Adenosine and hyaluronan promote lung fibrosis and pulmonary hypertension in combined pulmonary fibrosis and emphysema

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ABSTRACT

Combined pulmonary fibrosis and emphysema (CPFE) is a syndrome that predominantly affects male smokers or ex-smokers and it has a mortality rate of 55% and a median survival of 5 years. Pulmonary hypertension (PH) is a frequently fatal complication of CPFE. Despite this dismal prognosis, no curative therapies exist for patients with CPFE outside of lung transplantation and no therapies are recommended to treat PH. This highlights the need to develop novel treatment approaches for CPFE. Studies from our group have demonstrated that both adenosine and its receptor ADORA2B are elevated in chronic lung diseases. Activation of ADORA2B leads to elevated levels of hyaluronan synthases (HAS) and increased hyaluronan, a glycosaminoglycan that contributes to chronic lung injury. We hypothesize that ADORA2B and hyaluronan contribute to CPFE. Using isolated CPFE lung tissue, we characterized expression levels of ADORA2B and HAS. Next, using a unique mouse model of experimental lung injury that replicates features of CPFE, namely airspace enlargement, PH and fibrotic deposition, we investigated whether 4MU, a HAS inhibitor, was able to inhibit features of CPFE. Increased protein levels of ADORA2B and HAS3 were detected in CPFE and in our experimental model of CPFE. Treatment with 4MU was able to attenuate PH and fibrosis but not airspace enlargement. This was accompanied by a reduction of HAS3-positive macrophages. We have generated pre-clinical data demonstrating the capacity of 4MU, an FDA-approved drug, to attenuate features of CPFE in an experimental model of chronic lung injury.

This article has an associated First Person interview with the first author of the paper.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) embody the third leading cause of death in the US (https://www.cdc.gov/nchs/data/hus/2017/019.pdf). COPD and IPF represent two distinct multifactorial chronic lung diseases that are characterized by distinct clinical and pathological hallmarks. A defining pathological hallmark of COPD is the presence of emphysema, defined as airspace enlargement as a result of destruction of the alveoli (Hogg and Timens, 2009; Pauwels et al., 2001). IPF is characterized by varying degrees of inflammation, aberrant fibroblast proliferation and extracellular matrix deposition that result in distortion of pulmonary architecture and loss of pulmonary function (Lynch and Toews, 1998; Panos et al., 1990; Sime and O’Reilly, 2001; Thannickal et al., 2004).

Traditionally, COPD (specifically emphysema) and IPF are regarded as separate disease entities. Nonetheless, hallmarks of both COPD and IPF were first described in autopsies of lungs in 1974 (Auerbach et al., 1974); however, it was not until 2005 that this condition was classified as a well-defined syndrome termed ‘combined pulmonary fibrosis and emphysema’ (CPFE) (Cottin et al., 2005). Male smokers or ex-smokers most often present with CPFE (Cottin et al., 2005; Jankovich and Rounds, 2010, 2012) and it has a dismal prognosis, with only a 55% survival rate at 5 years (Cottin et al., 2005). CPFE is a distinct under-recognized syndrome for which no specific diagnostic guidelines are present, yet studies show that its presentation is much more frequent than was previously believed (Cottin, 2013). An important and frequently fatal co-morbidity in CPFE is the development of pulmonary hypertension (PH), which is defined by a mean pulmonary arterial pressure (mPAP) of ≥25 mmHg at rest (Archer et al., 2010). The prevalence of PH in CPFE ranges between 47-90% and is much higher than in patients with IPF or emphysema alone (Caminati et al., 2013; Cottin, 2013; Cottin et al., 2010; Seeger et al., 2013). Confirmation of PH by right-heart catheterization data reduces 1-year survival rates to only 60% (Cottin et al., 2010). Despite this dismal prognosis, no therapy options exist for patients with CPFE outside of smoking cessation, oxygen therapy and lung transplantation (Jankovich and Rounds, 2012; Lin and Jiang, 2015). Specifically for PH in CPFE, endothelin receptor antagonists, prostanoids and phosphodiesterase type 5 inhibitors have been used. However, these agents are not recommended for PH in CPFE because of their vasodilation profile that results in ventilation/perfusion mismatch...
Although several gene mutations have been associated with CPFE, including telomerase genes [TERT or TR (also known as TERC)], surfactant protein C (SFTPC) and ATP binding cassette subfamily A member 3 (ABCA3), these have been performed in very small familial cohorts (TERT/TR) or in case studies (SFTPC/ABCA3) (Lin and Jiang, 2015). As such, no single gene defect is known to be associated with CPFE, and the mechanisms leading to the development of CPFE remain vastly unknown (Jankowich and Rounds, 2012). Despite this, studies from our group in COPD and IPF patients with PH have revealed increased activation of the adenosinergic axis and enhanced levels of the glycosaminoglycan hyaluronan (Garcia-Morales et al., 2016; Karmouty-Quintana et al., 2013a, 2012). Furthermore, several studies from our group have implicated macrophages in the pathophysiology of lung fibrosis and PH. In these studies, deletion of myeloid ADORA2B was able to prevent BLM-induced PH and lung fibrosis (Karmouty-Quintana et al., 2015, 2013a, 2012). In these studies, increased activation of the adenosine A2B receptor (ADORA2B) was shown to enhance accumulation of extracellular adenosine that is associated with fibrotic deposition (Chunn et al., 2005; Karmouty-Quintana et al., 2013a), contributing to the development of CPFE. These experiments revealed a marked increase in fibrotic deposition in CPFE mice compared to controls, highlighting the need to develop novel treatment approaches for CPFE.

