Placental Growth Factor Contributes to Bronchial Neutrophilic Inflammation and Edema in Allergic Asthma

Sonja Bobic[1], Sven Seys[1], Vanessa De Vooght[2], Ina Callebaut[1], Valerie Hox[1], Christophe Dooms[3], Stefan Vinckier[4,5], Bart Jonckx[6], Jean-Marie Saint-Remy[7], Jean-Marie Stassen[6], Dominique M. A. Bullens[1,8], Jan L. Ceuppens[1], Peter Carmeliet[4,5], and Peter W. Hellings[1,9]

[1]Clinical Immunology, Department of Microbiology and Immunology, [2]Research Unit for Lung Toxicology, [3]Division of Pulmonology, [4]Laboratory of Angiogenesis and Neurovascular Link, Vesalius Research Center, VIB, and [5]Laboratory of Angiogenesis and Neurovascular Link, Vesalius Research Center, Catholic University of Leuven, Leuven, Belgium; [6]ThromboGenics NV, Leuven, Belgium; and [7]Department of Molecular and Cellular Medicine, [8]Clinical Department of Pediatrics, and [9]Clinical Department of Otorhinolaringology, Head and Neck Surgery, Catholic University Hospitals, Leuven, Belgium

Placental growth factor (PlGF) and its receptor vascular endothelial growth factor receptor 1 (VEGFR1) play an important role in pathological conditions related to angiogenesis, vascular leakage, and inflammation. This study investigated their contributions to inflammation and the formation of edema in allergic asthma. The expression of PlGF and VEGFR1 was measured in induced sputum of patients with asthma ($n = 11$) and healthy subjects ($n = 11$), and in bronchial biopsies of house dust mite (HDM)–allergic patients stimulated with HDM allergens. The effects of the endonasal administration of human PlGF-2 and PlGF deficiency on inflammation and edema were evaluated in a murine model of allergic asthma. The migration of human neutrophils in response to hPlGF-2 was tested *in vitro*. The expression of PlGF and VEGFR1 was significantly higher in the sputum of patients with asthma, and in Der p 1–induced PlGF in biopsies from HDM-allergic patients. PlGF was increased in the bronchi of ovalbumin (OVA)–challenged mice compared with control mice (65 ± 17 pg/mg versus 18 ± 1 pg/mg, respectively; $P < 0.01$), and VEGFR1 was expressed in bronchial epithelium, endothelium (control mice), and inflammatory cells (OVA-challenged mice). The endonasal instillation of hPlGF-2 in wild-type, OVA-challenged mice led to an increase in bronchial neutrophils, lung tissue wet/dry ratio, and IL-17. PlGF-deficient mice showed lower numbers of BAL-infiltrating neutrophils, a reduced lung wet/dry ratio, and lower production of IL-17, macrophage inflammatory protein-2, and granulocyte chemotactic protein-2/LPS-induced chemokine compared with wild-type, OVA-challenged mice. hPlGF-2 induced the migration of human neutrophils *in vitro* in a VEGFR1-dependent way. PlGF and its receptor VEGFR1 are up-regulated in allergic asthma and play a proinflammatory role by inducing tissue edema, and increasing tissue neutrophilia and the production of IL-17.

Keywords: placental growth factor; edema; neutrophils; interleukin 17

Airway edema constitutes one of the key features of allergic airway inflammation. Among the mediators that are considered to play a role in the development of bronchial edema, placental growth factor (PlGF) may be a key player. In contrast to studies demonstrating the role of vascular endothelial growth factor–A (VEGF-A) in inflammation and remodeling in asthma (1–4) and chronic obstructive pulmonary disease (COPD) (5, 6), little is known about the role of PlGF in airway inflammation. Apart from the role of PlGF in skin inflammation and edema (7), PlGF was found to be up-regulated in the serum and bronchoalveolar lavage (BAL) of patients with COPD (8), and was correlated with worse lung function (8). Nevertheless, the role of PlGF in bronchial edema and inflammation in allergic asthma remains elusive.

PlGF was originally discovered in the human placenta in 1991 (9). PlGF is a member of the VEGF family, and is related to VEGF-A, with approximately 50% of sequence similarity (9). Alternative splicing of the human PlGF gene generates four isoforms that differ in size and binding properties: PlGF-1 (PlGF131), PlGF-2 (PlGF152), PlGF-3 (PlGF203), and PlGF-4 (PlGF224). PlGF-1 and PlGF-2 are the major isoforms in humans. In mice, PlGF-2 is the only PlGF isoform identified so far (10). The structure of PlGF is dimeric, and monomers are held together by disulfide bonds. Moreover, PlGF is able to form heterodimers with VEGF, especially when these two proteins are coexpressed in the same cell (11). Many cell types express PlGF constitutively or upon stimulation, including endothelial cells (12), smooth muscle cells (12), fibroblasts (13), leukocytes (14), keratinocytes (15), retinal pigment epithelial cells (16), bronchial epithelial cells (8, 17), tumor cells, and others.

PlGF binds vascular endothelial growth factor receptor–1 (VEGFR1, or Flt-1, i.e., Fms-like tyrosine kinase–1), but not VEGFR2, which is the main receptor for VEGF-A signaling (18). VEGFR1 is a receptor tyrosine kinase, also existing in a soluble form that lacks a transmembrane domain and intracellular kinase domain, and is thus a decoy receptor for VEGF-A and PlGF (19, 20). Human cells of placental origin, called trophoblasts, express VEGFR1, which is activated upon PlGF binding (9). In human umbilical vein endothelial cells and uterine arteries, PlGF binding results in the release of nitric oxide and vasodilatation (21). Monocytes also express VEGFR1, and stimulation with PlGF induces the migration of monocytes (22, 23). VEGFR1 is also expressed on the surface of neutrophils (24), eosinophils, (25) and smooth muscle cells (26). The expression of VEGFR1 is directly regulated by hypoxia and hypoxia-induced factors (27).

