Placenta growth factor augments airway hyperresponsiveness via leukotrienes and IL-13

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Airway hyperresponsiveness (AHR) affects 55%–77% of children with sickle cell disease (SCD) and occurs even in the absence of asthma. While asthma increases SCD morbidity and mortality, the mechanisms underlying the high AHR prevalence in a hemoglobinopathy remain unknown. We hypothesized that placenta growth factor (PIGF), an erythroblast-secreted factor that is elevated in SCD, mediates AHR. In allergen-exposed mice, loss of Plgf dampened AHR, reduced inflammation and eosinophilia, and decreased expression of the Th2 cytokine IL-13 and the leukotriene-synthesizing enzymes 5-lipoxygenase and leukotriene-C4-synthase. Plgf−/− mice treated with leukotrienes phenocopied the WT response to allergen exposure; conversely, anti-PIGF Ab administration in WT animals blunted the AHR. Notably, Th2-mediated STAT6 activation further increased PIGF expression from lung epithelium, eosinophils, and macrophages, creating a PIGF/leukotriene/Th2-response positive feedback loop. Similarly, we found that the Th2 response in asthma patients is associated with increased expression of PIGF and its downstream genes in respiratory epithelial cells. In an SCD mouse model, we observed increased AHR and higher leukotriene levels that were abrogated by anti-PIGF Ab or the 5-lipoxygenase inhibitor zileuton. Overall, our findings indicate that PIGF exacerbates AHR and uniquely links the leukotriene and Th2 pathways in asthma. These data also suggest that zileuton and anti-PIGF Ab could be promising therapies to reduce pulmonary morbidity in SCD.

Introduction

Sickle cell disease (SCD) is one of the most prevalent genetic disorders worldwide. It is caused by a mutation in the β-globin gene that results in sickle-shaped rbc during deoxygenation (1, 2). Adhesion of rbc and leukocytes to the endothelium, rbc sickling, and adhesion of sickle rbc to wbc result in vascular occlusions and multiple end-organ complications. However, the oxygen tension in lungs is too high for sickle hemoglobin polymerization. Indeed, the lung is the only organ where sickled rbc returning from the venous vasculature can unsickle. Despite this, acute and chronic lung diseases are major complications of SCD, and independent risk factors for morbidity and mortality in these patients (3, 4) and mechanisms behind them are largely unknown. Airway hyperresponsiveness (AHR), a dynamic narrowing of the lower airways, is highly prevalent in patients with SCD, affecting 55%–77% of children (5–7) and 33% of adults with SCD (8, 9). In contrast, the reported prevalence of AHR is 6%–20% in the African-American and black Caribbean pediatric population (6, 10).

Clinically, AHR is a key feature of asthma and is assessed using a provocative challenge (e.g., methacholine challenge) to induce a measurable bronchoconstriction. In SCD, the incidence of AHR is much higher than that of clinically symptomatic asthma (5, 6, 10–12) and is present without clinical features of asthma (wheezing, exhaled NO, and positive skin test to aeroallergens). But unlike asthma, which is a known risk factor for morbidity in SCD (13–16), the mechanism behind high AHR and its clinical significance is unknown. In SCD, biomarkers of hemolysis rather than those of asthma show a strong correlation with AHR (7). This led us to hypothesize that the etiology of the high AHR prevalence in SCD may be related to erythroid cells.

Following allergen stimulation, AHR is driven by a potent Th2 immune response that primes B cells to release allergen-specific IgE and drives lung inflammation. Th2 cytokines, such as IL-13 and IL-4 (17), stimulate airway smooth muscle contraction, recruit inflammatory cells, and increase mucus production; congestion and constriction of the airway lumen contributes to its remodeling (18, 19). In SCD, AHR has been presumed to occur secondarily to the leukocytosis and inflammation present at steady state. Notably, SCD patients also have elevated levels of LTE4 and LTC4 at steady state (20, 21). LTC4, a potent neutrophil inflammatory molecule (22), and cysteinyl LTs (CysLT) have been implicated in different aspects of allergen-induced asthma (18, 23). CysLT receptor inhibitors of 5-lipoxygenase (5-LO, encoded by Alox5) inhibitors are currently licensed for use for the treatment of asthma in humans (24, 25).

Conflict of interest: B. Jonck is an employee and P. Carmeliet is on the board of ThromboGenics NV. ThromboGenics NV provided the anti-PIGF Ab.

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Figure 1. PlGF deficiency blunts AHR. (A) Experimental schema of induction of AHR in BALB/c mice. Mice were sensitized to HDM or saline i.p. and then exposed to HDM i.t. at the indicated time points; airway responsiveness was measured 48 hours after the last i.t. HDM exposure. (B) Airway responsiveness in Plgf+/+ or Plgf−/− mice was measured by airway pressure inflation after a challenge with acetylcholine. *P < 0.05 by 1-way ANOVA. (C) Airway responsiveness was also measured in response to increasing concentrations of methacholine using a forced oscillatory technique. HDM-treated Plgf+/+ mice either injected with vehicle (Saline) or αPlGF. *P < 0.05 Plgf+/+ HDM (vehicle) versus Plgf−/− HDM; #P < 0.05 Plgf+/+ HDM (vehicle) versus Plgf−/− HDM (αPlGF) by 2-way ANOVA. (D and E) Plasma IgE levels in Plgf+/+ and Plgf−/− mice measured in BALF after the last i.p. sensitization with HDM (D) or 48 hours after the last HDM airway exposure (E). *P < 0.05, **P < 0.01 by 1-way ANOVA. All data are presented as mean ± SEM; each symbol represents an individual mouse; n = 5 animals in the saline-treated groups and 6–10 animals in the HDM-treated groups. Results are from 1 experiment (B) and representative of 2 independent experiments (C–E).

