Chitinase 3–like–1 and its receptors in Hermansky-Pudlak syndrome–associated lung disease

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Hermansky-Pudlak syndrome (HPS) comprises a group of inherited disorders caused by mutations that alter the function of lysosome-related organelles. Pulmonary fibrosis is the major cause of morbidity and mortality in patients with subtypes HPS-1 and HPS-4, which both result from defects in biogenesis of lysosome-related organelle complex 3 (BLOC-3). The prototypic chitinase-like protein chitinase 3–like–1 (CHI3L1) plays a protective role in the lung by ameliorating cell death and stimulating fibroproliferative repair. Here, we demonstrated that circulating CHI3L1 levels are higher in HPS patients with pulmonary fibrosis compared with those who remain fibrosis free, and that these levels associate with disease severity. Using murine HPS models, we also determined that these animals have a defect in the ability of CHI3L1 to inhibit epithelial apoptosis but exhibit exaggerated CHI3L1-driven fibroproliferation, which together promote HPS fibrosis. These divergent responses resulted from differences in the trafficking and effector functions of two CHI3L1 receptors. Specifically, the enhanced sensitivity to apoptosis was due to abnormal localization of IL-13Rα2 as a consequence of dysfunctional BLOC-3–dependent membrane trafficking. In contrast, the fibrosis was due to interactions between CHI3L1 and the receptor CRTH2, which trafficked normally in BLOC-3 mutant HPS. These data demonstrate that CHI3L1-dependent pathways exacerbate pulmonary fibrosis and suggest CHI3L1 as a potential biomarker for pulmonary fibrosis progression and severity in HPS.

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Hermansky-Pudlak syndrome (HPS) comprises a group of inherited disorders caused by mutations that alter the function of lysosome-related organelles. Pulmonary fibrosis is the major cause of morbidity and mortality in patients with subtypes HPS-1 and HPS-4, which both result from defects in biogenesis of lysosome-related organelle complex 3 (BLOC-3). The prototypic chitinase-like protein chitinase 3–like–1 (CHI3L1) plays a protective role in the lung by ameliorating cell death and stimulating fibroproliferative repair. Here, we demonstrated that circulating CHI3L1 levels are higher in HPS patients with pulmonary fibrosis compared with those who remain fibrosis free, and that these levels associate with disease severity. Using murine HPS models, we also determined that these animals have a defect in the ability of CHI3L1 to inhibit epithelial apoptosis but exhibit exaggerated CHI3L1–driven fibroproliferation, which together promote HPS fibrosis. These divergent responses resulted from differences in the trafficking and effector functions of two CHI3L1 receptors. Specifically, the enhanced sensitivity to apoptosis was due to abnormal localization of IL-13Rα2 as a consequence of dysfunctional BLOC-3–dependent membrane trafficking. In contrast, the fibrosis was due to interactions between CHI3L1 and the receptor CRTH2, which trafficked normally in BLOC-3–mutant HPS. These data demonstrate that CHI3L1–dependent pathways exacerbate pulmonary fibrosis and suggest CHI3L1 as a potential biomarker for pulmonary fibrosis progression and severity in HPS.

Introduction

Hermansky-Pudlak syndrome (HPS) is a group of inherited autosomal recessive disorders that occur worldwide (1). Nine genetic subtypes (HPS1–9) have been described, with each mutation affecting the function of lysosome-related organelles (LROs). HPS-1 is particularly common in northwest Puerto Rico, where 1:1,800 people are affected, and the carrier frequency is 1 in 21 persons (1). The signs and symptoms of HPS are related to the dysfunction of a variety of LROs (2, 3). The dysfunction of melanosomes accounts for the ocuculocutaneous albinism and visual impairment found in all HPS patients (4). The dysfunction of platelet-dense granules accounts for the bleeding disorder that is often the presenting complaint of the disease (4, 5). Ceroid deposition also occurs in multiple organs, and inflammatory bowel disease has been reported in various subtypes of HPS (2, 6–8). More importantly, pulmonary fibrosis has been observed in HPS-1 and HPS-4 patients, whose genetic defects are in biogenesis of lysosome-related organelle complex 3 (BLOC-3), which includes HPS1 and HPS4 proteins; and, less commonly, in HPS-2 patients (3, 9–12). To date, pulmonary fibrosis has not been reported in patients with BLOC-2 defects (i.e., HPS-3, HPS-5, or HPS-6). Due to the untreated and progressive nature of the pulmonary fibrosis of HPS, this complication is the leading cause of death (13). However, there is no way to predict which HPS-1 or HPS-4 patients are at risk for lung disease, or which patients will progress most rapidly. In addition, although it is known that murine genetic models of HPS-1 manifest exaggerated injury and fibroproliferative repair responses to fibrogenic agents such as bleomycin (14), the mechanism(s) by which LRO-related defects in trafficking lead to injury and fibrosis have not been adequately defined. Furthermore, no plausible explanation for why fibrosis develops in patients with BLOC-3 mutations and not in patients with BLOC-2 mutations has been put forth. Thus, the field would benefit greatly from insights that clarify the mechanisms by which LRO dysfunction leads to injury and fibroproliferative repair and studies that highlight the relationship(s) between these mechanisms and the occurrence and severity of lung disease in HPS.