PlGF-deficient mice are viable and develop normally, but they exhibit impaired angiogenesis, impaired plasma extravasation and collateral growth during ischemia, inflammation, wound healing, and cancer (28). The absence of PlGF also reduces the vascular leakage induced by skin wounding, allergens, and neurogenic

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Correspondence and requests for reprints should be addressed to Peter W. Hellings, M.D., Ph.D., Department of Otorhinolaryngology and Head and Neck Surgery, Catholic University Hospitals, Kapucijnevoer 33, 3000 Leuven, Belgium. E-mail: Peter.Hellings@uzleuven.be

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inflammation (29). In humans, the *PlGF* gene is highly expressed in the placenta, whereas in adulthood, PlGF is mainly expressed in pathological conditions, for example, in keratinocytes during wound healing (15), in renal carcinoma cells (30), and in breast cancer cells (31). PlGF was extensively studied in tumors, based on its role in angiogenesis. Many studies evaluated the effects of anti-PlGF antibodies against tumor growth, because it has proven to be a much more selective target than VEGF, producing fewer side effects and less toxicity (32).

The present study investigated the contributions of PlGF to the inflammation and formation of edema in allergic asthma. Therefore, we compared PlGF expression in the sputum of allergic asthma patients and healthy subjects, and studied the production of PlGF by the bronchial mucosa of house dust mite (HDM)–allergic patients. In addition, a murine model of asthma was used to unravel the contributions of PlGF to bronchial inflammation, tissue edema, and the production of cytokines relevant to bronchial inflammation.

MATERIALS AND METHODS

Sputum Induction

Induced sputum was collected from 11 nonasthmatic volunteers and 11 allergic asthma patients, with a similar gender distribution. Asthma was diagnosed according to previous or current proof of the reversibility of forced expiratory volume in 1 second of greater than or equal to 12% after an inhalation of salbutamol or a positive histamine provocation test. Atopy was defined as a positive skin-prick test for a panel of 18 common inhalant allergens (HAL Allergy, Leiden, The Netherlands). Patients' characteristics are listed in Table 1. This study was approved by the Ethics Committee of the Catholic University Hospitals of Leuven. Sputum was induced as described previously (33) (*see* the online supplement).

Stimulation of Human Bronchial Biopsies

Bronchial tissue biopsies were taken from six HDM-allergic rhinitis patients without lower airways complaints and from nine nonallergic, nonasthmatic patients undergoing vocal cord surgery for unilateral lesions. The use of antiallergic treatment and smoking constituted exclusion criteria. Atopy was diagnosed as already described. Control subjects were not allergic to any of the tested allergens. Only one HDM-allergic patient was monosensitized, whereas the rest manifested different allergies to pollen and animal dander. The study was approved by the Ethics Committee of the Catholic University Hospitals of Leuven. Biopsies were cultured and stimulated as described in the online supplement.

Real-Time RT-PCR

The quantification of human PlGF, VEGFR1, and VEGF-A by real-time RT-PCR was performed as previously described (34) (for details, *see* the online supplement).

Murine Model of Allergic Asthma

An ovalbumin (OVA) murine model of allergic airway inflammation was used in this study (35–37) (for details, *see* the online supplement).

Measurement of Airway Hyperresponsiveness

Airway hyperresponsiveness in mice was measured by the forced oscillation technique (38) (for details, *see* the online supplement).

TABLE 1. PATIENTS' CHARACTERISTICS

	Control Subjects	Patients with Asthma
Number	11	11
Gender (female)	5/11	7/11
Inhaled corticosteroids	0/11	11/11
Atopy	8/11	11/11

Endonasal Administration of PlGF

Male Balb/c mice were sensitized and challenged with OVA, and received hPlGF-2 (ThromboGenics NV, Heverlee, Belgium), saline, or LPS endonasally during the challenge phase (for details, *see* the online supplement).

Experimental Asthma in PlGF-Deficient and OVA–T-Cell Receptor Transgenic Mice

Allergic bronchial inflammation was induced in PlGF-deficient (28) and wild-type (wt) C57BL/6N mice (three male and three female mice per group), as already described.

In addition, allergic inflammation was induced in mice transgenic for the T-cell receptor (TCR) specific to the OVA peptide (C.Cg-Tg (DO11.10)10Dlo/J, Balb/c background; Jackson Laboratory, Bar Harbor, ME). OVA-challenged mice were killed, and their mediastinal lymph nodes were collected (for details, *see* the online supplement).

Determination of Cytokine Production by ELISA

Murine lung tissue was homogenized, and cytokines were measured in the supernatants of homogenized lungs according to ELISA (for details, *see* the online supplement).

Immunohistochemistry for VEGFR1

VEGFR1 was stained on lung-tissue paraffin sections (for details, *see* the online supplement).

Human Neutrophil Migration Assay

Neutrophil migration in response to hPlGF-2 was tested in a Boyden chamber migration assay, as reported previously (39, 40) (for details, *see* the online supplement).

Data Analyses

Statistical analysis was performed with GraphPad Prism software, version 4 (http://www.graphpad.com/prism/Prism.htm). The Mann-Whitney U test was used for between-group comparisons. The Wilcoxon signed-rank test was performed for within-group comparisons in stimulation experiments. The nonparametric Spearman coefficient was used for correlations. The Kruskall-Wallis test with the Dunn *post hoc* test was used for multiple-group comparisons in the migration study. A difference was considered significant when $P < 0.05$. Data are expressed as scatter dot plots with mean values, or means ± standard errors of the mean.

RESULTS

Enhanced Expression of PlGF and VEGFR1 in Sputum Cells of Patients with Asthma

We first analyzed the mRNA concentrations of PlGF, VEGF-A, and VEGFR1 in the induced sputum of asthmatic and control subjects. The expression of human PlGF mRNA was significantly higher in the sputum cells of patients with asthma (8.6 ± 2.8, $n = 11$) compared with healthy subjects (1.1 ± 0.3, $n = 11$; $P < 0.01$; Figure 1A). Moreover, VEGFR1 mRNA expression was higher in patients with asthma (110.5 ± 34.1, $n = 8$) versus healthy subjects (37.1 ± 22.1, $n = 8$; $P < 0.05$; Figure 1B). In addition, VEGF-A mRNA expression was found to be almost four times higher in the sputum cells of asthmatic compared with healthy individuals ($P < 0.05$; data not shown).