Our group previously reported that placenta growth factor (PlGF), an erythroid cell–secreted growth factor that belongs to the vascular endothelial growth factor family, is increased in plasma of patients with SCD and contributes to the baseline inflammation and features of pulmonary hypertension (26, 27). Herein, we hypothesized that PlGF mediates the high prevalence of AHR in SCD. We utilized genetic and pharmacological ablation of Plgf in WT mice and Berkeley sickle mice (hereafter termed HbS mice) (28). We have previously reported that crosses between Plgf KO and HbS mice were born extremely rarely, and the few born were runted and infertile (29). Hence, we studied AHR in Plgf-deficient and HbS mice separately. Herein, we show that Plgf is a novel mediator of AHR.

Results

PlGF deficiency reduces allergen-induced AHR. To test the functional relevance of PlGF signaling in AHR development, we utilized BALB/c mice genetically lacking Plgf (Plgf−/−) and WT BALB/c controls (Plgf+/+). We first established that Plgf deficiency had no effect on lung mechanics at baseline (Supplemental Figure 1). To generate a model of allergic AHR, Plgf−/− and Plgf+/+ mice were sensitized by i.p. injections with house dust mite (HDM) allergen extract on days 0 and 7, followed by 2 rounds of airway exposure to HDM via intratracheal (i.t.) delivery on days 14 and 19 (Figure 1A). Airway sensitivity was measured on day 21 after the administration of a cholinergic agent using 2 well-established methodologies: the airway pressure time inflation and the forced oscillatory technique, which measure the AHR via airway pressure time index (APTI) (30) and airway resistance (R) (31), respectively.

Increased AHR (APTI ≥ 300 cm H2O × s) was detected in Plgf+/+ mice exposed to HDM, as compared with their saline-exposed counterparts (Figure 1B). However, Plgf−/− mice exposed to HDM showed a 2-fold reduction in AHR compared with HDM-exposed Plgf+/+ mice (683 ± 94 cm H2O × s vs. 1,283 ± 229 cm H2O × s, respectively, Figure 1B). We confirmed the decrease in AHR in Plgf−/− mice using the forced oscillatory technique and observed the same results (Figure 1C). PlGF did not directly induce AHR in Plgf+/+ mice, when delivered intranasally into the airways (Supplemental Figure 2). Taken together, our data show that PlGF indirectly exacerbates allergen-mediated AHR.

To determine if PlGF exacerbates allergen sensitization, we measured circulating allergen-specific IgE in the plasma of Plgf+/+ and Plgf−/− mice directly after i.p. allergen sensitization (day 14) or after i.t. allergen exposure (day 21). HDM-specific IgE was significantly lower in Plgf−/− mice than in Plgf+/+ mice within 2 weeks of
Plgf–/– decrease in goblet cells in (Figure 2B). The decreased mucus formation and a modest Plgf+/+ challenged significantly blunted asthma histological score of the HDM-challenged and perivascular and peribronchial infiltrates, which confirmed a tration, goblet cell hyperplasia, alveolar septal thickening, vascular smooth muscle hypertrophy/hyperplasia, inflammatory cell infil-

Figure 3). Asthma-related histological changes were graded for protein analysis diluted the goblet cell effect. Overall, we demonstrate significant changes in Clca3 after HDM exposure was also reflected in lower Muc5AC (Figure 3A) and Clca3 (Figure 3B) immunostaining of lung sections and a trend toward reduction in Muc5AC levels in BALF (P < 0.06, Supplemental Figure 4D). A clear reduction in goblet cell hyperplasia was observed in histology (Figure 2A), but Western blots for Clca3 on whole-lung homogenates from Plgf+/+ and Plgf–/– mice did not demonstrate significant changes in Clca3 levels (Supplemental Figure 4, A–C), likely because combining all the cells in the lungs for protein analysis diluted the goblet cell effect. Overall, we observed a modest reduction in goblet cell hyperplasia and mucus formation in HDM-exposed Plgf-null animals.

Analysis of the bronchoalveolar lavage fluid (BALF) revealed that the reduction in cellular infiltration into the airways in HDM-exposed Plgf+/+ animals was primarily due to reduced eosinophil infiltration in the lungs (Figure 3C). However, levels of the eosinophil chemokines eotaxin-1/2 in BALF were not significantly different from Plgf+/+ animals (P = 0.1, Supplemental Figure 4E). We therefore examined if part of the reason for the reduced eosinophilia in BALF was due to decreased eosinophil production in the Plgf+/+ animals. Flow cytometry for blood eosinophils (CD11b+CD11c SiglecF+ cells; Supplemental Figure 5A), in conjunction with leukocyte counts in Plgf+/+ and Plgf–/– animals, were performed to determine absolute eosinophil numbers in blood. As anticipated, there was no difference in circulating eosinophil levels in saline-challenged controls of either genotype. However, the decrease in BALF eosinophil numbers in HDM-challenged Plgf+/+ mice (Figure 3C) was associated with a significant decrease in total circulating eosinophils (Figure 3D), suggesting that impaired recruitment by eotaxin-1/2 may not be the primary reason for reduced BALF eosinophils and that PlGF deficiency affects eosinophil production/recruitment from the BM.