RESULTS
Characterization of our experimental model of CPFE
We used Ada−/− mice to model features of CPFE. Ada−/− mice received supplemental PEG-ADA, allowing them to live normally, from birth up to week 24. Starting on week 24, PEG-ADA was gradually reduced over 9 weeks and, starting on week 34, mice were provided with either control chow or were medicated with 4MU for 4 weeks. The gradual reduction of PEG-ADA results in accumulation of extracellular adenosine that is associated with chronic injury (Karmouty-Quintana et al., 2013b).

A hallmark of CPFE is the presence of fibrotic deposition and airspace enlargement. We determined the extent of fibrotic deposition and fibrotic lung injury (Philip et al., 2017). These results point at macrophages as a potential mechanism promoting lung fibrosis and PH; however, whether they play a role in CPFE is not known.

Using a unique model of experimental lung injury that replicates features of CPFE, namely airspace enlargement, PH and fibrotic deposition (Chunn et al., 2005; Karmouty-Quintana et al., 2013a), we investigated the capacity of an HAS inhibitor to attenuate fibrotic phenotypical accumulation of extracellular adenosine that is associated with chronic injury (Karmouty-Quintana et al., 2013b).
Ada−/− mice compared to Ada+ mice, which was significantly attenuated in Ada-deficient mice treated with the hyaluronan inhibitor 4-methylumbelliferone (4MU; Fig. 1A,B). These histological changes were consistent with expression levels of fibronectin that were elevated in Ada−/− mice compared to Ada-competent mice. Therapy with 4MU reduced Fn1 expression levels in Ada−/− mice (Fig. 1C). We next examined the extent of airspace enlargement, a key feature of CPFE, in our mouse model using black and white images of the lung parenchyma. Ada−/− mice presented with evidence of airspace enlargement, as observed histologically, and by mean chord length measurements, determined morphometrically, in comparison to Ada+ mice (Fig. 1D,E). Treatment with 4MU did not alter the emphysematous development in Ada-deficient mice (Fig. 1D,E), despite improved arterial oxygenation (SpO2) compared to Ada−/− mice exposed to control chow (Fig. 1F). The development of PH is a serious and common complication of CPFE (Cottin et al., 2010). A feature of PH associated with chronic lung disease is vascular remodeling and hyaluronan deposition (Collum et al., 2017; Karmouty-Quintana et al., 2013a, 2012). In order to assess the extent of vascular remodeling and hyaluronan deposition (Collum et al., 2017; Karmouty-Quintana et al., 2013a, 2012), we performed dual-immunohistochemistry (IHC) for alpha smooth muscle actin (αSMA; Acta2) and hyaluronan. These experiments revealed extensive muscularization of arterioles in Ada−/− compared to Ada+ mice that was significantly attenuated in 4MU-treated Ada−/− mice (Fig. 1G). Widespread hyaluronan deposition was observed surrounding remodeled vessels in Ada−/− mice, whereas no hyaluronan was present in parenchymal vessels of Ada-competent animals; 4MU treatment drastically reduced the levels of peri-vascular hyaluronan in mice lacking Ada (Fig. 1G). These observations were backed by morphometric evaluation of vascular wall remodeling and hyaluronan levels in bronchoalveolar lavage fluid (BALF) (Fig. 1H,I). These analyses demonstrated increased αSMA signals in the remodeled vessels of Ada−/− mice compared to Ada+ mice that were attenuated in Ada−/− mice exposed to 4MU (Fig. 1H). BALF hyaluronan levels revealed increased hyaluronan levels in Ada−/− compared to control mice that were markedly reduced in 4MU-treated Ada−/− mice (Fig. 1I). Taken together, our results show that our model of Ada−/− mice presents with cardinal features of CPFE: fibrotic deposition, airspace enlargement and vascular remodeling, a key component of PH. Furthermore, we demonstrate that treatment of these mice with 4MU is able to attenuate both the fibrotic deposition and vascular remodeling in Ada−/− mice. To ensure that mice ingested 4MU, we measured levels of its main metabolite, 4-methylumbelliferyl-β-D-glucuronide hydrate (4MUG), in plasma from 4MU-treated mice. These results demonstrate increased 4MUG levels in mice treated with 4MU (Fig. S1).

Vascular physiology, metabolism and hyaluronan levels in Ada−/− mice

Although vascular remodeling is a key feature of PH, right-heart catheterization and determination of right ventricle systolic pressure...
(RVSP) in mice is necessary to adequately evaluate the extent of PH. As such, RVSP measurements were determined in our experimental model. We report increased RVSP in Ada−/− mice compared to Ada+ mice, which was attenuated in 4MU-treated Ada−/− mice (Fig. 2A). Consistent with the phenomenon of PH, no differences in left ventricle systolic pressure (LVSP) were observed between treatment groups (Fig. 2B). Our RVSP data was accompanied by evidence of right ventricle hypertrophy (RVH) in Ada−/− mice compared to Ada+ littermates that did not appear to be significantly attenuated following 4MU therapy (Fig. 2C). In order to probe the mechanisms associated with the development of PH, we next evaluated the expression levels of the adenosine A2B receptor (Adora2b) and evidence of metabolic alterations. Enhanced adenosinergic axis and metabolic alterations have been reported in Group III PH (Garcia-Morales et al., 2016; Karmouty-Quintana et al., 2015, 2013a, 2012). These experiments revealed heightened Adora2b expression in Ada−/− mice but no expression in Ada+ or 4MU-treated Ada−/− mice (Fig. 2D). Consistent with dysregulated metabolism, we report reduced levels of Pparg and Sdha that were not rescued following 4MU therapy (Fig. 2E,F). We next evaluated the expression levels of HAS, which are known to be dysregulated metabolic profile, nor reverse RVH.