Induction of PlGF Expression by Der p 1 Stimulation of Bronchial Biopsies of HDM-Allergic Patients

Bronchial biopsies from healthy subjects and HDM-allergic rhinitis patients without asthma were cultured in medium with or without recombinant (r) Der p 1 or Der p 2 for 24 hours. rDer p 1 stimulation significantly increased concentrations of PlGF in

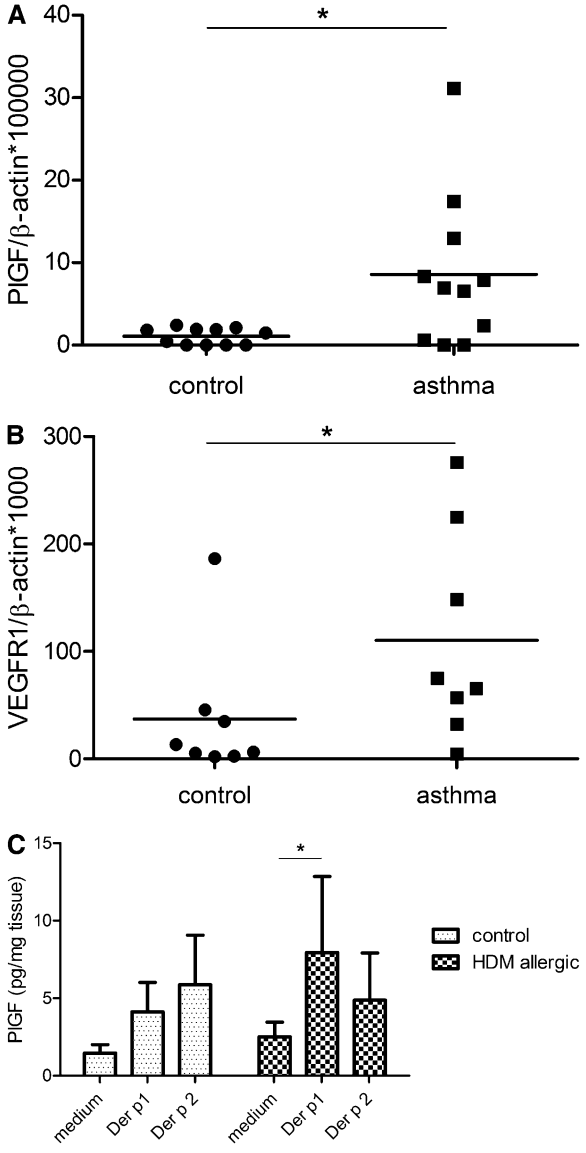

Figure 1. Expression of (*A*) placental growth factor (PlGF) and (*B*) vascular endothelial growth factor receptor–1 (VEGFR1) mRNA in the sputum cells of healthy and asthmatic individuals. PlGF and VEGFR1 demonstrated significantly higher mRNA expression in the sputum cells of patients with asthma compared with healthy subjects. Expression levels of corresponding genes were corrected for the expression of the housekeeping gene β-*actin*. PlGF: control group, $n = 11$; patients with asthma, $n = 11$. VEGFR1: control group, $n = 8$; patients with asthma, $n = 8$. *$P < 0.05$. (C) Effects of Der p 1 and Der p 2 stimulation on the production of PlGF in explant cultures of bronchial biopsies. Der p 1 significantly up-regulated PlGF expression in bronchial biopsies of house dust mite (HDM) –allergic patients. Control bronchial biopsies, $n = 9$; HDM bronchial biopsies, $n = 6$. *$P < 0.05$.

supernatants of bronchial biopsies from HDM-allergic patients ($n = 6$; Figure 1C), but had no effect on bronchial biopsies from healthy subjects ($n = 9$; data not shown). rDer p 2 stimulation did not induce the production of PlGF in biopsies from HDM-allergic patients and control subjects (Figure 1C). Baseline concentrations of PlGF did not differ between allergic and nonallergic subjects in bronchial biopsies after 24 hours of culture in medium (control subjects, 1.5 ± 0.6 pg/mg tissue versus HDM-allergic patients, 2.5 ± 1.0 pg/mg tissue; $P > 0.05$).

Murine Model of Allergic Asthma and Expression of PlGF and VEGFR1

Balb/c mice were sensitized with OVA by seven intraperitoneal injections, and were exposed to aerosolized OVA or saline for 8 subsequent days. Experimental asthma in the OVA model is associated with a marked increase in wet/dry ratio (Figure 2A). The total cell counts in bronchoalveolar lavage (BAL) fluid were increased after eight challenges, with higher eosinophil (278 ± 186 cells/ml versus 59,422 ± 9,211 cells/ml, $P < 0.01$), lymphocyte (6,067 ± 774 cells/ml versus 60,244 ± 7,395 cells/ml, $P < 0.01$), and neutrophil counts (3,689 ± 1,600 cells/ml versus 9,800 ± 1,980 cells/ml, $P < 0.05$) in BAL fluid (Figure 2B). Higher concentrations of Th2 cytokine IL-13 (Figure 2C) and IL-17 (Figure 2D) were found in OVA-challenged mice, compared with control mice.

The concentration of PlGF was determined in the supernatants of lung homogenates taken from OVA-challenged and control mice by ELISA. Expression levels were measured at different time points during the challenge phase of the model (after one, four, or eight challenges) (Figure 2E). PlGF was already found to be significantly up-regulated in murine lungs after four challenges with OVA. Concentrations of PlGF increased significantly further after eight challenges (Figure 2E), to a concentration of 65 ± 7 pg/mg (compared with 18 ± 1 pg/mg in mice challenged with saline; $P < 0.01$; Figure 2E). In addition, lymph node (LN) cells isolated from OVA-challenged mice and cultured with autologous splenocytes as a source of antigen-presenting cells were found to produce PlGF in response to stimulation with OVA (Figure 2F), whereas very low amounts of PlGF were produced by LN cells cultured with medium only. PlGF concentrations in lung tissue were significantly correlated with numbers of BAL infiltrating lymphocytes (Spearman $r = 0.66$, $P = 0.0438$) and neutrophils (Spearman $r = 0.78$, $P = 0.0105$), but not macrophages or eosinophils.