Collectively, these data demonstrate that PlGF enhances allergen-induced eosinophilia in the blood and lungs, modestly affects mucus production, and significantly increases the inflammatory features associated with asthma.
**PIGF modestly augments the Th2 immune response.** Analyses of Th2 cytokines in the BALF revealed significantly lower IL-13 levels in Plgf+/− mice (49.6 ± 16.3 pg/ml, n = 22) compared with Plgf+/+ mice exposed to HDM (89.8 ± 16.8 pg/ml, n = 26; P < 0.05) (Figure 4A). Although the average IL-4 levels in Plgf+/− mice were not statistically different from those of Plgf+/+ mice (P = 0.1), the median IL-4 levels in Plgf+/+ mice (18.7 pg/ml; n = 22) were nearly half of those seen in Plgf+/+ mice (33.2 pg/ml; n = 26) after HDM exposure (Figure 4B). No significant change was seen in IL-5 levels (Figure 4C) and, as noted earlier, there was a trend toward reduced eotaxin levels in HDM-exposed Plgf+/+ mice, albeit not statistically significantly (Supplemental Figure 4E). Additionally, no differences were observed in IFN-γ, IL-1β, MCP-1, IL-10, and IL-17A levels between Plgf+/+ and Plgf+/− mice (Supplemental Figure 6). Further characterization of lung immune cell populations revealed, that, although allergen exposure substantially increased lung Th2 lymphocytes and DCs, as was expected, no noticeable difference in these cell populations was seen between Plgf+/− and Plgf+/+ mice (Supplemental Figure 7). Taken together, these data suggest that PIGF increases Th2 immune response via IL-13 signaling—but this effect is modest at best—and that PIGF may be utilizing another pathway to exacerbate allergen-mediated pulmonary inflammation and AHR.

**Allergen exposure induces PIGF in a STAT6-dependent manner.** Interestingly, we noted that plasma PIGF levels were upregulated by several-fold in HDM-exposed Plgf+/+ mice compared with saline-exposed Plgf+/+ mice (Figure 5A). It has been reported that PIGF expression is restricted to lung macrophages and epithelial cells during murine (32) and human (33) lung development but is negligible postnatally at steady state (34, 35). Since PIGF modestly increased the Th2 cytokine responses, we tested the involvement of IL-13 and IL-4 signaling in allergen-mediated PIGF upregulation. STAT6 is a known downstream transcriptional effector of canonical IL-13 and IL-4 signaling. An silico analysis of the PIGF gene promoter showed presence of several STAT6 consensus sequences (Table 1). We therefore hypothesized that Th2 cytokine signaling reactivates PIGF expression postnatally in lungs. We determined Plgf mRNA expression in the lungs of HDM- or saline-exposed Stat6+/+ mice. Like the Plgf+/+ mice, the Stat6+/− and WT
types examined, as expected (Figure 5C and Supplemental Figure 8A). Hence, we show that PlGF can be expressed by respiratory epithelial cells, alveolar macrophages, and eosinophils following allergen challenge via STAT6 activation, suggesting a positive feedback loop between the Th2 response and PlGF.

PlGF-deficient mice have reduced activation of the leukotriene pathway. We have recently shown that PlGF induces expression of HIF-1α and Egr-1 transcription factors to mediate an increase in FLAP and Alox5 mRNA (key players in the leukotriene [LT] pathway), respectively, from human lung endothelial cells and monocytes in vitro (36, 37). Therefore, we investigated whether mice were also on a BALB/c background. Plgf mRNA expression was not upregulated in Stat6–/– mice exposed to HDM, in contrast to WT BALB/c mice exposed to HDM (Figure 5B). IHC showed minimal PlGF protein expression in the lungs of Stat6-deficient mice (Figure 5C). In contrast, IHC revealed that lung alveolar epithelial cells produced PlGF in WT mice after allergen exposure (Figure 5C). PlGF was minimally expressed by a few inflammatory cells/alveolar macrophages in Stat6–/– mice, in stark contrast to the high PlGF expression in epithelial cells, inflammatory cells, and alveolar macrophages observed in WT mice (Figure 5C). We sorted alveolar macrophages and eosinophils from lungs of allergen-challenged Plgf+/+ and Plgf–/– mice using flow cytometry (Supplemental Figure 5B) and performed quantitative PCR (qPCR) on RNA from these cell populations. BM (which contains erythroid cells, known to produce PlGF) was used as a control. Interestingly, while increased PlGF expression was seen in isolated alveolar macrophages, lung eosinophils also expressed high levels of PlGF in HDM-exposed Plgf+/+ mice (Supplemental Figure 8A). Plgf–/– mice had no detectable staining and did not show PlGF expression by qPCR on mRNA from any of the cell types examined, as expected (Figure 5C and Supplemental Figure 8A). Hence, we show that PlGF can be expressed by respiratory epithelial cells, alveolar macrophages, and eosinophils following allergen challenge via STAT6 activation, suggesting a positive feedback loop between the Th2 response and PlGF.