Macrophages in Ada−/− mice

An interesting feature of the Ada−/− mice was the presence of F4/80-positive cells in the lung (Fig. 3A), whereas treatment with 4MU resulted in an attenuation of F4/80-positive cells in Ada−/− mice (Fig. 3A,B). We next evaluated whether these cells were positive for HAS3. Indeed, dual-IHC for HAS3 and F4/80 revealed signals for HAS3 and F4/80 in Ada−/− mice that appeared to be reduced following treatment with 4MU (Fig. 3C,D). Interestingly, increased HAS3 signals were observed in vascular and epithelial cells. Further IHC demonstrated that these HAS3-positive cells also expressed hyaluronan and were observed close to vessels in Ada−/− mice (Fig. 4A). Treatment with 4MU resulted in an attenuation
of HAS3/hyaluronan-positive cells and a marked reduction of hyaluronan (Fig. 4A,B). These HAS3-positive cells were also observed in areas rich in myofibroblasts (observed using αSMA immunofluorescence) that were reduced following 4MU treatment (Fig. 4C,D). Our group has previously demonstrated that activation of hypoxic adenosinergic axis in macrophages is important for the development of lung fibrosis (Karmouty-Quintana et al., 2015; Philip et al., 2017). In these studies, reduced hyaluronan deposition was observed in mice that lacked Adora2b in myeloid cells (Karmouty-Quintana et al., 2015). Thus, using MH-S cells, a murine macrophage cell line, we next examined whether increased expression of the HAS isozymes was Adora2b-dependent. These experiments revealed that Has2 and Has3, but not Has1, were increased following treatment with the Adora2b agonist Bay60-6583, and that this response was inhibited by the presence of the Adora2b antagonist GS-6201 (Fig. 5A-C). This response was consistent with elevated BAY60-6583-induced interleukin 6 that was inhibited by GS-6201 (Fig. 5D). IL6 is a known pro-fibrotic mediator downstream of macrophage Adora2b stimulation (Karmouty-Quintana et al., 2015; Le et al., 2014; Philip et al., 2017). Taken together, these results point at an Adora2b-mediated increase of HAS3-expressing macrophages (F4/80-positive cells) that lead to increased hyaluronan deposition.

The adenosinergic response in CPFE

Using explanted lung tissue derived from upper or lower lung lobes from CPFE patients or from normal lungs that were discarded for transplantation, we evaluated mediators that are involved in the generation and degradation of adenosine, in addition to adenosine receptors. In CPFE, upper lobes often present with emphysema, whereas fibrotic deposition is more prevalent in lower lobes. Quantitative RT-PCR for the ectonucleotidases CD39 (ENTPD1) and CD73 (NT5E) (Fig. 6A,B) demonstrated increased expression of the rate limiting enzyme CD73 but not CD39 in lower lobes, but not in upper lobes, of CPFE compared to normal lung tissue (Fig. 6A,B). No differences were seen in Ada expression levels from lung tissue derived from upper or lower lobes (Fig. 6C). Equilibrative nucleoside transporters (ENT) also regulate extracellular adenosine levels whereby reduced expression is associated with increased adenosine accumulation and the development of lung injury (Luo et al., 2016). However, we report no difference in ENT expression in CPFE despite a downward trend for ENT2 (SLC29A2) in lower lobes of CPFE lungs (Fig. 6D). Expression levels of adenosine receptors did not show significant alterations in Adora1 or Adora2a between normal and CPFE lung tissue (Fig. 6E,F). However, increased expression of Adora2b was detected in lower lobes of CPFE compared to normal lungs, but not in tissue derived from upper lobes (Fig. 6G). Similarly to Adora1 and Adora2a, no significant difference in expression of Adora3 was observed between normal and CPFE-derived lung tissue (Fig. 6H). Taken together, these results suggest that in CPFE, as in other chronic lung diseases (Karmouty-Quintana et al., 2013b), adenosine synthesis is increased, together with expression of its low-affinity receptor, Adora2b.

The extracellular matrix in CPFE

Next, we evaluated the protein levels of Adora2b and Has3 in CPFE. Protein expression levels for Adora2b revealed increased expression in lower lobes from CPFE lung tissue versus normal controls, concomitant with increased signals for Hist3 (Fig. 7A-C). These observations were in line with IHC for αSMA, revealing hyaluronan signals in highly remodeled areas of lung that are characterized by dense myofibroblast presence and prominent vascular remodeling in CPFE lungs compared to normal tissue (Fig. 7D). Similarly, staining for Has3 and CD68 (a human monocytic phagocyte marker) revealed positive signals for CD68 and Has3 in lower lobes of CPFE lungs (Fig. 7E,F). Our results did not show alterations in Has1 or Has2 between normal and CPFE lung tissue derived from upper or lower lobes (Fig. 8A,B). Gene expression for fibrotic genes revealed increased expression of Fn1 in upper lobes of CPFE versus normal lungs but not in lower lobes (Fig. 8C). We saw significantly increased expression of Col1a1 in lung tissue derived from lower lobes from CPFE versus normal lung tissue (Fig. 8D). Col1a2 was significantly elevated in both upper

of HAS3/hyaluronan-positive cells and a marked reduction of hyaluronan (Fig. 4A,B). These HAS3-positive cells were also observed in areas rich in myofibroblasts (observed using αSMA immunofluorescence) that were reduced following 4MU treatment (Fig. 4C,D). Our group has previously demonstrated that activation of hypoxic adenosinergic axis in macrophages is important for the development of lung fibrosis (Karmouty-Quintana et al., 2015; Philip et al., 2017). In these studies, reduced hyaluronan deposition was observed in mice that lacked Adora2b in myeloid cells (Karmouty-Quintana et al., 2015). Thus, using MH-S cells, a murine macrophage cell line, we next examined whether increased expression of the HAS isozymes was Adora2b-dependent. These experiments revealed that Has2 and Has3, but not Has1, were increased following treatment with the Adora2b agonist Bay60-6583, and that this response was inhibited by the presence of the Adora2b antagonist GS-6201 (Fig. 5A-C). This response was consistent with elevated BAY60-6583-induced interleukin 6 that was inhibited by GS-6201 (Fig. 5D). IL6 is a known pro-fibrotic mediator downstream of macrophage Adora2b stimulation (Karmouty-Quintana et al., 2015; Le et al., 2014; Philip et al., 2017). Taken together, these results point at an Adora2b-mediated increase of HAS3-expressing macrophages (F4/80-positive cells) that lead to increased hyaluronan deposition.