To confirm that PlGF expression does not depend on the particular murine model used, we also validated the expression of PlGF in the lung tissue of OVA-challenged mice that were sensitized using alum as an adjuvant (data not shown). OVA/OVA mice demonstrated a significantly higher expression of PlGF in lung tissue compared with OVA/saline-treated (Sal) mice, similar to the results using a nonadjuvant model (data not shown).

Lung immunohistochemistry was performed after eight challenges. VEGFR1 was constitutively expressed in control mice, and was located in endothelial cell, epithelial cells, and some macrophages (Figures 3B and 3C). Allergen challenge with OVA induced the expression of VEGFR1 in infiltrating inflammatory cells, as shown in Figure 3D. The expression of soluble VEGFR1 was down-regulated in OVA-challenged mice (4,276 ± 104 pg/mg), compared with control mice (4,767 ± 160 pg/mg, $P < 0.05$).

Effects of Endonasal Instillation of PlGF on Bronchial Inflammation and Edema

To investigate further the role of PlGF and its effects on inflammation and edema in a murine model of allergic asthma, 5 μg of hPlGF-2 or saline were instilled endonasally before the second and sixth challenges with OVA. The instillation of hPlGF-2 resulted in a significantly higher amount of neutrophils in the BAL fluid (Figure 4A) and lung tissue (2.2 ± 0.2 cells in the Sal group, versus 3.1 ± 0.3 cells in the hPlGF-2 group; $P < 0.05$), but exerted no effect on the number of lymphocytes or eosinophils (data not shown). The number of macrophages was significantly higher in the BAL of mice that received endonasal hPlGF-2 compared with Sal, but this difference was not evident according to histology (data not shown). In parallel with the

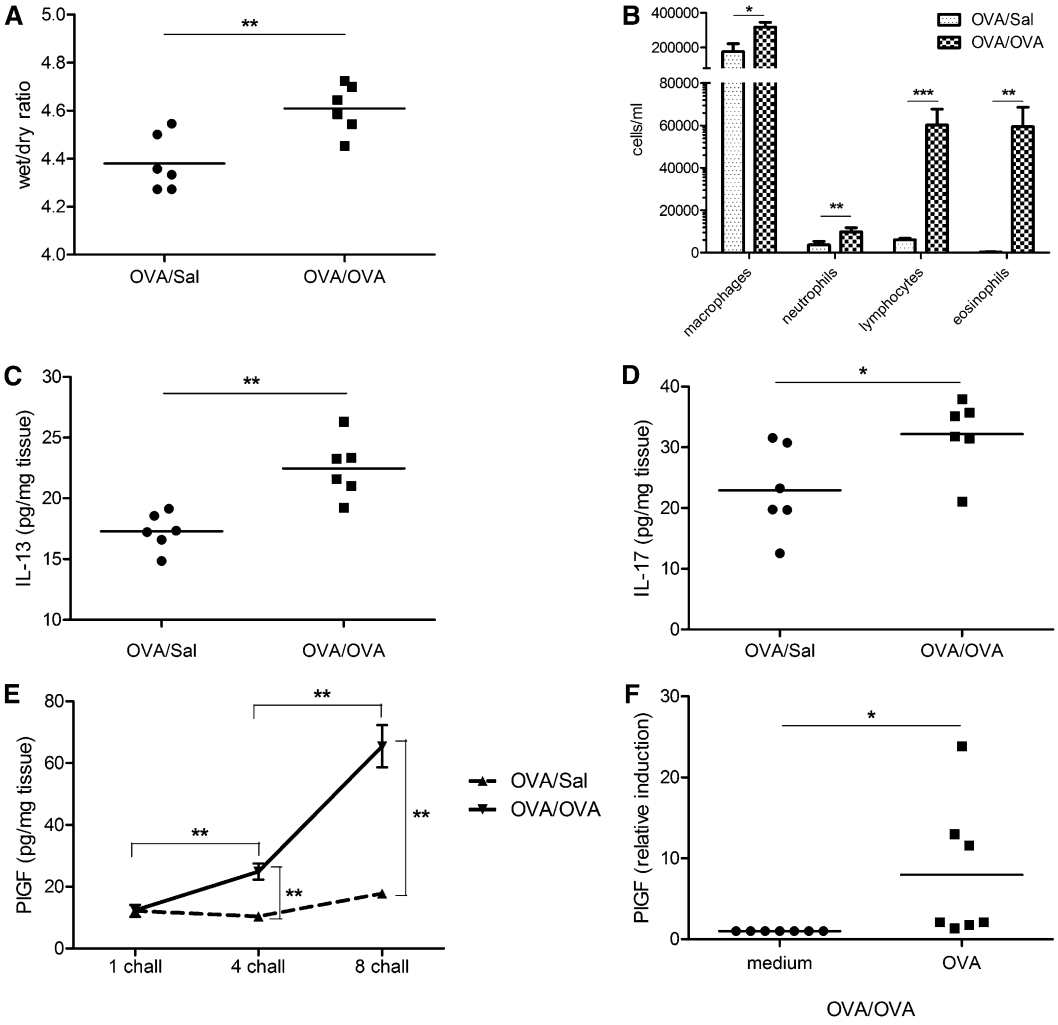

Figure 2. Expression of PlGF, IL-13, and IL-17 in a murine model of allergic airway inflammation. Mice were sensitized by repetitive intraperitoneal injections of ovalbumin (OVA), and challenged on 8 consecutive days by OVA aerosol or saline (Sal). Mice were killed 24 hours after the final challenge. For measuring the expression of PlGF, mice were killed after one, four, and eight challenges. (*A*) Wet/dry ratios of lung tissue. Wet/dry ratios were significantly higher in the OVA/OVA group, compared with the OVA/Sal group ($n = 6$). $^{**}P < 0.01$. (*B*) Differential cell counts in bronchoalveolar lavage (BAL) fluid. BAL cell counts were significantly higher in OVA/OVA mice, compared with OVA/Sal control mice ($n = 6$). $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. (*C*) Protein concentrations of IL-13 in lung-tissue homogenates. Th2 cytokine IL-13 was higher in the supernatants of homogenized lung tissue from OVA/OVA mice compared with OVA/Sal control mice ($n = 6$). $^{**}P < 0.01$. (*D*) Protein concentrations of IL-17 in lung-tissue homogenates. Concentrations of IL-17 were higher in the supernatants of homogenized lung tissue from OVA/OVA mice compared with OVA/Sal control mice ($n = 6$). $^{*}P < 0.05$. (*E*) Protein concentrations of PlGF in lung-tissue homogenates. PlGF was already significantly up-regulated in the lungs after four OVA challenges compared with Sal-challenged mice, and the expression further increased significantly after eight challenges ($n = 6$ mice/group). $^{**}P < 0.01$. (*F*) Induction of PlGF by OVA stimulation of lymph node (LN) cells taken from OVA-challenged mice. OVA stimulation induced significantly higher production of PlGF by LN cells, compared with medium condition. PlGF protein concentrations from the OVA-stimulated condition were divided by the values from the medium condition ($n = 7$). $^{*}P < 0.05$.