Figure 5. STAT6 mediates IL-13–induced increases in PlGF production. (A) HDM sensitization further increases PlGF production in Plgf–/– mice compared with control (saline-treated) mice; n = 3 animals in the saline-treated group and 5–9 animals in the HDM-treated group. **P < 0.01 by Mann-Whitney test. (B) Relative Plgf mRNA expression in the lungs of Stat6+/+ and Stat6–/– mice after HDM or saline exposure. ***P < 0.001 by Mann-Whitney U test; n = 6–8 mice per group. (C) Expression of PlGF protein in airway, alveolar epithelial cells, and alveolar macrophages in WT mice (top left panel), as compared with Plgf–/– mice (top right panel); ×20 images. A magnified view (×40) showing increased PlGF expression in alveolar epithelial cells and macrophages in WT mice following Th2-driven STAT6 activation (bottom left panel), compared with PlGF expression in Stat6–/– (bottom middle panel) and Plgf–/– (bottom right panel) alveolar epithelial cells and macrophages. All data are presented as mean ± SEM. Results are representative of 2 independent experiments.

Figure 4. PlGF mildly affects IL-13 BALF levels. (A–C) IL-13, IL-4, and IL-5 levels in the BALF of Plgf+/+ and Plgf–/– mice 48 hours following the last allergen challenge. *P < 0.05 using Mann Whitney U test between Plgf+/+ and Plgf–/– groups of mice. Each symbol represents 1 mouse; mean ± SEM of IL-13, IL-5, and IL-4 levels are indicated by the horizontal line and error bars. Results are from 3 independent experiments.
PIGF also exacerbated AHR via activation of the LT pathway in vivo in Plgf+/− and Plgf−/− mice.

Allergen-exposed PIGF-deficient mice have nearly half the Alox5 mRNA expression compared with their WT counterparts. Control (saline-exposed) animals showed no difference in Alox5 mRNA levels (Figure 6A). Surprisingly, no difference in Flap mRNA levels were observed in the lung tissue between Plgf+/− and Plgf−/− mice (Supplemental Figure 8B), unlike our in vitro findings in human endothelial cells (37). The transcriptional increase in Alox5 was also confirmed at the protein level, as IHC revealed markedly decreased staining for 5-LO in lung endothelial cells was also confirmed at the protein level, as IHC revealed markedly decreased staining for 5-LO in lung endothelial cells (37). The prevalence of AHR is also high in patients with SCD (41). RNA was extracted from nasal airway epithelial cells of patients that either had (i) no history of asthma and were confirmed to be nonatopic by skin tests to common allergens (normal controls) or (ii) asthma patients with a clinical history of asthma and a positive skin test to one of the common allergens. The details of the eligibility criteria and methodology of gene expression analysis have been previously described in detail (41). Expression of PLGF was significantly higher in patients with asthma compared with normal controls in nasal airway epithelial cells (Figure 7A). In addition, expression of the downstream target transcription factors that are upregulated by PIGF, Egr-1, and HIF-1α (36, 37) were increased in patients with asthma, as was the expression of Alox5, FLAP, and CYSLTR1 (Figure 7B). Although the expression of Alox5, FLAP, and CYSLTR1 could result from allergen-induced asthma and not be specific to PIGF, these data confirm our prior studies on PIGF and the murine studies performed herein on PIGF-deficient animals. Moreover, we have previously reported that PIGF upregulates endothelin-1 expression in the lung and increases EGR1 transcription (26, 27, 42); this data set also showed significantly increased endothelin-1 and EGR1 expression in respiratory epithelial cells, a further validation that the PIGF signaling pathway is activated in asthma. In experimental models of asthma, the LT pathway and Th2 cytokines are known to potentiate each other (43–46). Our data suggests that PIGF may be the novel link that connects these 2 pathways.

PIGF is critical for allergen-induced AHR in sickle mice. Of all hematopoietic cells, PIGF is elaborated from erythroid cells (26, 47). Due to the expanded erythropoiesis, hypoxia, and erythropoietin in SCD, blood PIGF levels are increased (26, 48). Similar to patients, mice with SCD have naturally elevated levels of circulating PIGF that secreted from the hyperplastic erythroblasts (27). The prevalence of AHR is also high in patients with SCD (5–9). We therefore sought to determine whether (i) PIGF is the molecule that results in AHR in SCD and (ii) AHR was solely due to patients, mice with SCD have naturally elevated levels of circulating PIGF that secreted from the hyperplastic erythroblasts (27). The prevalence of AHR is also high in patients with SCD (5–9). We therefore sought to determine whether (i) PIGF is the molecule that results in AHR in SCD and (ii) AHR was solely driven by sickle erythropoiesis.

To this end, we utilized the well-established Berkeley mouse model of SCD. Heritable components are known determinants of the susceptibility to allergen-induced AHR in different mouse strains, PlGF mediates increased blood and lung eosinophilia via increased eosinophil production and recruitment from BM, and this effect is mediated via both increased CysLT production and upregulation of CysLTR1. Collectively, we show that PIGF contributes to the upregulation of the production of both the ligand (CysLT) and receptor (CysLTR1) expression in the LT pathway to accentuate the allergen-induced AHR in mice.