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and lower lobes in CPFE versus normal lung tissue, whereas COL2A1 was elevated only in the lower lobes from CPFE lungs (Fig. 8E,F). Interestingly, these results demonstrate that purinergic remodeling and increased ADORA2B levels in CPFE are observed in the lower lobes, in which fibrotic deposition is most evident, and in areas of vascular remodeling, in line with the role of adenosine in promoting fibrotic deposition and vascular remodeling in chronic lung injury (Karmouty-Quintana et al., 2013b).

**DISCUSSION**

Despite reductions in mortality in cancer and cardiovascular disease, mortality rates for chronic lung diseases, including interstitial lung disease, have remained unaffected over the last decade (The Lancet Respiratory Medicine, 2016). This is a direct result of the disparate funding that is allocated towards research in chronic lung disease that in no way reflects the heavy societal burden of the disease (The Lancet Respiratory Medicine, 2016). This translates to an overwhelming lack of effective therapies for chronic lung diseases. CPFE represents a chronic lung disease syndrome that has higher mortality and morbidity rates than diseases such as COPD or IPF. As for other chronic lung diseases, limited therapeutic options exist outside lung transplantation (Jankowich and Rounds, 2010, 2012; Lin and Jiang, 2015). Thus, there is a need to identify novel treatments that can attenuate features of CPFE and improve the morbidity and mortality rates. In this study, we have characterized an experimental model of CPFE using male 6 month old Ada−/− mice and demonstrated that they present with features of CPFE, namely fibrotic deposition, airspace enlargement and PH. We have also generated preliminary results that show that 4MU, also known as hymecromone, a US Food and Drug Administration (FDA)-approved drug, is capable of attenuating fibrotic deposition and PH in a murine experimental model of chronic lung injury that recapitulates features of CPFE. Furthermore, our studies using lung tissues derived from upper or lower lobes from explanted lungs of patients with CPFE or from normal lungs that were discarded for transplantation reveal an upregulated adenosinergic response and evidence of increased hyaluronic acid deposition. Evaluation of the potential mechanisms leading to increased hyaluronan in our experimental model point at ADORA2B-mediated HAS3 expression in macrophages, which is supported by histological observation of dual F4/80- and HAS3-positive cells.

There is a lack of experimental models of lung disease that can accurately recapitulate the pathophysiology of chronic lung diseases (Vlahos and Bozinovski, 2015). This is particularly the case for CPFE, for which limited animal models that recapitulate its cardinal features (fibrotic deposition, airspace enlargement and PH) have been described. Features of CPFE have been modeled by treating mice with both the fibrotic agent bleomycin and elastase to induce airspace enlargement (Cattani-Cavalieri et al., 2017; Trajano et al., 2016). Experiments have also been performed treating cigarette-smoke exposed mice with bleomycin or herpesvirus, leading to airspace enlargement and fibrotic matrix deposition (Zhang et al., 2017). In addition, exposure to the halogen gas bromine has also been shown to induce combined airway fibrosis and emphysema (Aggarwal et al., 2018). Although these models are able to recapitulate features of CPFE, they rely on chemical-induced lung injury, which does not accurately represent the etiology of CPFE (Jankowich and Rounds, 2012). Furthermore, these models have not characterized the presence of PH, an important feature of CPFE (Caminati et al., 2013; Cottin, 2013; Cottin et al., 2010; Seeger et al., 2013).

The Ada−/− mouse model of lung injury was first reported by Blackburn et al. (1998, 2000) and has been demonstrated to recapitulate important features of chronic lung injury such as fibrotic deposition, airspace enlargement and PH (Karmouty-Quintana et al., 2013a; Schneider et al., 2010; Sun et al., 2006). In these studies both male and female littermate Ada−/− and Ada+ mice were used and PEG-ADA therapy was maintained typically up to postnatal day (PD) 21 whereupon it was discontinued, leading to a rapid increase in extracellular adenosine levels resulting in lung injury observed typically 15 to 20 days after cessation of PEG-ADA therapy (Karmouty-Quintana et al., 2013a; Schneider et al., 2010; Sun et al., 2006). To adequately model CPFE, a disease that primarily affects males (Cottin et al., 2005; Jankowich and Rounds, 2010, 2012), we used male 6 month old Ada−/− mice that were subjected to a progressive reduction in PEG-ADA therapy, leading to a gradual increase in extracellular adenosine. Using this approach, we aimed to mimic the repeated subclinical injury that is associated with chronic lung diseases. Mice presented with extensive remodeling of the lung parenchyma including airspace enlargement, fibrotic deposition and vascular remodeling, and PH consistent with previous publications (Karmouty-Quintana et al., 2013a; Schneider et al., 2010; Sun et al., 2006).
However, the extent of fibrotic injury and features of PH, such as RVSP, appeared to be enhanced in our model. An important therapeutic finding from our study was that 4MU was capable of inhibiting PH in Ada−/− mice, consistent with our previous studies using bleomycin-exposed mice that presented with fibrotic deposition and PH (Collum et al., 2017). However, contrary to the study of Collum et al. (2017), our study demonstrates that 4MU is capable of attenuating the fibrotic deposition in Ada−/− mice. This discrepancy could be explained by the different treatment regimen of 4MU. In the present study, 4MU was administered for 4 weeks whereas in our previous study it was only given for 2 weeks (Collum et al., 2017). The longer treatment regimen may explain the effect of 4MU at inhibiting both PH and fibrotic deposition; this effect is supported by a greater reduction of hyaluronan levels, observed by enzyme-linked immunosorbent assay (ELISA) in BALF, in our present study using Ada−/− mice compared to previous studies in the bleomycin model of lung fibrosis (Collum et al., 2017). These are important pre-clinical results that demonstrate an FDA-approved drug has the capacity of attenuating cardinal features of CPFE, a disease that currently lacks any effective therapy outside lung transplantation (Jankowich and Rounds, 2012; Lin and Jiang, 2015).