The 18 glycosyl hydrolase (GH 18) gene family contains true chitinases (Cs) that degrade chitin polysaccharides and chitinase-like proteins (CLPs) that bind but do not degrade chitin (15). GH 18 genes are members of an ancient gene family that exists in species as diverse as plants, insects, and humans, and whose evolution during speciation is characterized by a particularly impressive increase in CLPs coinciding with the appearance of mammals (16, 17). Retention of GH 18 genes across species and evolutionary time has led to the belief that some of these moieties play essential roles in biology. Recent studies have confirmed this speculation (15, 18–21), particularly for the prototypic CLP, chitinase 3-like-1 (CHI3L1; also called YKL-40 in humans and BRP-39 in mice). CHI3L1 has been shown by our laboratory and others to play major roles in anti-pathogen, antigen-induced, and oxidant-induced...
inflammation, repair, and remodeling responses by regulating a variety of essential biologic processes, including oxidant injury, apoptosis, pyroptosis, inflammasome activation, Th1/Th2 inflammatory balance, M2 macrophage differentiation, TGF-β1 elaboration, dendritic cell accumulation and activation, and MAPK and AKT signaling (18, 20–25). The potential importance of CHI3L1-induced responses can also be seen in the large number of diseases characterized by inflammation and remodeling in which CHI3L1 excess has been documented (reviewed in refs. 15, 26). In many of these disorders, CHI3L1 is likely produced as a protective response based on its ability to simultaneously decrease epithelial cell apoptosis while stimulating fibroproliferative repair (27). Recent studies from our laboratory have defined IL-13Ra2 as the first receptor for any GH 18 moiety and have demonstrated that it mediates many of the effects of CHI3L1 (28). However, the regulation and roles of CHI3L1 and its receptors in HPS have not to our knowledge been addressed.

We hypothesized that CHI3L1 is dysregulated in HPS patients with BLOC-3 defects and plays an important role(s) in the pathogenesis of HPS lung disease. To test this, we measured the levels of CHI3L1 in plasma from patients with various types of HPS and assessed the relationships between these levels and the presence and severity of lung disease. We also used WT and Hps1 mutant mice to characterize the roles of CHI3L1 and its receptors in the injury and fibroproliferative repair responses induced by intratracheal bleomycin. Our results demonstrate that levels of circulating CHI3L1 are increased in patients with HPS-1 and HPS-4 when compared with controls and non–BLOC-3 HPS patients and that, in the BLOC-3 patients, these levels correlate with the presence and severity of lung disease. CHI3L1 levels are increased in Hps1 mutant mice at baseline and after bleomycin treatment, and null mutations of Hps1 cause exaggerated bleomycin-induced epithelial cell apoptosis and fibrotic responses. These murine studies also demonstrate that CHI3L1 inhibits injury and stimulates repair in WT mice. In contrast, in Hps1 mutant mice, the ability of CHI3L1 to stimulate fibroproliferation is preserved, but its ability to control epithelial apoptosis is markedly diminished. Last, we provide insights into the mechanisms that underlie these seemingly opposed responses by demonstrating that they are due to differences in the trafficking, localization, and function of two different CHI3L1 receptors. Specifically, the abnormal regulation of apoptosis is due to abnormal BLOC-3– and Rab32/38–dependent plasma membrane trafficking and localization of IL-13Ra2, and can be overcome with IL-13Ra2 overexpression. In contrast, the exaggerated collagen accumulation is mediated by CHI3L1 interaction with CRTH2, which traffics normally in Hps1 mutant cells and tissues, and CRTH2 inhibition significantly diminishes this CHI3L1-induced fibrotic response.

Results
Levels of CHI3L1 are increased in the circulation of HPS-1 and HPS-4 patients. To determine whether CHI3L1 is dysregulated in HPS, we measured the levels of circulating CHI3L1 in patients older than 18 years of age with HPS and controls who had been assessed for the presence or absence of pulmonary fibrosis. One hundred forty-seven plasma samples from HPS patients were obtained. Of these, 129 had BLOC-3–related HPS (125 HPS-1 and 4 HPS-4) and 18 had BLOC-2–related HPS (12 HPS-3, 4 HPS-5, and 2 HPS-6). No other demographic information was available for the HPS subjects. Plasma from age-matched healthy controls was used as a control (n = 38). These assays revealed that CHI3L1 is elevated in the circulation of HPS-1 and HPS-4 (BLOC-3) patients compared with age-matched normal controls (Figure 1A). Moreover, the levels of CHI3L1 were elevated in the circulation of HPS-1 and HPS-4 patients compared with HPS-3, HPS-5, and HPS-6 (BLOC-2) patients (Figure 1A), though the values in these subtypes also mildly exceeded those of normal controls (Figure 1A). Interestingly, among the HPS-1 patients, the levels of CHI3L1 did not differ between individuals with the common Puerto Rican mutation (a 16-base-pair duplication in exon 15) and those with less common mutations (Supplemental Figure 1A). These studies demonstrate that the levels of CHI3L1 are elevated in the circulation of patients with BLOC-3–related HPS, who are more likely to develop pulmonary fibrosis, compared with normal controls and with BLOC-2–related HPS patients, who will not develop pulmonary fibrosis.

Levels of CHI3L1 are elevated in HPS-1 and HPS-4 patients with pulmonary fibrosis. Having found that HPS-1 and HPS-4 patients had elevated concentrations of circulating CHI3L1, we next stratified these patients into those with and those without documented lung disease. We found that HPS-1 and HPS-4 patients with lung disease had significantly elevated levels of circulating CHI3L1 compared with HPS-1 and HPS-4 patients without known lung disease (Figure 1B). These results demonstrate that circulating CHI3L1 is highest in those HPS patients with BLOC-3 mutations who have known pulmonary fibrosis. Immunohistochemistry on lung tissues from HPS-1 patients further revealed that airway epithelial cells and macrophages were the major source of CHI3L1 (Supplemental Figure 1B).

Levels of CHI3L1 correlate with disease severity. We next sought to determine whether circulating CHI3L1 was associated with clinically relevant parameters of disease severity. For this analysis we stratified HPS-1 and HPS-4 patients with pulmonary fibrosis into mild disease (defined as forced vital capacity [FVC] ≥80% predicted), mild-moderate disease (defined as FVC between 60% and 79% predicted), and severe disease (defined as FVC ≤59% predicted). We found that the levels of circulating CHI3L1 were similar in patients with mild and moderate disease (Figure 1C). In contrast, although there was some overlap, patients with severe disease had levels of CHI3L1 that were elevated compared with mild and moderate disease patients (Figure 1C). The levels of CHI3L1 followed a similar pattern when assessed using the diffusing capacity of lung for carbon monoxide (DLCO). These evaluations demonstrated that patients with severe disease had the highest levels of CHI3L1 (Figure 1D). In accordance with these findings, the levels of CHI3L1 displayed a modest but significant negative correlation with FVC (Figure 1E) and a negative correlation with DLCO (Figure 1F).