**Table 1. In silico analysis of the mouse PlGF locus showing putative STAT6 binding sites**

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STAT6 binding sites are highlighted. Parentheses indicate the DNA strand; TSS, transcription start site.
strains (30, 49, 50), and the HbS mice are relatively outbred; they are originally derived from a mixture of 5 parental strains (28, 51), and in this mixed background, unidentified heritable loci prevent AHR development (Supplemental Figure 12, A and B). Therefore, we utilized a BM transplant approach to generate mice that were completely chimeric for sickle hematopoiesis on a homogeneous-mouse genetic background. The predominant strain in HbS mice is C57BL/6, and therefore HbS BM was transplanted into C57BL/6 mice (Figure 8A). This approach allowed us to test whether sickle erythroid cells/rbc (erythropoietic cells) resulted in AHR and also allowed studies on numerous age-matched chimeric HbS mice concurrently, which were not otherwise possible with the fragile and difficult-to-breed HbS mice. Sickle BM cells from HbS mice (that were screened to express the CD45.2 + isoform on their leukocytes) were transplanted into lethally irradiated WT C57BL/6 ByJ (BL/6; CD45.1+) mice (Figure 8A). BM from WT C57BL/6 (BL/6; CD45.2) mice transplanted into congenic C57BL/6 ByJ mice (CD45.1 on the same BL/6 background) served as controls. We confirmed that Hbs/WT mice were fully chimeric for donor-derived sickle or normal hematopoiesis that completely replaced the normal recipient hematopoiesis in the blood and the lungs of recipient mice. Sixteen weeks after transplantation, full reconstitution of the recipient hematopoietic system by the donor hematopoietic cells was confirmed by flow cytometry for the exclusive presence of donor leukocytes (CD45.2) in the lung and human sickle hemoglobin in the blood (Supplemental Figure 11, A and B).

Chimeric HbS-BL/6 mice (Chi-HbS mice) developed hematological parameters characteristic of SCD: anemia, reticulocytosis, and leukocytosis (Supplemental Figure 11C). Like the native HbS mice (27), Chi-HbS mice developed elevated PlGF levels compared with chimeric BL/6 mice (Chi-BL/6; Figure 8B), confirming that sickle erythroid cells resulted in elevated PlGF in SCD. We next determined whether the transplanted mice, especially the Chi-HbS mice, were able to mount a robust Th2 response, and indeed, they exhibited classic features of allergen-induced Th2 response, including the induction of Th2 cytokines and the expansion of Th2 lymphocytes in the lung (Figure 8, C–F, and Supplemental Figure 13, A–F).

Next, we exposed Chi-HbS mice to HDM and determined HDM-specific B cell responses and airway reactivity: Both Chi-BL/6 and Chi-HbS mice were able to mount allergen-specific B cell responses. HDM-specific IgE trended higher in HDM-exposed Chi-HbS mice compared with Chi-BL/6 controls, albeit not statistically significantly ($P = 0.1$; Supplemental Figure 14C).

After HDM airway exposure, Chi-HbS mice developed more severe AHR than their corresponding Chi-BL/6 controls exposed to HDM (Figure 9A). Notably, increased AHR was present in Chi-HbS mice, even at baseline, without exposure to methacholine (Figure 9A). However, this was not due to a higher Th2 response in these Chi-HbS mice: Chi-BL/6 and Chi-HbS mice had very similar Th2 cytokine levels in saline- and allergen-exposed animals. IL-13 levels trended lower in Chi-HbS mice ($P = 0.06$) but were not significantly different. Notably, the number of lung DCs and Th2...
lymphocytes were also similar between Chi-BL/6 and Chi-HbS mice (Supplemental Figure 13, A–F, and Supplemental Figure 14, A and B), excluding the possibility that Chi-HbS mice have an intrinsically skewed Th2 immune response favoring allergic responses.

Nonetheless, the exaggerated AHR in Chi-HbS mice compared with Chi-BL/6 mice was mediated by PlGF, since treatment of HDM-exposed Chi-HbS mice with aPlGF blocked the airway reactivity to levels seen in vehicle-treated Chi-HbS mice and vehicle-treated Chi-BL/6 mice (Figure 9B). These data highlight the critical role of PlGF in AHR in SCD and suggest a mechanism of action other than the classic allergic Th2 pathway.

**PlGF mediates AHR in HbS sickle mice through a LT-dependent pathway.** Chi-HbS mice had higher PlGF levels in circulation, as expected, due to a markedly expanded sickle erythropoiesis, which increases 5-LO and CysLT1 expression. We found that zileuton treatment reduced AHR in HDM-exposed Chi-HbS mice to levels seen in vehicle-treated Chi-HbS mice and vehicle-treated Chi-BL/6 mice (Figure 9B). This was accompanied with a marked reduction in urinary CysLTs (Figure 9C), more so with zileuton than with aPlGF. It was noteworthy that, similar to BALB/c Plgf−/− mice, Chi-HbS mice showed increased LT production when compared with Chi-BL/6 mice exposed to allergen (Figure 9C). In both animal models, blocking PlGF function by an Ab or blocking LT production by zileuton resulted in reduced AHR (Figure 9B).

Since zileuton had a greater effect than PlGF Ab, in addition to 5-LO, we also evaluated expression of LTC4 synthase (Ltc4s), a key CysLT synthetic enzyme downstream of 5-LO. There was enhanced expression of Ltc4s in non–allergen-exposed Chi-HbS mice compared with Chi-BL/6 controls, which explains the AHR in Chi-HbS mice at baseline. The expression of Ltc4s increased further after allergen challenge in Chi-HbS mice (Supplemental Figure 15A), but no such effect was seen in Chi-BL/6 mice, suggesting that this was a sickle-specific effect. Remarkably, blockade of PlGF with a PlGF-neutralizing mAb significantly reduced Ltc4s expression in HDM-challenged Chi-HbS to the level of AHR seen in allergen/saline-treated Chi-BL/6 mice, suggesting LTC4S is also regulated by PlGF (Supplemental Figure 15A). We also studied Ltc4s gene expression in PlGF-deficient and WT mice. Mean Ltc4s expression trended lower in saline-treated or HDM-challenged Plgf−/− mice compared with WT mice (Supplemental Figure 15B). Our data suggests that there may be PlGF-dependent regulation of LTC4S (Figure 10) and the greater effect of zileuton in lowering CysLT levels in Chi-HbS mice is a PlGF-independent effect. Collectively, our data demonstrate that PlGF enhances allergen-induced AHR in sickle mice through 5-LO- and CysLTR1-mediated LT production.

