Infectious Diseases (NIAID) and Taconic Farms. *Il1a* − / − mice were originally derived by Y. Iwakura (Tokyo University, Tokyo, Japan) and are maintained on a B6 background under specific pathogen-free conditions at the National Institutes of Health in an American Association for the Accreditation of Laboratory Animal Care–approved facility. The NIAID animal care and use committee approved all experimental procedures and mice were used at 8–10 weeks of age for experiments.

Bleomycin. Single intratracheal instillation of saline (30 μl) or bleomycin sulfate (0.15 U in 30 μl of saline) were administered to C57Bl/6, *Il1a* − / −, and *Il1r1* − / − mice. Half of the mice were killed by pentobarbital overdose on day 7 and their tracheas cannulated with an Insyte venous catheter (BD Bioscience). BAL was performed with ice-cold PBS supplemented with 5 mM EDTA and the frequency of neutrophils determined using flow cytometry. The remaining mice were harvested at day 21. For histological analyses, matched lung lobes were washed with PBS, inflated with Bouin's fixative, and stained with Masson's trichrome. For assessment of fibrosis, matched lung lobes were weighed and digested with 2 ml of 6 N HCl to measure the quantity of hydroxyproline as described in Wynn *et al.*[30]

Immunoblotting. Proteins were separated using 4–12% gradient sodium dodecyl sulfate–polyacrylamide gels (Invitrogen). The separated proteins were transferred overnight to polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ), immunoblotted with an appropriate primary antibody, and probed with a corresponding horseradish peroxidase-linked secondary antibody.

Enzyme-linked immunosorbent assay. *Sandwich ELISA*: IL-6 and IL-8 concentrations in cell culture media were measured with sandwich ELISA (R&D Systems) according to the manufacturer's instructions. Detection limits for these assays were 10 pg ml^{-1}.

HMGB1 direct ELISA: HMGB-1 concentrations in conditioned media were measured with a direct, home-made ELISA. A total of 100 μl of neat sample or human recombinant HMGB1 (R&D Systems) was added to a 96-well plate and incubated overnight at room temperature. The plates were blocked with 3% bovine serum albumin in PBS and the antigen detection was performed using 2 μg ml^{-1} of anti-human HMGB1 antibody (mouse monoclonal; R&D Systems) followed by an anti-mouse horseradish peroxidase-linked IgG (1:2,000; Sigma). Plates were developed with substrate reagent (R&D Systems) and the reactions were stopped with 1 M H_2SO_4. Optical density was measured at the wavelength of 450 nm using MRX microplate reader (Dynex Technologies, Chantilly, VA). HMGB1 concentration in samples was calculated against a seven-point standard curve prepared by performing twofold serial dilutions of human recombinant HMGB1 with the highest point of 1 μg ml^{-1}.

PCR. *Quantitative reverse transcription–PCR*: RNA extraction was performed using Absolutely RNA MicroPrep Kit (Stratagen, La Jolla, CA) or the MagMAX Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reverse transcription–PCR was carried out with AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene).

Relative gene expression was measured using SYBR Green JumpStart Taq ReadyMix.

XBP-1 splicing assay: Total RNA (1 μg) was reverse-transcribed with AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene) and 20 ng of complementary DNA was amplified with JumpStart Taq polymerase using a pair of primers covering a 26-nucleotide intron (nucleotides 531–556) that is spliced out by inositol-requiring enzyme 1 following ER stress induction. DNA fragments were separated on 2% agarose gel.

Immunofluorescence. Following sample fixation and permeabilization, antigens were detected using appropriate primary antibodies. Proteins were visualized using a corresponding secondary antibody conjugated with FITC, TRITC (DAKO, Glostrup, Denmark), or Alexa Fluor 633 (Jackson Immuno Research Laboratories, West Grove, PA). The cell nuclei were stained with DAPI (Vectra Shield, Vector Labs, Burlingame, CA). Samples were analyzed using a Leica TCS-SP-2UV confocal microscope (Milton Keynes, UK).