Levels of CHI3L1 are elevated in pale ear mice, a mouse model of HPS-1. The pale ear mouse, which has a null mutation of the Hps1 gene (Hps1<sup>−/−</sup>), shares many aspects of the human disease phenotype (14, 29). To determine whether CHI3L1 is dysregulated in pale ear mice, we evaluated lung lysate Chi3l1 mRNA and bronchoalveolar lavage (BAL) CHI3L1 protein in WT and pale ear mice at baseline and after bleomycin challenge. These assessments were
Levels of epithelial apoptosis and tissue fibrosis are elevated in pale ear mice. Studies from our laboratory and others have demonstrated that injury is a prerequisite for the development of tissue fibrosis (30). Thus, studies were undertaken to evaluate both of these responses in WT mice and pale ear mice after bleomycin administration. Dose response evaluations demonstrated that differences between these mice were most readily appreciated with 1.25 U/kg of bleomycin (Supplemental Figure 3A). Studies using this dose demonstrated that, in WT mice, bleomycin caused an acute injury response characterized by inflammation (Supplemental Figure 3B) and alveolar type II epithelial cell apoptosis on day 7 that was followed by enhanced collagen accumulation and tissue fibrosis on days 7 and 14 (Figure 2, C and D, Supplemental Figure 3, C and D). At baseline pale ear mice had a modest increase in epithelial TUNEL staining (Figure 2C and Supplemental Figure 3, C and D, and Supplemental Figure 4A). At these time points immunohistochemistry demonstrated that epithelial cells and macrophages were the major sources of CHI3L1 in pale ear mice (Supplemental Figure 2 and data not shown). These studies demonstrate that the expression of CHI3L1 is increased at baseline and during bleomycin-induced injury and repair in pale ear mice.

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compared with the WT controls, bleomycin-challenged Chi3l1-null mice and pale ear mice manifested exaggerated levels of apoptosis and BAL protein (Figure 2E and Supplemental Figure 5A). In contrast, the levels of collagen accumulation in Chi3l1 mutant and pale ear mice were comparable to and exceeded, respectively, those of WT controls (Figure 2F). Importantly, the exaggerated bleomycin-induced epithelial cell death and lung injury response in pale ear mice was not altered by an absence of Chi3l1 (Figure 2E and Supplemental Figure 5A). In contrast, the enhanced fibrotic response in pale ear mice and Chi3l1 mutant mice compared with the WT controls, bleomycin-challenged null mice (Figure 2G and Supplemental Figure 6C). In these experiments, transgenic CHI3L1 significantly increased collagen accumulation in lungs from both WT and pale ear mice (Figure 2H and Supplemental Figure 6D). In accordance with these findings, transient CHI3L1 exaggerates bleomycin-induced fibroproliferative repair in WT and pale ear mice. We also used the CHI3L1 Tg mice to further define the roles of CHI3L1 in bleomycin-induced fibroproliferative repair. We administered bleomycin to WT and pale ear mice and activated the CHI3L1 transgene only during the fibroproliferative phase (days 5–14) of this response. This resulted in similar levels of BAL CHI3L1 (between 550 and 650 ng/ml) in the WT and pale ear mice (Supplemental Figure 6C). In these experiments, transgenic CHI3L1 significantly increased collagen accumulation in lungs from both WT and pale ear mice (Figure 2H and Supplemental Figure 6D). In accordance with these findings,
The transgenic expression of CHI3L1 also increased the levels of mRNA encoding α1-procollagen and fibronectin, and the levels of BAL TGF-β1, in WT mice and caused even more substantial increases in these parameters in pale ear mice (Supplemental Figure 6, E–G). These studies demonstrate that, when overexpressed only during the fibroproliferative repair phase of the bleomycin-induced response, CHI3L1 stimulates fibrosis and matrix gene expression in WT mice and mice with null mutations of Hps1.

IL-13Rα2 membrane localization is impaired in pale ear mice. The studies noted above highlight a defect in the ability of CHI3L1 to control epithelial cell apoptosis in pale ear mice. Recent studies from our laboratory have described the first receptor for CHI3L1 or any GH 18 moiety. These studies demonstrated that CHI3L1 binds to, signals, and controls apoptosis via IL-13Rα2 (28). Hence, we sought to determine whether IL-13Rα2 localization is altered in the setting of BLOC-3 mutations. In these experiments lungs were obtained from WT and pale ear mice that had been treated with bleomycin or from controls, and IL-13Rα2 immunofluorescence staining was performed using pan-cadherin or early endosome antigen 1 (EEA1) markers to highlight the plasma membrane and intracellular/endo-somal compartments, respectively. In keeping with the literature, in WT mice at baseline, IL-13Rα2 was expressed both intracellularly and on the cell membrane (Figure 3, A and E). In contrast, in pale ear mouse lung, IL-13Rα2 was expressed at lower levels, and the cytoplasmic pool was readily noted, but the membrane pool could not be detected (Figure 3, B and F). Importantly, bleomycin treatment caused substantial IL-13Rα2 membrane translocation in WT lungs, as indicated by its colocalization with cadherin (Figure 3, C and G). In contrast, IL-13Rα2 remained in the intracellular space in bleomycin-treated pale ear mouse lungs, demonstrating that its localization to plasma membrane was impaired (Figure 3, D and H). Western blot analysis confirmed these observations. In
WT lung lysates, IL-13Rα2 could be detected in the cytosol, intracellular membrane fraction, and plasma membrane fraction, while membrane localization of IL-13Rα2 was markedly diminished in pale ear mouse lungs (Figure 4).

IL-13Rα2 localization and trafficking is different in cells from WT and pale ear mice. To further understand the role(s) of IL-13Rα2 in the defective CHI3L1 regulation of epithelial apoptosis in pale ear mice, we isolated alveolar type II cells from lungs of WT and pale ear mice and assessed the localization and trafficking of IL-13Rα2 in these cells. Consistent with our in vivo findings, IL-13Rα2 membrane translocation was readily detected in cells from WT mice following bleomycin stimulation (Figure 5, A and B). In contrast, IL-13Rα2 remained in the intracellular fraction in bleomycin-treated pale ear cells (Figure 5, A and B).