**Discussion**

Using genetic and pharmacological approaches, we show a distinct and significant exacerbation of AHR by PlGF. This effect occurs primarily via its upregulation of 5-LO and LT production and, to a lesser extent, upregulation of IL-13-mediated Th2 response, which further upregulates PlGF production from lung epithelial cells, eosinophils, and alveolar macrophages. Indeed, respiratory epithelial cells from patients with asthma showed significantly increased Plgf mRNA and an increase expression of PlGF target genes. Since PlGF is also an erythroid cell–derived factor that is increased in SCD, we transplanted sickle hematopoiesis into WT mice and showed that the high AHR is mediated through sickle erythroid cell–induced high PlGF and is therefore transplantable and completely abrogated by either inhibiting PlGF or the LT pathway.

Our results contrast to a previous report, which suggested that PlGF has no pathogenic effect on AHR in ovalbumin allergen-induced experimental AHR (52). This apparent discrepancy may be due to differences in allergens and mouse strains. The activity of specific pathways required for AHR severity has been shown to be mouse strain dependent (30, 49). Given that HDM inhalation is more relevant to human pathology, our finding of the pathogenic role of PlGF in the context of HDM-induced AHR is clinically relevant to human disease. Our data support and extend the prior work by Pritchard et al., who showed that chimeric sickle mice have baseline lung pathology characterized by increased R (53). In agreement with their study, we showed that sickle mice have an increased R at baseline, in the absence of the bronchoconstrictor challenge with methacholine. Our study further demonstrates the mechanism of increased AHR in SCD is via CysLT induction, as abrogation of the augmented AHR occurred by aPlGF or zileu-
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Figure 8. PlGF and cytokine levels in chimeric SCD mice. (A) Experimental schema showing generation of chimeric HbS (Chi-HbS) and BL/6 (Chi-BL/6) mice by BM transplantation. (B) Plasma PlGF levels of HDM- and saline-exposed Chi-HbS and Chi-BL/6 mice. *P < 0.05 by Mann Whitney U test; n = 17–19 animals per group. (C-F) Eotaxin, IL-13, IL-4, and IL-5 levels in the BALF of saline- and HDM-exposed chimeric mice. All data are presented as mean ± SEM; each symbol represents 1 mouse. Results are from 3 independent experiments (B) or representative of 1 experiment (C and D).

The same phenotype in PlGF-deficient mice suggests that PlGF is an upstream activator of 5-LO. Besides augmenting the LT pathway, we observed that levels of the Th2 cytokine IL-13 were also significantly more exaggerated in HDM-exposed Plgf+/+ mice compared with Plgf–/– mice. IL-13 has been shown to upregulate CysLT receptor expression (43, 44); conversely, there are also reports of CysLT-mediated increased Th2 response and eosinophilic infiltration in the lung (23, 57). Indeed, CysLTR1 expression was significantly lower in Plgf–/– mice. CysLT are also known potent contractile agonists of the airway smooth muscle cells (58) and vascular mural cells (59). Together, these interconnected LT and Th2 cytokine pathways are accentuated to increase AHR and inflammation in Plgf+/+ mice. IL-13 levels were not significantly different in HbS mice, suggesting that either IL-13 does not play a role in AHR in SCD or that this is a mouse strain–specific effect.

Importantly, both models show the primary involvement of the LT pathway for AHR based on the results in Plgf–/– mice, or in Plgf+/+ and HbS mice given αPlGF. Other studies have reported the development of CysLT-induced AHR in the presence of normal IL-13 induction (54, 55).
Our data show that the LT signaling axis is interrupted in the absence of PlGF and is exaggerated in SCD, where PlGF concentrations are increased. Notably, zileuton had the same effect as αPlGF. Moreover, the PlGF-null mouse AHR phenotype was rescued to levels similar to *Plgf<sup>+/−</sup>* mice by i.t. instillation of CysLT. Collectively, our data shows that the LT pathway was the primary downstream effector pathway of PlGF, with secondary activation of an IL-13–mediated T cell (Th2) and B cell (IgE) response. Taken together, this study provides novel mechanistic evidence of activation of both LT and Th2 pathways by PlGF.

In BALB/c mice, PlGF effect on Th2 cytokines was limited to IL-13, but not IL-4, IL-5, or eotaxin, the latter being the same despite high eosinophils. While there may have been a kinetic delay between the peak of eotaxin production in the lung (10 hours) following allergen exposure (60) and our sampling time (48 hours), IL-5 and IL-4–independent allergen-induced lung eosinophilia and airway hyperreactivity have been described in BALB/c mice (61) and asthma patients (62). More importantly, loss- and gain-of-function studies show that PlGF mediates AHR and blood and lung eosinophilia primarily via CysLT that are known to increase eosinophil production from BM and induce blood and airway eosinophilia in mice and in humans (38–40), and this effect is abolished by a blockade of CysLTR1 (40). Moreover, the IL-13–restricted effect of PlGF may suggest participation of PlGF in previously described IL-13–mediated IL-4/eotaxin–independent AHR (63), which is directed at augmenting the airway smooth muscle responsiveness (63, 64).