Flow cytometry. *Human epithelial cells*: Flow cytometric analysis was performed to assess the viability of epithelial cells. Cells were pelleted, resuspended in 300 μl Dulbecco's modified Eagle's medium phenol red-free media (Invitrogen), and incubated for 30 min at 37 °C. Immediately before analysis, the cells were stained with propidium iodide at a final concentration of 100 $\mu g\,ml^{-1}$. Analysis was performed using a FACScan Cytometer (BD Bioscience) and data were processed with CellQuest Software (BD Bioscience).

Mouse BAL cells: To assess the frequency of neutrophils in BAL, cells were stained with antibodies specific for CD45 (104), CD11b (M1/70), and Ly6G (1A8) (all from Biolegend, San Diego, CA). Ultraviolet fixable live–dead cell stain was purchased from Molecular Probes–Invitrogen (Carlsbad, CA) and used in accordance with the manufacturer's protocol. All samples were acquired on a LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, San Carlos, CA).

Statistical analysis. *In vitro* data were analyzed using Kruskal–Wallis test followed by Mann–Whitney *U*-test or one-way analysis of variance test followed by unpaired Student's *t*-test as appropriate. *In vivo* data were analyzed using Mann–Whitney *U*-test. All results are reported as mean ± s.e.m. $P<0.05$ was considered to be statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

DISCLAIMER

The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

REFERENCES

1. Chtarbanova, S. & Imler, J.L. Microbial sensing by toll receptors: a historical perspective. *Arterioscler. Thromb. Vasc. Biol.* **31**, 1734–1738 (2011).
2. Davies, D.E. The role of the epithelium in airway remodeling in asthma. *Proc. Am. Thorac. Soc.* **6**, 678–682 (2009).
3. Kersul, A.L. *et al.* Molecular mechanisms of inflammation during exacerbations of chronic obstructive pulmonary disease. *Arch. Bronconeumol.* **47**, 176–183 (2011).
4. Jaeschke, H. Reactive oxygen and mechanisms of inflammatory liver injury: present concepts. *J. Gastroenterol. Hepatol.* **26** (Suppl 1), 173–179 (2011).
5. Kumar, V. & Sharma, A. Neutrophils: cinderella of innate immune system. *Int. Immunopharmacol.* **10**, 1325–1334 (2010).
6. Jordana, M., Sarnstrand, B., Sime, P.J. & Ramis, I. Immune-inflammatory functions of fibroblasts. *Eur. Respir. J.* **7**, 2212–2222 (1994).
7. Caramori, G. *et al.* Unbalanced oxidant-induced DNA damage and repair in copd: a link towards lung cancer. *Thorax* **66**, 521–527 (2011).
8. Wright, D.T., Cohn, L.A., Li, H., Fischer, B., Li, C.M. & Adler, K.B. Interactions of oxygen radicals with airway epithelium. *Environ. Health Perspect.* **102** (Suppl 10), 85–90 (1994).
9. Malhotra, D. *et al.* Heightened endoplasmic reticulum stress in the lungs of patients with chronic obstructive pulmonary disease: the role of nrf2-regulated proteasomal activity. *Am. J. Respir. Crit. Care Med.* **180**, 1196–1207 (2009).
10. Korfei, M. *et al.* Epithelial endoplasmic reticulum stress and apoptosis in sporadic idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* **178**, 838–846 (2008).
11. Somborac-Bacura, A. *et al.* Cigarette smoke induces endoplasmic reticulum stress response and proteasomal dysfunction in human alveolar epithelial cells. *Exp. Physiol.* **98**, 316–325 (2013).
12. Hassan, I.H. *et al.* Influenza a viral replication is blocked by inhibition of the inositol-requiring enzyme 1 (ire1) stress pathway. *J. Biol. Chem.* **287**, 4679–4689 (2012).
13. Schroder, M. & Kaufman, R.J. ER stress and the unfolded protein response. *Mutat. Res.* **569**, 29–63 (2005).
14. Holt, P.G. & Sly, P.D. Interaction between adaptive and innate immune pathways in the pathogenesis of atopic asthma: operation of a lung/bone marrow axis. *Chest* **139**, 1165–1171 (2011).
15. Wallach-Dayan, S.B., Izbicki, G., Cohen, P.Y., Gerstl-Golan, R., Fine, A. & Breuer, R. Bleomycin initiates apoptosis of lung epithelial cells by ROS but not by Fas/FasL pathway. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **290**, L790–L796 (2006).
16. Teixeira, K.C. *et al.* Attenuation of bleomycin-induced lung injury and oxidative stress by N-acetylcysteine plus deferoxamine. *Pulm. Pharmacol. Ther.* **21**, 309–316 (2008).
17. Ogushi, F. *et al.* Autoantibodies to IL-1 alpha in sera from rapidly progressive idiopathic pulmonary fibrosis. *J. Med. Invest.* **48**, 181–189 (2001).
18. Belperio, J.A. *et al.* Interleukin-1 receptor antagonist as a biomarker for bronchiolitis obliterans syndrome in lung transplant recipients. *Transplantation* **73**, 591–599 (2002).
19. Korfei, M. *et al.* Epithelial endoplasmic reticulum stress and apoptosis in sporadic idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **178**, 838–846 (2008).
20. Wright, D.T., Cohn, L.A., Li, H., Fischer, B., Li, C.M. & Adler, K.B. Interactions of oxygen radicals with airway epithelium. *Environ. Health Perspect.* **102** (Suppl 10), 85–90 (1994).
21. Kelly, F.L. *et al.* Epithelial clara cell injury occurs in bronchiolitis obliterans syndrome after human lung transplantation. *Am. J. Transplant.* **12**, 3076–3084 (2012).
22. Hohki, S. *et al.* Blockade of interleukin-6 signaling suppresses experimental autoimmune uveoretinitis by the inhibition of inflammatory Th17 responses. *Exp. Eye Res.* **91**, 162–170 (2010).
23. Chomarat, P., Banchereau, J., Davoust, J. & Palucka, A.K. Il-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat. Immunol* **1**, 510–514 (2000).
24. Pap, T., Muller-Ladner, U., Gay, R.E. & Gay, S. Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. *Arthritis Res.* **2**, 361–367 (2000).