In order to track cellular IL-13Rα2 protein movements, we employed a green fluorescent protein label. In these experiments an IL-13Rα2–GFP fusion protein expression plasmid was constructed and expressed in primary WT and primary pale ear type II alveolar epithelial cells. Live cell confocal imaging was then employed to localize the tagged IL-13Rα2 over time. These studies demonstrated that bleomycin stimulated IL-13Rα2–GFP fusion protein membrane translocation in WT cells (Figure 5C). In contrast, under identical circumstances, substantial membrane translocation was not detected, and the majority of the IL-13Rα2 remained in the intracellular compartment in pale ear cells (Figure 5D). These results demonstrate that IL-13Rα2 exists in an intracellular pool and can be mobilized to the plasma membrane after stimulation with agents such as bleomycin. In addition, they demonstrate that IL-13Rα2 trafficking to the plasma membrane is impaired in pale ear cells and thus is dependent on a pathway that involves the BLOC-3 complex.

The levels of IL-13Rα2 expression are similar at baseline and modestly decreased after bleomycin treatment in pale ear mice. The studies noted above demonstrate that IL-13Rα2 accumulation and localization are different in pale ear and WT mice. To further understand these differences, we evaluated the levels of mRNA encoding IL-13Rα2 in these mice at baseline and after bleomycin treatment. As also noted above, at baseline, pale ear mice had elevated levels of apoptosis and BAL CHI3L1. Despite these changes, at baseline, pale ear and WT mice had similar levels of mRNA encoding IL-13Rα2 (Supplemental Figure 7A). This demonstrates that differences in Il13ra2 gene expression did not make a major contribution to the differences between WT and pale ear mice in the absence of bleomycin treatment. In contrast, the levels of mRNA encoding IL-13Rα2 were significantly increased in WT mice 7 to 14 days after bleomycin administration, and this response was modestly blunted in pale ear mice (Supplemental Figure 7A). Western blot analysis using whole lung lysates confirmed these findings (Supplemental Figure 7B). The findings demonstrate that differences in Il13ra2 gene expression might also contribute in a modest manner to the IL-13Rα2 abnormalities that are seen in pale ear mice.

IL-13Rα2 rescues the antiapoptotic effects of CHI3L1 in primary type II epithelial cells from pale ear mice. Studies were next undertaken to define the functional consequences of the IL-13Rα2 abnormalities in pale ear mice. In these experiments we characterized the ability of CHI3L1 to inhibit the basal and bleomycin-induced apoptosis responses in alveolar type II epithelial cells from WT and pale ear mice. The cells from WT mice manifested a low level of basal apoptosis, which was markedly increased by bleomycin treatment (Figure 6A). The basal and bleomycin-induced cell death responses were exaggerated in cells from pale ear mice (Figure 6A). Recombinant CHI3L1 was a powerful inhibitor of bleomycin-induced apoptosis in cells from WT mice (Figure 6A). In contrast, it did not decrease the apoptosis in similarly treated cells from pale ear mice (Figure 6A). Transfection of cells from pale ear mice with high concentrations of a construct that expressed IL-13Rα2 allowed a membrane pool of IL-13Rα2 to be detected (Supplemental Figure 8). This treatment decreased the levels of basal apoptosis in cells from pale ear mice (Figure 6B). It also rescued the antiapoptotic effects of rCHI3L1 after bleomycin treatment (Figure 6B). In accordance with these findings, Il13ra2-null mutant mice had similarly increased basal levels of apoptosis (Supplemental Figure 9). These studies demonstrate that the abnormalities in IL-13Rα2 in pale ear mice play a critical role in the pathogenesis of the exaggerated apoptosis in these animals. They also demonstrate that an intervention that restores the membrane localization and expression of IL-13Rα2 rescues the antiapoptotic effects of CHI3L1 in unstimulated and bleomycin-treated pale ear epithelial cells.

The BLOC-3/Rab32/38 axis plays an important role in the antiapoptotic effects of IL-13Rα2. BLOC-3 is a guanine nucleotide exchange factor for the Rab small GTPase family member Rab32/38 (31). As a result, HPS1 and HPS4 are required for the normal trafficking and activation of Rab32/38. The studies noted above highlight a critical role of BLOC-3 in IL-13Rα2 membrane trafficking and its antiapoptotic response. To further understand this trafficking pathway, we treated type II epithelial A549 cells with siRNA that specifically silenced HPS1, HPS4, Rab32, or Rab38 (Supplemental Figure 10, A–D). Bleomycin-induced IL-13Rα2 membrane accumulation was markedly diminished when the expression
of any of these moieties was diminished (Figure 6C), while the accumulation was not altered when HPS3 or RAB4A was knocked down (Supplemental Figure 10E). In accordance with the findings noted above, bleomycin-induced apoptotic responses were exaggerated when the expression of HPS1, HPS4, Rab32, and/or Rab38 was diminished (Figure 6D), and IL-13Rα2 transfection could rescue this exaggerated apoptotic response (Figure 6E). These studies demonstrate that BLOC-3 and its target Rab32/38 are required for IL-13Rα2 membrane localization and its antiapoptotic effects.