Consistent with previous publications from our group (Karmouty-Quintana et al., 2013a, 2012), we demonstrate that both elevated ADORA2B expression and hyaluronan accumulation are present in our experimental model of lung injury. A novel finding of our study was that HAS3 was the main HAS that was upregulated in our experimental model, and not HAS1 or HAS2, as had previously been reported in models of lung injury (Collum et al., 2017; Karmouty-Quintana et al., 2013a, 2012). Our results are consistent with studies on cigarette-exposed mice, in which increased lung hyaluronan and HAS3 expression was observed (Bracke et al., 2010). Interestingly, elevated HAS3 signals were primarily observed in macrophages in Ada−/− mice. In addition, increased HAS3 expression in macrophages was ADORA2B-dependent, consistent with previous publications using mice that lacked ADORA2B-expression in the monocyte lineage (Karmouty-Quintana et al., 2015) and from studies demonstrating an ADORA2B-dependent HAS expression in pulmonary artery smooth muscle cells (Collum et al., 2017; Karmouty-Quintana et al., 2012). Treatment with 4MU was able to attenuate HAS3 expression levels, which is consistent with reduced hyaluronan accumulation and macrophages. These results point at macrophages as an important contributor to the development of lung fibrosis and PH, which is consistent with previous studies (Chen et al., 2016; Karmouty-Quintana et al., 2015; Pedroza et al., 2011; Philip et al., 2017) in which alternatively activated macrophages were shown to promote the development of features of chronic lung injury. It is important to mention that increased HAS3 signals were also observed in epithelial and vascular cells in Ada−/− mice, which could also contribute to increased hyaluronan accumulation. Increased vascular or perivascular expression of HAS3 and subsequent hyaluronan deposition could also contribute directly to vascular remodeling and PH by promoting vascular stiffness and proliferation, as has been previously demonstrated by our group (Collum et al., 2017). Pulmonary artery smooth muscle cell expression of HAS has been shown to be modulated by activation of ADORA2B (Karmouty-Quintana et al., 2012; Mertens et al., 2018). This is in line with our studies showing ADORA2B-mediated macrophage HAS3 expression.

A novel finding of our study was that HAS3-expressing macrophages appeared to contribute to chronic lung injury. This is
significant, as typically HAS3 is associated with the generation of low-molecular-weight (LMW) hyaluronan fragments (Itano et al., 1999). These LMW hyaluronan fragments are associated with pathogenic effects (Bracke et al., 2010; Lennon and Singleton, 2011). Importantly, results from our experimental model were consistent with the presence of HAS3-expressing CD68 cells in CPFE that was associated with massive hyaluronan deposition surrounding fibrotic areas and remodeled vessels from CPFE explanted lungs. These results are highly significant as little is known about the pathogenic mediators involved in the pathophysiology of CPFE.

It is important to mention that adenosine is a danger-associated molecular pattern that is generated following cell injury and the subsequent extracellular release of ATP that is converted to adenosine by CD39 and CD73, with the latter being the rate-limiting enzyme (Colgan et al., 2006). Adenosine can then activate any of its four receptors: ADORA1, ADORA2A, ADORA2B and ADORA3 (Fredholm et al., 2001). Adenosine is known to have many protective effects acutely; however, chronically elevated levels of adenosine and subsequent activation of its low-affinity receptor, ADORA2B, has been extensively associated with chronic lung injury (Karmouty-Quintana et al., 2013a, 2012; Mustafa et al., 2007; Sun et al., 2006).

Remarkably, treatment with 4MU was not able to attenuate airspace enlargement, RVH or metabolic changes in Ada−/− mice. RVH is typically a response to increased RVSP; as such, it is conceivable for changes to be observed over time following normalization of blood pressure. Airspace enlargement may be due, however, to other adenosinergic mechanisms that promote alveolar cell destruction. These potential mechanisms may include elevations in deoxy-adenosine (dAdo), a molecule that is also metabolized by ADA and accumulates in Ada−/− mice (Weng et al., 2013). Increased phosphorylation of dAdo by deoxy-cytosine kinase (DCK) has been shown to contribute to airspace enlargement in Ada−/− mice and increased DCK signals have been detected in stage 4 COPD (Weng et al., 2013). Taken together, these studies suggest that treatment of 4MU may not alter DCK or dAdo levels to alter airspace enlargement. Similarly, alterations in metabolism have also been reported in Ada−/− mice (Blackburn et al., 2000) that may not be affected by 4MU, but this compound may be effective at treating lung fibrosis and PH in CPFE.