neutrophilic influx, concentrations of myeloperoxidase (MPO) were higher in the lungs of PlGF-instilled mice (Figure 4B), and correlated with the number of infiltrating neutrophils (Spearman $r = 0.71$, $P < 0.0001$). The instillation of PlGF resulted in the increased edema of lung tissue, as determined by wet/dry ratios (Figure 4C), but had no effect on bronchial hyperresponsiveness in response to methacholine (data not shown).

Protein concentrations of IL-17 were found to be up-regulated in the lungs of mice that received endonasal hPlGF-2, compared with those that received endonasal saline (Figure 4D), but endonasal hPlGF-2 exerted no effect on IL-13 or VEGF concentrations (data not shown). Chemokines inducing neutrophilic migration in mice, such as macrophage inflammatory protein (MIP)-2 and granulocyte chemotactic protein-2 (GCP-2)/LPS-induced CXC chemokine (LIX), were also tested, but their concentrations were not affected by PlGF instillation (data not shown).

Effects of PlGF Deficiency on Experimental Asthma

The role of PlGF in allergic asthma was further investigated by inducing allergic airway inflammation in PlGF-deficient and control mice. OVA-sensitized and OVA-challenged PlGF-deficient mice presented a significantly lower number of neutrophils in lung tissue (histology) than wt OVA/OVA mice (2.3 ± 0.2 cells in OVA/OVA PlGF knockout [KO] mice, versus 3.7 ± 0.2 cells in OVA/OVA wt mice; $P < 0.001$; Figure 5A), and concentrations of MPO in the lungs were significantly lower in OVA/OVA PlGF KO mice than in wt mice (Figure 5B). Total cell counts, as well as neutrophil, eosinophil, macrophages, and lymphocyte counts in BAL fluid, did not differ between PlGF-deficient and wt mice (data not shown). PlGF KO mice did not show the same significant increase in wet/dry ratios between OVA/Sal and OVA/OVA mice as was observed in wt mice (Figure 5C). Wet/dry ratios of lung tissue in OVA/OVA $PlGF^{-/-}$ mice were lower than in OVA/OVA wt mice, without reaching levels of significance ($P = 0.064$; Figure 5C). No difference in bronchial hyperresponsiveness was evident between OVA-challenged PlGF KO and wt mice (data not shown).

PlGF-deficient mice exhibited lower protein concentrations of IL-17 in the lungs, compared with wt mice (Figure 5D). Furthermore, PlGF deficiency also affected the production of MIP-2 and GCP-2/LIX, which were significantly down-regulated in OVA-challenged, PlGF-deficient mice compared with OVA-challenged wt mice (Figures 5E and 5F). No significant effects of PlGF deficiency on VEGF concentrations in lung tissue were evident (data not shown).

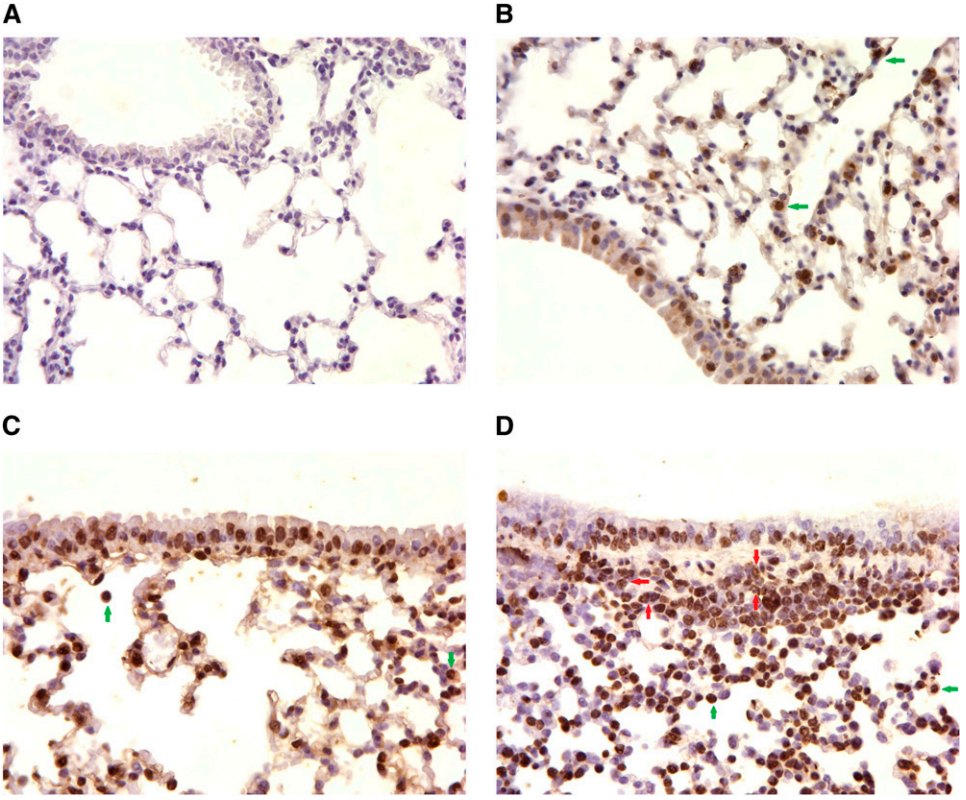

Figure 3. Expression of VEGFR1 in lungs of mice with allergic airway inflammation, according to immunohistochemistry. Images depict isotype control staining (*A*) and lung tissue stained with anti-VEGFR1 antibody from naive mice (*B*), from OVA/Sal mice (*C*), and from OVA/OVA mice (*D*). VEGFR1 was constitutively expressed in endothelial and epithelial cells and some macrophages (*green arrows*) in naive mice, whereas in OVA/OVA mice, the expression of VEGFR1 was also detected in infiltrating inflammatory cells (*red arrows*). Magnification, ×40.