**CHI3L1 promotes fibroproliferation via CRTH2.** The above studies demonstrate that CHI3L1 is induced and plays an antiapoptotic role during the injury phase of bleomycin administration. They also demonstrate that this cytoprotective effect is mediated via IL-13Rα2 and that, in *pale ear* mice, the ability of CHI3L1 to exert its antiapoptotic effects is diminished due to the abnormal localization of IL-13Rα2. In contrast, the fibrogenic effects of CHI3L1 were intact in WT and *pale ear* mice and did not utilize IL-13Rα2 in either setting. This suggested that CHI3L1 might mediate its fibroproliferative effects via a different receptor. To address this possibility, yeast two-hybrid evaluations were undertaken to define the binding partners of CHI3L1. This approach was the first to define IL-13Rα2 as a receptor for CHI3L1 (28). In addition to IL-13Rα2, one of the most intriguing positive clones encoded the prostaglandin D2 receptor CRTH2 (Supplemental Figure 11). The ability of CHI3L1 and CRTH2 to interact with one another was then assessed using co-immunoprecipitation (Co-IP) evaluations. These studies demonstrated that the two moieties physically bind one another, because the immunoprecipitation of one always brought down the other (Figure 7A). Immunoprecipitation with antibodies against unrelated controls such as IgG or galectin-3 did not yield similar results (Figure 7A and data not shown). Fluorescence-activated cell sorting (FACS) evaluations of non-permeabilized cells also demonstrated that CHI3L1 and CRTH2 are both expressed on the surface of the cell (Figure 7B). The role(s) of CRTH2 in imple-
menting the fibroproliferative effects of bleomycin in WT, pale ear, and CHI3L1 Tg mice were then evaluated. In these experiments, inhibition of CRTH2 decreased the levels of bleomycin-induced collagen accumulation in WT and pale ear mice (Figure 7C). In addition, the exaggerated fibrosis that was caused by activation of the CHI3L1 transgene activation during fibroproliferative repair was also abrogated by CRTH2 inhibition (Figure 7D). Because M2 macrophages play an important role in fibrogenesis and studies from our laboratory have demonstrated that CHI3L1 is a powerful stimulator of M2 macrophage differentiation (27), studies were undertaken to determine whether CRTH2 played an important role in this response. As shown in Figure 7, E–G, CRTH2 plays an essential role in CHI3L1-induced M2 macrophage differentiation in vivo and in vitro. In accordance with these findings, the localization of CRTH2 was similar in lungs from WT and pale ear mice following bleomycin treatment (Figure 8, A and B). Furthermore, the levels of mRNA encoding CRTH2 (Supplemental Figure 12A) and the levels of CRTH2 protein in whole lung lysates (Supplemental Figure 12B) were similar at baseline and after bleomycin challenge in WT and pale ear mice. These studies demonstrate that the membrane localization of CRTH2 is similar in WT and Hps1 mutant cells and tissues, and that CRTH2 plays a critical role in CHI3L1-induced fibroproliferative responses.

IL-13Rα2 membrane expression is decreased in HPS lung tissues. The studies noted above demonstrate that the trafficking of IL-13Rα2 to the cell membrane is decreased in bleomycin-treated lungs from pale ear versus WT mice. Studies were next undertaken to see whether similar results would be seen in lung tissues from HPS patients. In these experiments we compared the expression of IL-13Rα2 in tissues from normal individuals, HPS patients, and patients with idiopathic pulmonary fibrosis (IPF), who have fibrosis but do not have a HPS1 defect. As shown in Figure 8C, these studies highlight a modest IL-13Rα2 signal in lungs from normal individuals, most of which was intracellular. In keeping with the literature (32, 33), an enhanced IL-13Rα2 signal, some of which was localized to cell membranes, was seen in the samples from the IPF patients (Figure 8C). In contrast, in HPS lungs IL-13Rα2 was expressed at lower levels, and the cytoplasmic pool was readily noted but the membrane pool could not be detected (Figure 8C). These findings are consistent with our findings in mice demonstrating that IL-13Rα2 trafficking to the plasma membrane is impaired in HPS lung tissues.

Discussion

HPS-related pulmonary fibrosis develops in the fourth or fifth decade of life and is the leading cause of death in HPS-1 and HPS-4 patients (3, 9, 10, 13). Despite considerable effort to understand this disorder, the mechanisms that drive the lung injury and the progressive fibrotic response in HPS patients are not well defined, and no therapeutics successfully intervene in these responses. Our studies demonstrate that the levels of CHI3L1 are elevated in the circulation of BLOC-3 HPS patients (who are more likely to develop pulmonary fibrosis) compared with BLOC-2 HPS patients and controls and that, in BLOC-3 patients, the levels correlate with physiologic parameters of disease severity. Our results also demonstrate that CHI3L1 plays an important role...
in the pathogenesis of HPS pulmonary fibrosis. Specifically, in bleomycin-treated normal mice, CHI3L1 decreased epithelial injury while stimulating fibroproliferative repair. In contrast, in the pale ear mouse model of HPS-1, the ability of CHI3L1 to inhibit epithelial apoptosis was markedly blunted, while the fibroproliferative effects of CHI3L1 were augmented. This demonstrates that a defect in the CHI3L1 axis is a major contributor to the exaggerated sensitivity of HPS epithelium to injury-inducing and apoptotic stimuli (14, 34). We also provide insights into the mechanisms that underlie the defect in CHI3L1 inhibition of epithelial apoptosis and the augmented fibroproliferative repair in HPS. These studies demonstrate that IL-13Ra2, the receptor that CHI3L1 uses to mediate its antiapoptotic effect (28), does not localize properly to the cell membrane in pale ear Hps1-null cells and tissues. They also demonstrate that overexpression of IL-13Ra2 rescues this antiapoptotic effect. Simultaneously, they demonstrate that CHI3L1 also binds to CRTH2, that CRTH2 localizes normally in cells and tissues from pale ear Hps1-null mice. Hence, interventions that control epithelial apoptosis in the setting of oxidant and other injuries. Therefore, the discovery of a protein with the ability to exert compartment-specific effects upon different components of the fibrotic response is exciting. Our studies suggest that CHI3L1 is just such a moiety, with distinct roles in injury and repair. Specifically, in these and other studies in WT mice, CHI3L1 has protective effects that can be mediated by its ability to decrease epithelial apoptosis, inhibit inflammation, and decrease oxidant injury (36). CHI3L1 can also drive fibroproliferative repair by augmenting alternative macrophage activation, fibroblast proliferation, and extracellular matrix gene expression during the repair response. As noted above and in studies from other groups, HPS mouse models do not spontaneously develop fibrosis (37). However, they consistently manifest exaggerated sensitivity to fibrogenic, injurious, and apoptotic stimuli, and these exaggerated injury responses are believed to lead to the pulmonary fibrosis that follows (14, 29, 34, 37-42). Our studies add to this body of data by demonstrating, for the first time to our knowledge, that the CHI3L1 axis plays an essential role in the regulation of epithelial apoptosis and that this response is blunted in pale ear mice. Hence, interventions that
of IL-13Rα2 exists in an intracellular cytoplasmic pool and traffics to the plasma membrane after appropriate cellular activation (53, 54). However, the mechanism(s) of IL-13Rα2 mobilization to the plasma membrane have not been defined. Our current studies demonstrate that IL-13Rα2 trafficking to the cell surface is mediated by a BLOC-3 complex–dependent pathway. In fact, insufficient IL-13Rα2 membrane localization is responsible for the enhanced apoptotic response in pale ear mice, and overexpression of IL-13Rα2 rescues the antiapoptotic effects of CHI3L1 in pale ear cells. These studies define, for the first time to our knowledge, the important role that abnormalities in IL-13Rα2 trafficking play in HPS. Studies by others have demonstrated that IL-13Rα2 is a critical downregulator of IL-13–mediated tissue fibrotic responses (55, 56). Additional investigation will be required to determine augment CHI3L1 can be therapeutically useful in controlling the injury phase of a variety of pulmonary disorders. However, this would not be as useful in patients with HPS-1 and HPS-4, where IL-13Rα2 trafficking is also altered (see below).