In conclusion, our study has demonstrated that a gradual reduction of PEG-ADA to male 6 month old Ada−/− mice results in the evidence of purinergic remodeling consistent with increased ADORA2B expression. Taken together, these observations advocate the use of an ADORA2B antagonist to treat chronic lung diseases. Indeed, preclinical studies using both Ada−/− and bleomycin-treated mice have demonstrated that genetic or pharmacological abrogation of ADORA2B signaling is capable of attenuating features of chronic lung injury (Karmouty-Quintana et al., 2013a, 2012; Mustafa et al., 2007; Sun et al., 2006).

Fig. 7. ADORA2B and HAS3 are elevated in CPFE.
(A) Western blot for ADORA2B, HAS3 and GAPDH from upper and lower lobes from normal or CPFE lungs. (B,C) Densitometries for HAS3 (B) and ADORA2B (C) from upper lobes from normal lungs (white bars), upper lobes from CPFE lungs (light blue bars), lower lobes from normal lungs (gray bars) and lower lobes from CPFE lungs (dark blue bars). (D) Dual-IHC for αSMA (red/brown) and hyaluronan (blue) in normal (upper panels) or CPFE (lower panels) lungs. The red V denotes vessels and the green asterisks indicate myofibroblasts. (E) IHC for CD68 (blue) and HAS3 (red) from lower lobes from normal (left) and CPFE (right) lungs. Red arrowheads represent CD68-positive (HAS3-negative) signals. Black arrowheads indicate dual HAS3 and CD68 signals. (F) Double HAS3- and CD68-positive cells from ten micropictographs from the upper and lower lobes from CPFE lungs (dark blue bars). Data are mean+s.e.m. (n=3 per group). *P<0.05 (comparisons between normal and CPFE groups within upper or lower lung lobes); unpaired two-tailed Student’s t-test with a Welch correction. Scale bars: 50 µm.
development of hallmarks of CPFE: airspace enlargement, fibrotic deposition and PH. In addition, we demonstrate, using a therapeutic approach, that the FDA-approved drug 4MU is capable of reducing the extent of fibrotic injury and the features of PH. Our study also proposes a novel mechanism whereby HAS3-positive macrophages contribute to chronic lung injury in Ada−/− mice. Importantly, observations in our experimental model of CPFE were recapitulated in lung explants from patients with CPFE, in which increased hyaluronan was detected in remodeled areas of the lung, consistent with HAS3-expressing CD68-positive cells. These results are significant as very little is known about the mechanisms that promote injury in CPFE and, as a result, limited therapies exist for this deadly condition. Our study proposes that inhibition of hyaluronan accumulation is a novel potential therapy for CPFE.

MATERIALS AND METHODS

Animals
Ada−/− were generated as previously described (Blackburn et al., 1998). Briefly, to generate Ada−/− postnatal mice, an ADA minigene that targeted expression specifically to the trophoblast lineage was introduced onto the Ada−/− background. This was accomplished by intercrossing mice carrying the trophoblast-specific ADA minigene (Tg) with mice heterozygous for the −null allele. Subsequent intercrosses yielded litters that contained mice harboring the ADA minigene (Tg) that were also heterozygous for the null Ada allele (m1/m1). Given that the regulatory elements used targeted Ada expression only to trophoblasts, once born, and with the loss of the placenta, Tg-ml/m1 mice lacked ADA enzymatic activity. Ada−/− mice were genotyped as previously described (Blackburn et al., 2003; Sun et al., 2006). Mice homozygous for the Ada-null allele were designated Ada−/−, whereas control mice, designated Ada−, were heterozygous for the Ada-null allele. Ada−/− and Ada− mice were congenic on a C57BLk/6J background. Male 24 week old Ada−/− mice or littermate Ada− controls were used for all experiments. All mice were housed in ventilated cages equipped with microisolator lids and kept at an ambient temperature of 22°C and a 12 h dark/light cycle. All mice were ear-tagged and researchers were blinded to the treatment group. Animal care was in accordance with institutional and National Institutes of Health guidelines. All studies were reviewed and approved by the University of Texas Health Science Center at Houston Animal Welfare Committee.

Following consultation with a statistician, our experimental n number was set as 5, based on a power analysis (F-tests one-way ANOVA) with the following criteria: alpha error, 0.05; power, 0.95; number of groups, 4; f, 1.189. The power and f values were calculated post-hoc using previous data generated by our lab, including pulseox values, RVSP, Fulton Indices and gene expression data for fibrotic markers. O*Power 3.1.9.2 (Universität Düsseldorf, Germany) was used for all the analysis.

Experimental design
Ada−/− mice were identified at birth and maintained on ADA enzyme therapy from PD2 until PD25 as follows: PD5, 1.25 UI intramuscular (IM); PD9-PD17, 2.5 UI IM; PD21, 2.5 UI intraperitoneal (IP); PD25, 5 UI IP. Starting on PD25, mice received weekly doses of PEG-ADA (5UI IP) until PD168 (week 24). Mice were randomized to group treatment using a random number generator using www.graphpad.com/quickcalcs. Starting on week 24, PEG-ADA was gradually reduced over the course of 9 weeks to 0.125 UI IP as follows: week 1, 5UI IP; week 2, 2.5UI IP; weeks 3 and 4, 1.25UI IP; weeks 5 and 6, 0.625UI IP; weeks 7 and 8, 0.312 UI IP; week 9, 0.125UI IP. On week 34, mice were provided with medicated chow containing 4MU (Sigma-Aldrich) at 20 mg kg−1 body weight (BW) per day or normal chow (vehicle; Teklad) for 4 weeks. During the course of these 4 weeks, mice were kept on 0.0125 UI IP of PEG-ADA. The dose was chosen based on previous experiments from our group that demonstrated a beneficial effect at reducing PH in bleomycin-treated mice (Collum et al., 2017). On PD266 (week 38), physiological readouts were performed and the animals were sacrificed for the collection of tissues and fluids for analysis (details below).