Effects of PlGF on Neutrophil Migration *In Vitro*

We further investigated the direct effects of PlGF on neutrophilic migration in an *in vitro* Boyden chamber migration assay. The migration of granulocytes isolated from the peripheral blood of four HDM-allergic donors was tested in response to different doses of hPlGF-2 (1,000, 200, and 40 ng/ml), and the numbers of migrated neutrophils were counted. One thousand nanograms/milliliters of hPlGF-2 induced the migration of neutrophils, with a migration index of 2.5 ± 0.3 (Kruskal-Wallis test, $P = 0.0267$; Figure 6A). In addition, we tested the migration of neutrophils in response to hPlGF-2 (1,000 ng/ml) in the presence or absence of VEGFR1 blockade. Anti-VEGFR1 antibody completely abolished the migration of neutrophils in response to hPlGF-2, whereas control antibodies had no effect on neutrophilic migration (Kruskal-Wallis test, $P = 0.0071$; Figure 6B).

LN Stimulation

Mediastinal LN cells from OVA–TCR transgenic mice, sensitized and challenged with OVA, were stimulated *in vitro* with OVA, PlGF, and anti-VEGFR1 antibody in the presence of autologous splenocytes. OVA stimulation induced the significant production of IL-17 by LN cells (OVA, 121 ± 41 pg/ml, versus medium, 1.0 ± 0.4 pg/ml; $P < 0.01$). PlGF, alone or in combination with OVA, exerted no effect on the production of

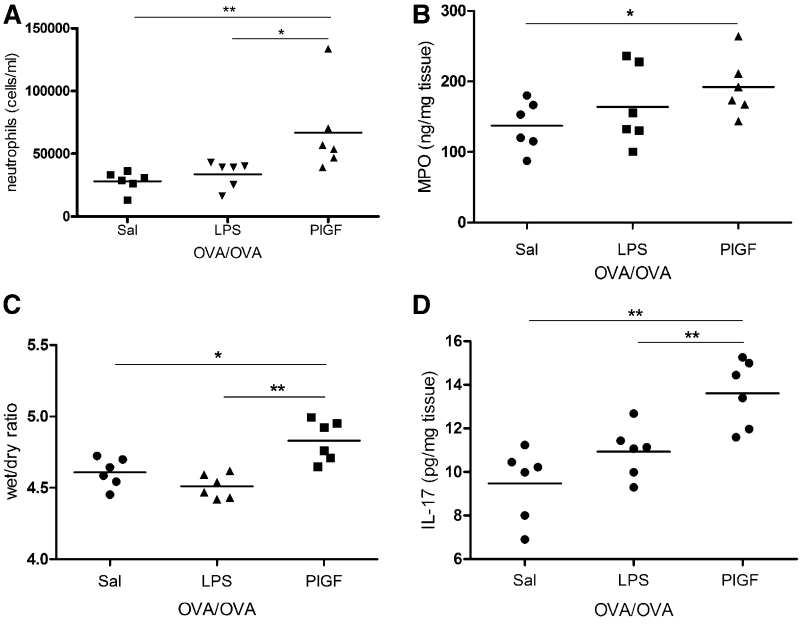

Figure 4. Effects of endonasal human PlGF-2 administration during the OVA challenge phase on (*A*) neutrophil counts in BAL, (*B*) myeloperoxidase (MPO) concentrations in lung tissue, (*C*) lung wet/dry ratios, and (*D*) IL-17 protein in lung tissue. The administration of hPlGF-2 significantly increased wet/dry ratios of lung tissue, neutrophil counts in the BAL, and concentrations of IL-17 in lung tissue. An increase in the number of neutrophils was confirmed by MPO assay ($n = 6$ mice/group). *$P < 0.05$. **$P < 0.01$.