IL-13Rα2 was described as a high-affinity receptor for IL-13 that is distinct from the IL-13Rα1–IL-4Rα receptor dimer that IL-13 shares with IL-4 (43, 44). It was initially believed to be a decoy receptor (45). However, other studies demonstrated that IL-13 signals and regulates a variety of cellular and tissue responses via IL-13Rα2 (44, 46–52). Recent studies from our laboratory identified the first receptor for any GH 18 moiety by demonstrating that IL-13Rα2 binds to and is activated by CHI3L1 (28). These studies also showed that CHI3L1 mediates its antiapoptotic effects via this ligand-receptor interaction (28). Importantly, the majority of IL-13Rα2 exists in an intracellular cytoplasmic pool and traffics to the plasma membrane after appropriate cellular activation (53, 54). However, the mechanism(s) of IL-13Rα2 mobilization to the plasma membrane have not been defined. Our current studies demonstrate that IL-13Rα2 trafficking to the cell surface is mediated by a BLOC-3 complex–dependent pathway. In fact, insufficient IL-13Rα2 membrane localization is responsible for the enhanced apoptotic response in pale ear mice, and overexpression of IL-13Rα2 rescues the antiapoptotic effects of CHI3L1 in pale ear cells. These studies define, for the first time to our knowledge, the important role that abnormalities in IL-13Rα2 trafficking play in HPS. Studies by others have demonstrated that IL-13Rα2 is a critical downregulator of IL-13–mediated tissue fibrotic responses (55, 56). Additional investigation will be required to determine
Nevertheless, our studies suggest that interventions that increase CHI3L1 or if IL-13 signaling contributes to fibrogenesis in HPS. α whether these protective effects of IL-13Rα2 binding to and signals via CRTH2. They also demonstrate, for epithelial apoptosis. The present studies demonstrate that CHI3L1 is a critical regulator of the levels of basal and bleomycin-stimulated enzyme activity in melanocytes (31, 37). In the present studies we demonstrate, for the first time to our knowledge, that Rab32/38 also play critical roles in IL-13Rα2 trafficking and that loss of BLOC-3-mediated Rab32/38 activity can explain the type II lung epithelial cell dysfunction and apoptosis seen in pale ear mice. These studies allow for the interesting speculation that interventions that augment Rab32/38 can improve IL-13Rα2 trafficking and prove therapeutically useful in BLOC-3 HPS patients.

Recent studies from our laboratories have also demonstrated that galectin-3, a β-galactoside-binding lectin, is also dysregulated in HPS (58, 59). These studies demonstrated that the levels of galectin-3 are increased in the BAL and lungs of HPS patients. They also demonstrated that its accumulation was decreased in plasma membranes of fibroblasts from HPS patients. These findings suggest that galectin-3, like CHI3L1, also traffics abnormally in HPS. When viewed in combination, these observations allow for the possibility that the abnormal trafficking of moieties such as the 14 mammalian galectins and receptors of 18 glycosyl hydrolases may be a general feature of HPS. The appreciation that these moieties have far reaching effects on essential biologic processes, including cell adhesion activation growth and differentiation, apoptosis, and tissue repair, also allow for the interesting possibility that these trafficking abnormalities play essential roles in the pathogenesis of HPS. Additional investigation will be needed to assess the validity of these speculations. It will also be important to determine whether these moieties interact with one another and if BLOC-3-mediated Rab32/38 activity is also responsible for galectin-3 trafficking to the membrane.

CHI3L1 is produced by a wide variety of cells, including macrophages, epithelial cells, and chondrocytes and is markedly induced in a wide variety of diseases characterized by inflammation, remodeling, and aging (17, 60–62). Recent studies have provided insights into the roles that CHI3L1 plays in these settings by demonstrating that CHI3L1 is a critical regulator of cell death, inflammation, dendritic cell accumulation and activation, oxidant injury, macrophage differentiation, bacterial clearance, innate immunity, and host tolerance to infection (18, 20, 25, 36, 63, 64). They also highlighted the mechanism(s) that CHI3L1 uses in many of these settings by demonstrating that CHI3L1 binds to and signals via IL-13Rα2 (28). The ability of CHI3L1 to regulate apoptosis via IL-13Rα2 was a prominent finding in the earlier studies of CHI3L1-IL-13Rα2 interactions (28) and is reinforced in the current studies, which demonstrate that the CHI3L1/IL-13Rα2 axis is a critical regulator of the levels of basal and bleomycin-stimulated epithelial apoptosis. The present studies demonstrate that CHI3L1 also binds to and signals via CRTH2. They also demonstrate, for the first time to our knowledge, that this binding plays a critical role in CHI3L1-induced fibroproliferative repair. In accordance with the importance of M2 macrophages and fibroblast activation in fibrogenesis (27), these studies also demonstrate that CRTH2 plays a key role in CHI3L1-induced M2 macrophage differentiation and allow for the speculation that CRTH2 also plays a role in fibroblast proliferation and activation and other fibroproliferative events. Overall, these findings demonstrate that there is more than one CHI3L1 receptor and that they differ in the mechanisms they use to traffic to the cell membrane and the effector responses they activate. Importantly, they also demonstrate that the lung pathology in HPS is mediated, at least in part, by the differential regulation of these receptors and their receptor responses. When viewed in combination, these studies demonstrate that dysregulation of CHI3L1, abnormal IL-13Rα2 trafficking, and normal CRTH2 trafficking are fundamental events in BLOC-3 HPS lung disease and allow for the conceptualization presented in Figure 8D. These findings are similar in many ways to the well-documented relationship between glucose, hepatic lipid responses, and insulin resistance in diabetes. In these patients, whole-body insulin resistance results in hyperglycemia despite increased levels of circulating insulin, with the increased levels of insulin driving hepatic lipogenesis and the development of fatty liver (65, 66). Overall, these studies suggest that interventions that increase IL-13Rα2 membrane localization and block CRTH2 function, alone or in combination, could be used to treat BLOC-3 HPS patients. Additional investigation will be required to test this speculation.