Another finding was that PlGF expression was further upregulated in the lung epithelial cells, eosinophils, and macrophages via a Th2-driven STAT6 induction, creating a positive feedback loop (shown schematically in Figure 10). The human PlGF promoter showed several putative STAT6 binding consensus sequences (Table 1). However, further studies are needed to determine if PlGF is a direct downstream target of STAT6. Regardless, induction of PlGF via a Th2 pathway and vice versa has not been previously reported and has much broader implications than SCD, since the Th2 response is classically upregulated in asthma. Indeed, interrogation of PlGF expression in respiratory epithelial cells in a well-characterized asthma and control cohort we have previously published (41) showed significantly increased *PLGF* mRNA expression in patients with asthma compared with controls. High PlGF levels in asthma patients were associated with the upregulation of expression of PlGF signaling partners and target genes we have previously identified or those identified herein: *EGRI*, *HIFA*, *endothelin-1* (*ETI*), and *Alox5* (36, 37, 42). PlGF is expressed largely in the placenta. We have previously shown that, in SCD, excessive expansion of the erythroid precursors, erythropoietin, and hypoxia drive the rise in erythroid cell–secreted PlGF (26, 48). While PlGF expression has been shown to be upregulated in lung cancer and a variety of other cancers (65, 66), its expression in the normal lung has only been documented during mouse and human lung development (33–35). Herein, we show that PlGF expression can be upregulated in the postnatal/adult lung alveolar macrophages/ eosinophils and epithelial cells, as well as via allergen-mediated Th2 cytokines/STAT6 in experimentally induced asthma.

Transgenic mice that overexpress PlGF develop emphysema (35), an effect abrogated in elastase-treated *Plgf<sup>−/−</sup>* mice (32). PlGF is elevated in patients with chronic obstructive pulmonary disease (COPD), where emphysema is a key component, and its levels were shown to be associated with higher pulmonary morbidity (67). Furthermore, high PlGF levels have been reported in the sputum of COPD patients with lower FEV1 (52, 67). However, its expression in normal adult (postnatal) pulmonary eosinophils, alveolar macrophages, and epithelial cells; its role in exacerbating AHR and asthma; and its mechanism of action were hitherto unexplored. To our knowledge, this is the first report showing a mechanism of increased AHR by PlGF in SCD that also explains its association with acute asthma, and conceivably in obstructive airway physiology in COPD.
We have previously shown that PlGF contributes to the chronic inflammatory state in SCD via activation of monocytes and increased inflammatory cells (48, 68, 69). Indeed, SCD is characterized by increased blood leukocytes at baseline (70, 71), a major source of LTs in vivo. Targeting the LT pathway using αPlGF/zileuton may decrease both PlGF-dependent and PlGF-independent AHR and pulmonary morbidity in patients with SCD. Furthermore, LT plays a role in inflammation (58), a major player in SCD pathophysiology. Hence, LT-targeted therapy such as zileuton may provide benefits beyond the AHR lung disease spectrum in SCD.

Increased hemolysis triggers increased erythropoiesis and may be associated with increased PlGF production, priming the lung to AHR. Importantly, acute chest syndrome (ACS), a life-threatening complication of SCD, has been shown to be associated with asthma in children with SCD (72). However, ACS also occurs in a vast majority of SCD patients who do not have clinically diagnosed asthma. It is conceivable that individuals with high PlGF levels predisposed to ACS by increasing AHR in the absence of asthma, and here, pharmacological blockade of either PlGF or LT may help prevent this life-threatening complication of SCD. Pulmonary complications are important risk factors for the high morbidity in SCD (3, 4, 73); therefore, targeting PlGF or LT can improve the overall SCD outcome. We have previously reported elevated PlGF expression in β-thalassemia (26), another chronic hemolytic anemia disorder. It is possible PlGF may play a similar detrimental role in β-thalassemia that remains to be explored.

Interestingly, in SCD, the transition from childhood to adulthood is associated with a decline in the incidence of AHR (5, 6, 8, 9) and obstructive lung disease (74, 75) and an increase in restrictive lung disease. While asthma and AHR decline with age due to the increased size of the airways in adults, other factors — including transfusion hemodiasis, vascular pruning, and pulmonary artery hypertension that develop in adults with SCD — may cause a profibrotic phenotype. More broadly, our studies show a distinct role of PlGF in asthma. Therefore, targeting PlGF may prove beneficial for asthma by blunting the Th2 and LT pathways and for other airway diseases featuring an AHR component, such as COPD and asthma.

In conclusion, we report an erythropoiesis-induced, PlGF-dependent IgE-IL-13-LT axis that contributes to increased AHR in SCD, which in turn further upregulates PlGF production from lungs via Th2 cytokine upregulation. PlGF-exacerbated AHR can be pharmacologically targeted by the licensed 5-LO inhibitor, zileuton, or αPlGF, which is currently in clinical trials for cancer. Thus, this study provides an opportunity for targeting PlGF as an early clinical intervention to effectively reduce the pulmonary morbidity associated with SCD and asthma.

Methods

Supplemental Methods are available online with this article.