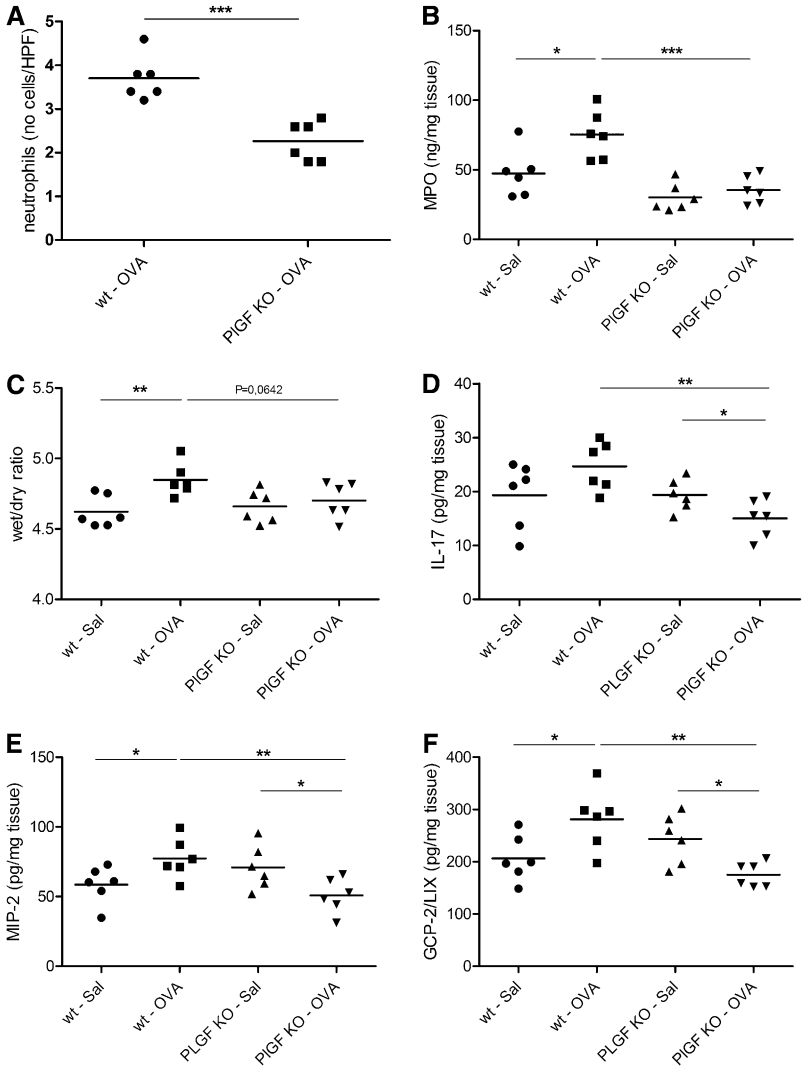

Figure 5. Effects of PlGF deficiency on (*A*) neutrophil counts in lung tissue, (*B*) MPO concentrations in lung tissue, (*C*) lung wet/dry ratios, (*D*) IL-17 protein, and (*E*) neutrophil chemoattractants macrophage inflammatory protein (MIP)-2 and (*F*) granulocyte chemotactic protein (GCP)-2/LPS-induced CXC chemokine (LIX) in lung tissue. PlGF deficiency reduced the wet/dry ratios of lung tissue and the numbers of infiltrating neutrophils in OVA/OVA mice. Concentrations of MPO produced by neutrophils, IL-17, and the chemokines that induce neutrophil migration (MIP-2 and GCP-2/LIX) were significantly lower in OVA/OVA PlGF-deficient mice compared with OVA/OVA wild-type (wt) mice ($n = 6$ mice/group). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

IL-17 by lymph node T cells (PlGF + OVA, 108 ± 39 pg/ml; $P > 0.05$, compared with OVA stimulation). Accordingly, VEGFR1 blockage in the presence of OVA and PlGF did not affect the production of IL-17 (94 ± 25 pg/ml, $P > 0.05$, compared with OVA or OVA + PlGF stimulation). Control antibody exerted the same effect on IL-17 production as anti-VEGFR1 neutralizing antibody (96 ± 22 pg/ml, $P > 0.05$, compared with OVA + PlGF + anti-VEGFR1 antibody).

DISCUSSION

We report for the first time, to the best of our knowledge, on a significant up-regulation of PlGF in the sputum of allergic asthma patients, and the *in vitro* production of PlGF in bronchial mucosa upon allergen stimulation. The role of PlGF in allergic inflammation was further studied in a murine model of asthma, revealing links between PlGF, bronchial IL-17, and neutrophilic inflammation.

Previously, PlGF was shown to be up-regulated in the BAL fluid and serum of patients with COPD (8), without knowledge of the role PlGF plays in asthma and allergic inflammation. We found a significantly higher expression of PlGF mRNA in the sputum of allergic asthma patients, compared with healthy subjects. Cheng and colleagues (8) showed that bronchial epithelial cells are the source of PlGF in patients with COPD, although other cell types, such as endothelial cells or inflammatory cells, may be responsible for the production of PlGF. The populations of sputum cells in asthmatic and healthy individuals differ, and this should be taken into account when interpreting expression data. A similar up-regulation in expression was found for VEGF-A, the member closest to PlGF in the VEGF family, and for VEGFR1, as was also reported by other groups (2–4). In addition, the allergen stimulation of bronchial mucosa explants from HDM-allergic and nonallergic subjects induced the *in vitro* production of PlGF. Der p 1 stimulation significantly increased concentrations of PlGF in bronchial biopsies of HDM-allergic donors, but not in the control group. Stimulation with IL-1β, TNF-α, transforming growth factor (TGF)–β, and epidermal growth factor (EGF) (8), or by cyclic stretch (17), was shown to induce the production of PlGF by bronchial epithelial cells. EGF and TGF-α/β can also induce the production of PlGF in keratinocytes. The production of PlGF is further induced by hypoxia in fibroblasts (13) and renal epithelial cells (16). In our experiment, the production of PlGF seemed to be associated with persistent airway inflammation, as is the case in asthma or upon allergen challenge in sensitized individuals.

To investigate the further role of PlGF in allergic inflammation of the lower airways, a murine model of allergic airway inflammation was used (35–37). Expression studies showed that PlGF was expressed in the airways of mice, and was significantly up-regulated in OVA-challenged mice compared with control mice. PlGF was up-regulated not only in the lung tissue of OVA-challenged

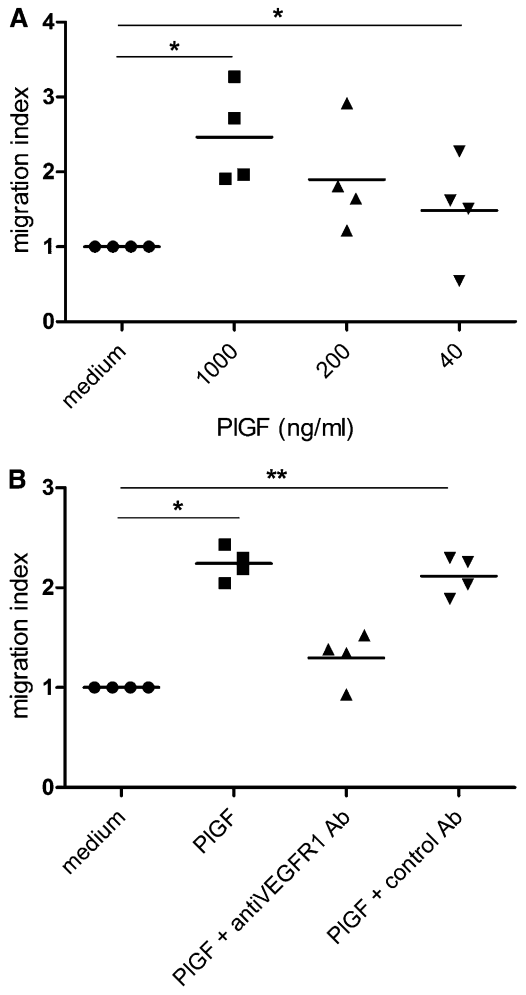

Figure 6. (*A*) Effects of hPlGF-2 on human neutrophil migration *in vitro*. hPlGF-2 (1,000 ng/ml) induced strong migration of human neutrophils with a migration index higher than 2, compared with the medium condition. (*B*) Neutrophilic migration in response to hPlGF-2 (1,000 ng/ml), in the presence or absence of VEGFR1 blockade by goat anti-human VEGFR1 antibody. Control goat antibodies did not exert an effect on neutrophilic migration in response to hPlGF-2. Number of patients, $n = 4$. *$P < 0.05$. **$P < 0.01$.