Bleomycin is well known to induce acute injury and later fibroproliferative responses in the lung. One of the interesting findings in these and previous (27) studies was that the levels of CHI3L1 decreased during the former and increased remarkably during the latter. On superficial analysis it is hard to understand how these divergent responses both contribute to the generation of pulmonary fibrosis. Since WT mice and pale ear mice manifest similar biphasic responses, it is also difficult to understand how this biphasic response contributes to the exaggerated responses in pale ear mice. However, the answers to these questions bring forth a number of important points. First, studies from our laboratory have demonstrated that oxidant injury is a powerful inhibitor of CHI3L1 and that if oxidant inhibition of CHI3L1 is prevented, oxidant injury and lung epithelial cell apoptosis are abrogated (36). Similar results were found in experiments with bleomycin (27). This suggests that a decrease in CHI3L1 appears to be a prerequisite for agents such as hyperoxia or bleomycin to induce the acute and severe tissue injury and alveolar epithelial cell death responses that they generate. Since it is well known that injury is a prerequisite for the development of tissue fibrosis (30), one can see how this decrease in CHI3L1 is required to allow fibrosis to be generated. As regards the exaggerated injury and fibrotic responses in pale ear mice, there are a number of important differences between the responses in these mice that need to be highlighted. First, in the pale ear mice there are exaggerated levels of CHI3L1 at baseline and at days 7 to 14, when these levels exceed those in WT mice. Interestingly, although CHI3L1 was a powerful inhibitor of apoptosis in WT mice and the levels of CHI3L1 were increase in pale ear mice, CHI3L1 did not inhibit apoptosis in the Hps1 mutant animals. This apparent lack of CHI3L1 effect in the pale ear mice appears
to be due, at least in part, to alterations in IL-13Rα2 biology. The most important alteration appears to be the impressive defect in trafficking and membrane localization that can be seen at baseline and after bleomycin administration. However, we cannot rule out a modest contribution by the decrease in bleomycin-induced gene expression that is seen during the fibroproliferative repair phase in *pale ear* versus WT lungs. From these observations it is clear that the ability of bleomycin to decrease CHI3L1 is a prerequisite for the development of pulmonary fibrosis in WT and *pale ear* mice. It is also clear that the exaggerated levels of CHI3L1 and abnormal trafficking and possibly gene expression of IL-13Rα2 play critical roles in the exaggerated apoptotic/injury and fibrotic responses that are seen. Additional investigation will be required to identify the mechanisms that drive this biphasic response.

Our studies demonstrate that the levels of circulating CHI3L1 are increased in BLOC-3 HPS patients, where they associate with disease severity. They also demonstrate that the levels that are seen are higher than in patients with HPS mutations that are not associated with lung disease. At present, we do not know whether this increase is due solely to the ongoing injury response caused the abnormal trafficking of IL-13Rα2. Alternatively, it could represent a feedback mechanism that is attempting to control tissue injury. Nonetheless, these observations suggest that the levels of circulating CHI3L1 might be a clinically useful biomarker that predicts the development or rate of progression of lung disease in BLOC-3 HPS patients. It is important to point out, however, that we do not have longitudinal studies that address these possibilities. We are lacking extensive demographic information, and we also cannot rule out a confounder effect based on the number of patients of Puerto Rican ancestry in our patient cohort. Thus, additional investigation will be required to determine whether the levels of circulating CHI3L1 are truly a useful predictor of disease progression in HPS.

In summary, the present studies demonstrate that BLOC-3 HPS is associated with higher levels of CHI3L1 and suggest that CHI3L1 is likely produced as a protective response based on its ability to simultaneously decrease epithelial cell apoptosis and stimulate fibroproliferative repair in normal individuals. However, in BLOC-3 HPS, the ability of CHI3L1 to inhibit epithelial cell apoptosis is impaired due, at least in part, to faulty trafficking and insufficient membrane expression of the CHI3L1 receptor IL-13Rα2. On the other hand, the sustained and enhanced production of CHI3L1 interacts with CRT1H2, which traffics normally, and causes an exaggerated fibroproliferative repair response and fibrotic excess in BLOC-3 HPS patients. Additional investigations of the roles of CHI3L1 and its receptors in HPS and related diseases are warranted.