Figures

Figure 10. Schematic showing the mechanism of PlGF-induced AHR and the positive feedback loop between IL-13 and PlGF. PlGF is secreted by erythroid progenitors from the BM in response to hypoxia and erythropoietin (Epo) in SCD. PlGF stimulates the expression of 5-LO, which generates CysLT, and increases CysLTR1 expression. Sickle hematopoietic cells additionally increase LTC4S expression, which further contributes to CysLT generation. LT and IL-13 are key effectors of AHR. PlGF blockade or 5-LO inhibition reduces AHR. IL-13 levels activate its downstream effector molecule, STAT6, which in turn further augments PlGF production from pulmonary airway epithelial cells, eosinophils, and alveolar macrophages. The latter explains the elevated PlGF levels in allergen-challenged WT mice and in patients with asthma.
120 breaths per minute with a constant tidal volume (0.2 ml), while mice were kept paralyzed with decamethonium bromide (25 mg/kg). After a stable baseline was achieved, acetylcholine (50 mg/kg) was injected into the inferior vena cava and dynamic airway pressure (cm H2O × s) was monitored for 5 minutes. In addition, AHR was also measured in parallel experiments using an independent method: the forced oscillation technique, as described previously (76). Mice were anesthetized with a triple sedative solution (ketamine/zylaxine/acepromazine; 4:1:1) and connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ) by intubation. R was assessed following delivery of nebulized saline and increasing doses of the broncho-constrictor agent methacholine (50 mg/ml and 100 mg/ml; acetyl-β-methylcholine chloride, Sigma-Aldrich). For each concentration of methacholine, the maximal R (Rs) values were averaged. Following studies on lung mechanics, blood, bronchoalveolar lavage fluid, and organs were collected for ELISA assays and pathology.

PlGF Ab and zileuton regimens. PlGF neutralizing Ab (PLSD11D4) was a gift from Bart Jonckx (ThromboGenics NV). The mouse monoclonal anti-PlGF Ab was developed against all mouse PlGF proteins (65). A dose of 50 mg/kg of PlGF-neutralizing Ab or saline was administrated i.p. on days 14, 16, 17, 19, and 20 (relative to the day of the initial administration of HDM) to mice. In one experiment, Plgf+/+ animals challenged with HDM were given either mouse IgG1 (the isotype of αPlGF) or saline i.p. to ensure there was no difference in pulmonary phenotype with either. Doses of 70 mg/kg or 150 mg/kg of zileuton (Zyflo CR) were delivered by gavage into mice 5 hours prior to the first i.t. administration of HDM and an hour after the first HDM challenge. Subsequently, mice received 70 mg/kg or 150 mg/kg (200 μl) of zileuton twice daily until the termination of the experiment on day 21. Zileuton was dissolved in a diluent solution: 10% DMSO in water.

Asthma cohort analysis. Nasal respiratory epithelial cells from asthmatic and nonasthmatic subjects, younger than 18 years of age, were obtained. The selection criteria were described in detail before (41). Briefly, subjects included in the control group had (i) no acute infection or major illnesses and (ii) a negative result to the skin-prick test to a panel of environmental allergens. Subjects in the asthma group included both patients with (i) a physician diagnosis of asthma with no current wheezing episodes and (ii) a positive skin-prick test to environmental allergens.

Nasal epithelial brushings were found to contain 97% respiratory epithelial cells (41). RNA from 4 individuals in the nonasthmatic group and 8 individuals in the asthma cohort was obtained from these cells and subjected to microarray analysis using the HG-U133A GeneChip (Affymetrix) (41). The expression profile was interrogated for PlGF and its target genes using GeneSpring software. Expression profiles were derived from a microarray database utilized in a previous publication (77).

In silico analysis of STAT6 binding sites. A search of putative STAT6 binding sites in the murine Plgf locus was performed using MatInspector software (Genomatix); a stringent cut-off allowing a single mismatch was applied. The 2011 mouse genome version GRCh38/mm10 Assembly (www.genome.ucsc.edu; University of California Santa Cruz, Santa Cruz, California, USA) was used for the analysis.

Statistics. The software GraphPad Prism was used for all statistical analyses. Statistical tests used include 1-way Dunnet’s ANOVA, Sidak pairwise ANOVA, unpaired t test, and Mann-Whitney U test wherever applicable. Data are presented as mean ± SEM and P < 0.05 was considered significant.

Study approval. Animal protocols were approved by the Institutional Animal Care and Use Committee at CCHMC (IACUC Protocol numbers: ZE07051; ZD07050). For the human studies, patients were recruited after appropriate Institutional Review Board approval at CCHMC (IRB; protocol number 2008-1384). Written informed consent was obtained from all study participants or their guardians.

Author contributions

MSEMM, EBB, PIA, ST, AL, and DP conducted the experiments; MSEMM, PM, EBB, and GKKH designed research studies; MSEMM coordinated all research experiments; MSEMM and EBB plotted and analyzed the data; SKS performed the histopathological staining and interpretation; TR performed the IHC; ML performed the microarray and bioinformatics analysis; BJ provided α-PlGF Ab; PC generated and provided the placenta growth factor–deficient mice; VKK, TDLC, NR, MWK, GKKH, and PM provided intellectual input into the conduct and analysis of the experiments; MSEMM, EBB, GKKH, and PM analyzed and interpreted the data; PM conceived of the project; MSEMM and PM wrote the manuscript; and all authors read and edited the manuscript.

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