25. Tracy, E.C., Bowman, M.J., Henderson, B.W. & Baumann, H. Interleukin-1alpha is the major alarmin of lung epithelial cells released during photodynamic therapy to induce inflammatory mediators in fibroblasts. *Br. J. Cancer* **107**, 1534–1546 (2012).
26. Pauwels, N.S. *et al.* Role of IL-1alpha and the Nlrp3/caspase-1/IL-1beta axis in cigarette smoke-induced pulmonary inflammation and COPD. *Eur. Respir. J.* **38**, 1019–1028 (2011).
27. Zhang, J., Wu, L., Qu, J.-m., Bai, C.-x., Merrilees, M.J. & Black, P.N. Pro-inflammatory phenotype of COPD fibroblasts not compatible with repair in COPD lung. *J. Cell Mol. Med.* **16**, 1522–1532 (2012).
28. Sims, K.D. & Blumberg, E.A. Common infections in the lung transplant recipient. *Clin. Chest. Med.* **32**, 327–341 (2011).
29. Song, J.W., Hong, S.B., Lim, C.M., Koh, Y. & Kim, D.S. Acute exacerbation of idiopathic pulmonary fibrosis: incidence, risk factors and outcome. *Eur. Respir. J.* **37**, 356–363 (2011).
30. Wynn, T.A. *et al.* Quantitative assessment of macrophage functions in repair and fibrosis. *Curr. Protoc. Immunol.* **Chapter 14**, 22 (2011).