Arterial oxygen saturation
Physiological assessment measuring arterial oxygen saturation was conducted on conscious mice using the pulse MouseOx software analysis (STARR Life Sciences). The hair around the neck was removed from mice in order to use the collar clip light sensor. The MouseOx provides real-time percent oxygen saturation of functional arterial hemoglobin...
using pulse oximetry measurements of light absorption from the red and infrared LEDs.

**Hemodynamic measurements**

RVSP, heart rate and RV hypertrophy measurement was performed as previously described (Karmouty-Quintana et al., 2012). Briefly, mice were given 0.75 mg kg⁻¹ of 5% Avertin (a mixture of tert-amyl alcohol and 2-2-2 tribromoethanol, Sigma-Aldrich) to induce a surgical plane of anesthesia. Mice were placed on a heated surgical workstation (Molecular Imaging Products) and secured with surgical tape. Mice were then tracheotomised with a 19G blunt needle (Brio Medical Supplies), attached to a small animal ventilator (MiniVent, Hugo-Sachs Elektronik) and ventilated at 250 µl at 200 strokes per minute. The surgical site was viewed using a surgical microscope (SMZ-2B, Nikon). An incision of ~1 cm in length was made just below the xiphoid process. An aln retractor (ALM-112, Braintrace Scientific) was used to expose the abdominal cavity to visualize the diaphragm and the liver. An incision was then made on the diaphragm to expose the heart, and the pericardium was removed. The right ventricle (RV) was then identified and a puncture was made with a 28G needle. A 1 French pressure catheter (SPR-1000, Millar) was then inserted through the puncture. Using the same procedure, we recorded left ventricular systolic pressure (LVSP) immediately after RVSP measurements. The heart rate results were continuously recorded using a Powerlab 8-SP A/D (AD Instruments) converter acquired at 1000 Hz. All RVSP results were recorded to a PC using Chart8 software. After completion of the measurements, blood was collected, and the lungs were excised and formalin fixed paraffin embedded (FFPE). The heart was excised and the atria were removed. The RV was then surgically removed and the dry weights of the RV were used to determine the extent of RV-hypertrophy [RV/left ventricle (LV)+septum]. A heart rate below 250 beats per minute (BPM) during the determination of the RVSP or LVSP was considered a violation of a predetermined criteria.

**Human tissue collection**

The use of human material for this study was reviewed by the University of Texas Health Science Center at Houston Committee for the Protection of Human Subjects (Institutional Review Board #HSC-MS-08-0354). Informed consent was obtained to collect all tissues in these studies. The demographic details of the population are summarized in Table S1. Explanted lung tissue from patients with a diagnosis of CFPE or from discarded donor lungs for transplantation were processed on site within 20 min. A 1 cm transverse center cut was obtained from each lobe. This section was divided into portions that were FFPE or flash frozen as previously described (Garcia-Morales et al., 2016).

**RT-PCR and protein expression**

Total RNA was isolated from frozen lung tissue using Trizol reagent (Life Technologies) for human samples. Recover All (Thermo Fisher Scientific) was used to isolate RNA from FFPE mouse samples. RNA samples were then DNase treated (ArticZymes) and subjected to quantitative RT-PCR. See Table S2 for primer sequences.

Results were normalized to 18srRNA. Here, two technical replicates were performed per sample. Before RT-PCR experiments, RNA quality was evaluated using the 260 nm/280 nm absorbance ratios below 1.90 or above 2.10 were considered to have poor RNA quality and thus violated a predetermined criteria.

**Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of 4MUG concentrations in mouse plasma**

For sample preparation, 4-methylumbelliflorone-13C4 (Toronto Research Chemicals) and 7-hydroxy coumarin β-D-glucuronide (as sodium salt) (Toronto Research Chemicals) were used as the internal standard (IS) for 4MU and 4MUG, respectively. The neat stock solutions of a mixture of 4MU and 4MUG were diluted in 50% methanol to prepare the spiking solutions ranging from 1 ng/ml to 5000 ng/ml. For calibration standards, 25 µl of blank plasma was mixed with 25 µl of the spiking solutions. For unknown samples, 25 µl of test plasma was mixed with 25 µl of 50% methanol to make up the volume; 25 µl of a mixture of the two IS (1000 ng/ml in 50% methanol) was also added. After vortexing all standards and samples, 150 µl of methanol/acetonitrile 20:80 (v/v) was added to the mixture, which was further vortexed vigorously for 1 min followed by centrifugation at 800 g for 10 min. Following this, 100 µl of the supernatant was diluted with 200 µl of Milli Q water.

The LC-MS/MS system consisted of an AB SCIEX QTRAP 4000 mass spectrometer coupled to a Shimadzu UFLC system. Mobile phase A is high performance liquid chromatography (HPLC)-grade water. Mobile phase B is HPLC-grade acetonitrile. Liquid chromatography separation was carried out using a Phenomenex Luna PFP(2) column (3 µm, 150×2 mm) with isocratic elution using 45% mobile phase B and a flow rate of 0.4 ml/min at room temperature. The analysis time was 2.5 min. We injected 5 µl of the extracted sample. The mass spectrometer was operated in the negative mode with multiple-reaction monitoring (MRM). The following MRM transitions were used: 4MU (m/z 174.7→132.9), IS for 4MU (m/z 178.7→134.9), 4MUG (m/z 350.8→174.9) and IS for 4MUG (m/z 336.9→160.9). Data acquisition and analysis were performed using the Analyst 1.6.1 software (AB SCIEX).