mice, but was also produced *in vitro* in LN cell cultures upon stimulation with the relevant allergen (i.e., OVA). We also correlated concentrations of PlGF produced in lung tissue with the number of BAL-infiltrating cells, and found that PlGF was significantly correlated with the numbers of neutrophils and lymphocytes, but not with the numbers of macrophages or eosinophils.

Apart from PlGF, VEGFR1, the only receptor for PlGF, was also up-regulated in inflammatory cells infiltrating the lungs of OVA-challenged mice. In addition, VEGFR1 was present in epithelial and endothelial cells in both control and inflamed tissue. The soluble form of VEGFR1, which is inhibitory (19, 20), was significantly down-regulated in the lungs of OVA-challenged mice compared with the control group, suggesting that there is less inhibition of PlGF signaling through membrane VEGFR1.

The effects of PlGF on inflammation and edema were further studied in this murine model of allergic asthma, in which OVA-sensitized and OVA-challenged mice received endonasal hPlGF-2. The instillation of hPlGF-2 at the time of OVA challenge induced a significantly stronger influx of neutrophils and macrophages to the lungs of OVA-sensitized and OVA-challenged mice, compared with control mice. According to a previous report, PlGF directly induces the migration of macrophages to the site of inflammation (22. This finding has not been directly confirmed for neutrophils, although neutrophils are known to express VEGFR1 on their cell surface (24). In addition, the instillation of PlGF significantly increased edema of the lung tissue, but exerted no effect of bronchial hyperresponsiveness. Cell infiltration and vascular leakage contribute to tissue edema, and PlGF was reported to play a role in maintaining vascular permeability and inducing vascular leakage (28, 29). The instillation of PlGF also increased concentrations of lung IL-17, which is known to be involved in neutrophilic recruitment (33, 41). A link between VEGF-A and IL-17 was reported previously (42, 43), but this link has never been described for PlGF and IL-17. IL-17 does not directly induce the migration of neutrophils, but acts on epithelial cells, which respond by producing MIP-2 and GCP-2/LIX (i.e., IL-8 analogues in mice that attract neutrophils to the site of inflammation) (44, 45). However, in our experiments, the instillation of PlGF did not affect concentrations of MIP-2 and GCP-2/LIX, even though IL-17 was up-regulated. These effects of the endonasal administration of hPlGF-2 could not be attributed to low amounts of LPS present in the hPLGF-2 preparation, because no effect of endonasal LPS administration was evident in the measured parameters (Figure 4). Another link between PlGF, neutrophilic inflammation, and IL-17 production was provided by experiments in PlGF-deficient mice. OVA-challenged, PlGF KO mice showed lower concentrations of neutrophils in their lung tissue (although this was not observed with BAL-infiltrating neutrophils), compared with wt OVA-challenged mice, along with significantly lower MPO concentrations. The wet/dry ratio of lung tissue, a marker of tissue edema, was also markedly reduced in PlGF-deficient mice. PlGF KO mice also had reduced concentrations of IL-17 and neutrophil chemoattractants MIP-2 and GCP-2, suggesting a potent etiologic role of PlGF in bronchial neutrophilic inflammation.

Murine experiments revealed that the addition of hPlGF-2 increased the number of bronchial neutrophils, whereas PlGF-deficient mice demonstrated a lower production of MPO by lung neutrophils. Thus, we performed *in vitro* experiments to study the effect of PlGF on neutrophilic migration in a Boyden chamber migration assay. Interestingly, hPlGF-2 directly induced the migration of neutrophils isolated from the blood of HDM-allergic donors in a VEGFR1-dependent way. Therefore, PlGF seems to exert a direct effect on neutrophils via the VEGFR1 expressed on their surface, but it is not clear how PlGF induces a higher production of IL-17. The stimulation of LN cells with splenocytes and allergen (OVA) induced the production of IL-17, but the addition of PlGF had no effect on concentrations of IL-17 *in vitro*. The mechanism by which PlGF affects concentrations of IL-17 remains to be elucidated. The intracellular staining of lung cells of OVA-challenged mice revealed that only T cells are the source of IL-17, and IL-17 concentrations were not affected by *in vitro* PlGF stimulation (data not published). Not only T cells, but also macrophages (46) and neutrophils (47), were reported to produce IL-17. Furthermore, a novel subset of Th2 memory effector cells coexpressing IL-17 and Th2 cytokines was recently described in a murine model of experimental asthma (48). Part of the explanation for the link between PlGF and the production of IL-17 may involve the role of VEGFR1. VEGFR1 (which is also a receptor for VEGF-A) was shown to contribute to Th17 cell development (49). It also plays an important role in dendritic cell migration and maturation and T-cell priming in an asthma model induced with LPS-containing allergens (OVA) (49).

Despite the strong association between PlGF and neutrophilic inflammation, both the endonasal administration of PlGF and PlGF genetic deficiency did not alter airway hyperresponsiveness in our model. This observation regarding airway hyperresponsiveness underscores once more the dissociation between

inflammation (characterized by Th2 cytokines), edema, and granulocyte influx and airway hyperresponsiveness.

In conclusion, PlGF is significantly up-regulated in the sputum of allergic asthma patients, and is induced in bronchial tissue upon allergen stimulation. PlGF plays a proinflammatory role in allergic airway inflammation, and induces neutrophilic chemotaxis, tissue edema, and the production of IL-17. The effects of PlGF are mediated via VEGFR1, which is expressed in endothelial cells, neutrophils, and macrophages.

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