**Methods**

**Patient characteristics.** One hundred and forty-seven samples were obtained from molecularly confirmed HPS patients ≥18 years of age with or without known lung disease (based on high-resolution CT [HRCT] evaluation) that were enrolled in a clinical protocol approved by the NHGRI Institutional Review Board. Of the 147 patients, 129 had BLOC-3-related HPS (125 HPS-1 and 4 HPS-4) and 18 had BLOC-2–related disease (12 HPS-3, 4 HPS-5, and 2 HPS-6). The mean age of BLOC-3-related patients was 37.7 ± 0.9 years, while the mean age of the BLOC-3–related patients was 37.7 ± 0.9 years, while the mean age of BLOC-3–related HPS (125 HPS-1 and 4 HPS-4) and 18 had BLOC-2–related disease (12 HPS-3, 4 HPS-5, and 2 HPS-6). The mean age of HPS-3–related patients was 37.7 ± 0.9 years, while the mean age of HPS-3–related disease (12 HPS-3, 4 HPS-5, and 2 HPS-6). The mean age of
Immunofluorescence staining and immunohistochemistry. To localize the expression of IL-13Rα2 and CRTH2, double-label immunofluorescence staining was undertaken using paraffin-embedded lungs from WT and pale ear mice. Monoclonal anti-IL-13Rα2 (R&D Systems, #MAB539), anti-CRTH2 (Santa Cruz Biotechnology Inc., #sc-23093), and antibodies against pan-cadherin (Abcam, #ab16505) and EEA1 (Abcam #ab2900) and Cell Signaling Technology #3288 were used in these evaluations. To localize the expression of IL-13Rα2 in humans, lung biopsies from normal, IPF (27), and HPS-1 patients were obtained, and immunofluorescence staining was undertaken. Polyclonal anti-IL-13Rα2 antibody (Milipore, #06-1091) was used in these evaluations. Immunohistochemical analysis of HPS lungs was performed as previously described (27).

Live cell confocal imaging. WT and pale ear mouse lungs were digested with collagenase and DNase I. The resulting suspension was filtered through Falcon cell strainers, and the cells were negatively selected with anti-CD16/CD32 antibodies (eBioscience, #14-0161). Cells were maintained in BEGM + 5% charcoal-stripped FBS and 10 ng/ml KGF. Mouse IL-13Rα2-EGFP fusion protein plasmid was constructed (Origene) and expressed in WT and pale ear primary type II cells (500 ng/well). Cells were stimulated with 100 μg/ml bleomycin and imaged every five minutes. DRAQ5 was used to stain the nuclei (Thermo-Fisher).

Cell culture. Primary type II cells were pretreated with 500 ng/ml recombinant CHI3L1, transfected with IL-13Rα2 construct (3 μg/well), and stimulated with 100 μg/ml bleomycin. A549 cells were obtained from ATCC and maintained in DMEM + 2 mM glutamine + 10% FBS. HPS1, HPS4, Rab32, and/or Rab38 siRNA were cotransfected with IL-13Rα2 construct (3 μg/well) and stimulated with 100 μg/ml bleomycin. Cells were cytopsined onto slides. Immunofluorescence and/or TUNEL staining was performed as previously described. Mouse peritoneal macrophages were obtained as previously described (28). Cells were transfected with control or CRTH2 siRNA and treated with recombinant CHI3L1 (500 ng/ml) for 24 hours.

Western blot analysis. Lung and cell lysates were fractioned using a Plasma Membrane Protein Extraction Kit (Abcam), and Western blot analysis was completed with antibodies that react selectively with IL-13Rα2 (R&D Systems, #MAB539), EEA1 (Abcam, #ab2900), pan-cadherin (Abcam, #ab16505), and GAPDH (Cell Signaling Technology, #36683) as described previously (30).

Yeast two-hybrid screening. Yeast two-hybrid screening was performed as previously described (28). Briefly, full-length murine Chi3l1 DNA was cloned into the yeast two-hybrid BD vector at the BamHI and SalI sites. The Matchmaker System 3 two-hybrid assay using Saccharomyces cerevisiae (Clontech) was used to detect interactions between CHI3L1 and other cellular proteins. S. cerevisiae strain AH109 (Clontech) containing the four reporter genes ADE2, HIS3, MELI, and lacZ was cotransfected with the pGBK7-CHI3L1 bait plasmid and the mouse lung cDNA library (Clontech) constructed into the vector pAC2 by the lithium acetate method. All positive constructs were rescreened for their ability to grow on complete dropout medium when transfected together with the CHI3L1 bait or with the empty pGBK7 plasmid to verify potential interactions and to eliminate false positives.

Co-IP. Proteins from the lung lysate of WT mice or Il13 Tg mice were clarified by centrifugation for 10 minutes at 4°C. CHI3L1 and CRTH2 were immunoprecipitated with anti-CHI3L1 rabbit polyclonal antibody (MedImmune) or anti-CRTH2 monoclonal antibody (Santa Cruz Biotechnology Inc.), respectively, using Catch and Release v2.0 Reversible Immunoprecipitation System (EMD Millipore). The precipitates were subjected to immunoblotting with antibodies against CRTH2 or CHI3L1, respectively.

FACS analysis. THP-1 cells (ATCC) were incubated in the presence or absence of anti-CHI3L1–biotin antibody and anti-CRTH2 IgG antibody without permeabilization. Cells were then washed and stained with streptavidin–PE and anti-IgG–APC and subjected to flow cytometric analysis.

CRTH2 inhibition. Sex-matched, 8-week-old WT, pale ear, and CHI3L1 Tg mice (≥5 mice/group) were exposed to a single bleomycin injection (1.25 U/kg; Teva Parenteral Medicines) via intratracheal administration. Mice were treated with 30 mg/kg CAY10471 (Cayman Chemical) (i.p., every other day) or DMSO control from day 5 to day 14.

Statistics. Human data are presented as dot plots with median and interquartile ranges unless stated otherwise. Data distribution was assessed with the D’Agostino and Pearson omnibus test. Normally distributed data were compared using ANOVA with Bonferroni’s post hoc test as appropriate. Non-normally distributed data in two groups were compared using the nonparametric two-tailed Mann-Whitney U test. Correlations between pulmonary function and plasma CHI3L1 concentrations were performed for those subjects on whom clinical data were available using Spearman correlations. GraphPad Prism 5.0 (GraphPad Software) and SPSS 13.0 (SPSS Inc.) were used for the analysis. Mouse data are expressed as mean ± SEM. As appropriate, groups were compared by ANOVA with Bonferroni’s post hoc test; follow-up comparisons between groups were conducted using a two-tailed Student t test. A P value of ≤0.05 was considered significant.

Study approval. HPS patients were enrolled in a clinical protocol approved by the NHGRI Institutional Review Board. All patients gave written informed consent for protocols 95-HG-0193 and/or 04-HG-0211. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Yale School of Medicine and Brown University in accordance with federal guidelines.

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