**Western blots and ELISA**

For western blots, protein from lung tissue lysates or pulmonary artery smooth muscle cells (PASMCC) was extracted using RIPA buffer (Thermo Fisher Scientific) containing 1 mM of protease and phosphate inhibitor (Sigma-Aldrich). We then loaded 30 µg of protein per sample onto 4-12% Mini-Protein TGX gels (Bio-Rad) for electrophoresis, which was then transferred on to polyvinylidene difluoride (PVDF) membranes (0.45 µm, GE Healthcare). Membranes were blocked in 5% milk (Bio-Rad) for 1 h at room temperature and then incubated with the appropriate primary antibody overnight. Secondary antibodies and an ECL Prime Western Blotting kit from GE Healthcare were applied to generate chemiluminescent signals. The following antibodies were used: ADORA2B (rabbit polyclonal, Novus Biologicals, NB2-41312; 1:2000); HAS3 (rabbit polyclonal, Proteintech, 15609-1-AP; 1:1000); GAPDH (mouse monoclonal, Invtrogen, AM4300; 1:500).

BALF was extracted by flushing the lungs with 0.5 ml of ice-cold PBS twice and collecting the fluid using a 1 ml syringe. The presence of blood in BALF was considered a violation of pre-established criteria. BALF hyaluronan levels were measured by ELISA using a kit from Echelon Biosciences and two technical replicates were performed per sample.

**Histology and IHC**

Formalin-fixed-paraffin embedded (FFPE) lung sections were prepared and stained as previously described (Karmouty-Quintana et al., 2013a) and developed using Vector Laboratories NovaRED peroxidase, Vector Red and Vector Blue Alkaline Phosphatase substrate kits. The following primary antibodies were used: smooth muscle actin (mouse monoclonal, clone 1a1, Sigma-Aldrich; 1:1000); HAS3 (rabbit polyclonal, Proteintech, 15609-1-AP; 1:200); rat anti-mouse F4/80 (Bio-Rad, MCA497, clone CI:A3-1; 1:200); rabbit anti-CD68 (Abcam, ab125212; 1:100). To detect hyaluronic acid, sections were incubated with 4 µg/ml of biotinylated HA-binding protein (HABP, Calbiochem; 1:125) overnight at 4°C, incubated with the ABC kit (Vector Laboratories) and developed using Vector Laboratories NovaRED peroxidase, Vector Red and samples, 150 µl of methanol/acetonitrile 20:80 (v/v) was added to the mixture, which was further vortexed vigorously for 1 min followed by centrifugation at 800 g for 10 min. Following this, 100 µl of the supernatant was diluted with 200 µl of Milli Q water.

To determine fibrotic deposition, lung sections were stained for Masson’s Trichrome and analyzed using a modified Ashcroft scale optimized for mouse lung sections (Hubner et al., 2008). Ten images per animal were analyzed by two individuals blinded to group status.

To quantify airspace enlargement, alveolar airspace size was determined in pressure-infused lungs by measuring mean chord lengths on Hematoxylin and Eosin-stained lung sections. Representative images (black and white) were digitized, and a grid consisting of 53 black lines at 10.5 µm intervals was overlaid on each image. This line grid was subtracted from the black and white lung images using Image-Pro Plus image analysis software (version 2.0, MediaCybernetics). The resultant lines were measured (in µm) and averaged to give the mean chord length of the airspace enlargement. The final mean chord lengths represent averages from ten non-overlapping images of each lung specimen. All quantitative studies were performed...
blinded with regard to animal genotype and treatment. It is important to note that, for the quantification of airspace enlargement, the images taken were from the parenchyma, devoid of large airway, vascular structures or fibrotic deposition. The exclusion of these structures is necessary to quantify airspace enlargement. However, these exclusions were not performed for the determination of Ashcroft scores.

**Morphometric evaluation of vascular αSMA deposition**

Muscularized arterioles of the lung parenchyma were observed under 20× magnification and noted as being different from both airways and non-muscularized arterioles. They were then photographed under 40× magnification and the microphotographs were analyzed using Image Pro-Plus software (MediaCybernetics). In short, the overall area of the muscularized portion was measured for each arteriole. To account for size, the largest diameter for each arteriole was also measured. The area of the arteriole was then divided by the largest diameter to give a relative measurement of muscularization. In additional observations, ten microphotographs of the parenchymal area were taken at 20× magnification. The number of muscularized vessels (those positive for αSMA) were counted for each microphotograph and the total number per animal was obtained and averaged within the group.

**Cell culture experiments**

The mouse alveolar macrophage cell line MH-S (ATCC) was maintained at 37°C and 5% CO₂. Cells were tested for contamination before experimentation. MH-S cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin. For experimental use, MH-S were serum-starved overnight in inactivated fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin. MH-S cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin. For experimental use, MH-S were serum-starved overnight in inactivated fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin.

**Statistical analysis**

All analyses were blinded to the experimenter. A two-way ANOVA with the α-test with a Welch correction for group differences was performed. Statistical analysis was performed using the GraphPad Prism 7.0 or higher was used to analyze the data. All software used in this study is available commercially.

**Acknowledgements**

We acknowledge Kelly Volcik PhD, McGovern Medical School at UTHealth, for proofreading the manuscript.

**Competing interests**

The authors declare no competing or financial interests.

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**Funding**

We acknowledge funding from: the American Heart Association (14SDG18550039 to H.K.-Q.), the American Lung Association (RG-414673 to H.K.-Q.), the National Institutes of Health (NHI) (5R01 HL138510-01 to H.K.-Q and 5P01 HL114457-02 to M.R.B.) and the University of Texas Health Science Center at Houston Pulmonary Center of Excellence Discovery Awards (to H.K.-Q. and Y.K.).
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