Elevated levels of placental growth factor represent an adaptive host response in sepsis

Kiichiro Yano,[1,2] Yoshiaki Okada,[1] Guido Beldi,[2] Shou-Ching Shih,[1] Natalya Bodyak,[1] Hitomi Okada,[1] Peter M. Kang,[1,2] William Luscinskas,[3] Simon C. Robson,[2] Peter Carmeliet,[4,5] S. Ananth Karumanchi,[1,2] and William C. Aird[1,2]

[1]The Center for Vascular Biology Research and Division of Molecular and Vascular Medicine and [2]Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA 02215
[3]Center for Excellence in Vascular Biology, Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115
[4]Department for Transgene Technology and Gene Therapy, VIB, 3000 Leuven, Belgium
[5]Center for Transgene Technology and Gene Therapy, Katholieke Universiteit Leuven, 3000 Leuven, Belgium

Recently, we demonstrated that circulating levels of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) are increased in sepsis (Yano, K., P.C. Liaw, J.M. Mullington, S.C. Shih, H. Okada, N. Bodyak, P.M. Kang, L. Toltl, B. Belikoff, J. Buras, et al. 2006. *J. Exp. Med.* 203:1447–1458). Moreover, enhanced VEGF/Flk-1 signaling was shown to contribute to sepsis morbidity and mortality. We tested the hypothesis that PlGF also contributes to sepsis outcome. In mouse models of endotoxemia and cecal ligation puncture, the genetic absence of PlGF or the systemic administration of neutralizing anti-PlGF antibodies resulted in higher mortality compared with wild-type or immunoglobulin G–injected controls, respectively. The increased mortality associated with genetic deficiency of PlGF was reversed by adenovirus (Ad)-mediated overexpression of PlGF. In the endotoxemia model, PlGF deficiency was associated with elevated circulating levels of VEGF, induction of VEGF expression in the liver, impaired cardiac function, and organ-specific accentuation of barrier dysfunction and inflammation. Mortality of endotoxemic PlGF-deficient mice was increased by Ad-mediated overexpression of VEGF and was blocked by expression of soluble Flt-1. Collectively, these data suggest that up-regulation of PlGF in sepsis is an adaptive host response that exerts its benefit, at least in part, by attenuating VEGF signaling.

CORRESPONDENCE
William C. Aird:
waird@bidmc.harvard.edu

Abbreviations used: Ad, adenovirus; CLP, cecal ligation puncture; COX, cyclooxygenase; ICAM, intercellular adhesion molecule; MPO, myeloperoxidase; PlGF, placental growth factor; sFlt-1, soluble Flt-1; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

Vascular endothelial growth factor (VEGF; also termed VEGF-A) is a member of a growing family of related proteins that include VEGF-B, -C, and -D, and placental growth factor (PlGF; for review see reference 1). VEGF/vascular permeability factor was first identified and characterized by Senger et al. as a potent stimulator of endothelial permeability (2). VEGF was subsequently reported to promote proliferation, migration, and survival of endothelial cells (3). In addition, VEGF has been implicated in inflammatory processes, including rheumatoid arthritis, cancer, and inflammatory bowel disease (4–6). Several studies have demonstrated increased VEGF levels in animal and human models of sepsis (7–11). Importantly, inhibition of VEGF signaling with soluble Flt-1 (sFlt-1) or antibodies against Flk-1 improved morbidity and mortality in septic mice (7, 11).

PlGF occurs in four isoforms in humans (PlGF-1–4), and in one isoform in mice (PlGF-2) (12–15). PlGF was originally identified in the placenta (13, 16) but has subsequently been shown to be expressed in other tissues, including the heart, lung, thyroid gland, and skeletal muscle (for review see reference 17). Circulating levels of PlGF are normally undetectable. However, increased PlGF levels have been described in several conditions, including cancer (18–20), cutaneous wound and bone fracture healing (21–24), atherosclerosis, and sickle cell disease (25). Recently, we demonstrated that circulating levels of PlGF are elevated in mouse and human models of sepsis (7). Previous studies have implicated a role for PlGF in inflammation (21, 23, 25–31). Thus, we

The online version of this paper contains supplemental material.

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 205 No. 11 2623-2631
www.jem.org/cgi/doi/10.1084/jem.20080398

The Journal of Experimental Medicine

hypothesized that elevated levels of PlGF in sepsis might contribute (as do increased VEGF levels) to the pathophysiology of the host response to infection. On the contrary, we found that PlGF protects against sepsis morbidity and mortality.

RESULTS

PlGF protein and mRNA levels are induced in animal models of sepsis

We recently demonstrated that circulating PlGF levels are increased in C57BL/6 mice injected with LPS or subjected to cecal ligation puncture (CLP) (7). The PlGF$^{-/-}$ mice used in this study were backcrossed to an FVB background. Previous studies have demonstrated strain-specific sensitivity to LPS (32–34). Thus, we wished to confirm these findings in FVB mice. The i.p. administration of 18 mg/kg LPS resulted in a time-dependent increase in plasma PlGF concentrations, with peak levels (3,447.4 pg/ml) occurring at 24 h (Fig. 1 A). Similarly, in a CLP model of sepsis, peak levels of PlGF (111.4 pg/ml) occurred at 24 h (Fig. 1 A). Previously, we demonstrated that PlGF protein levels were increased in all tissues examined,

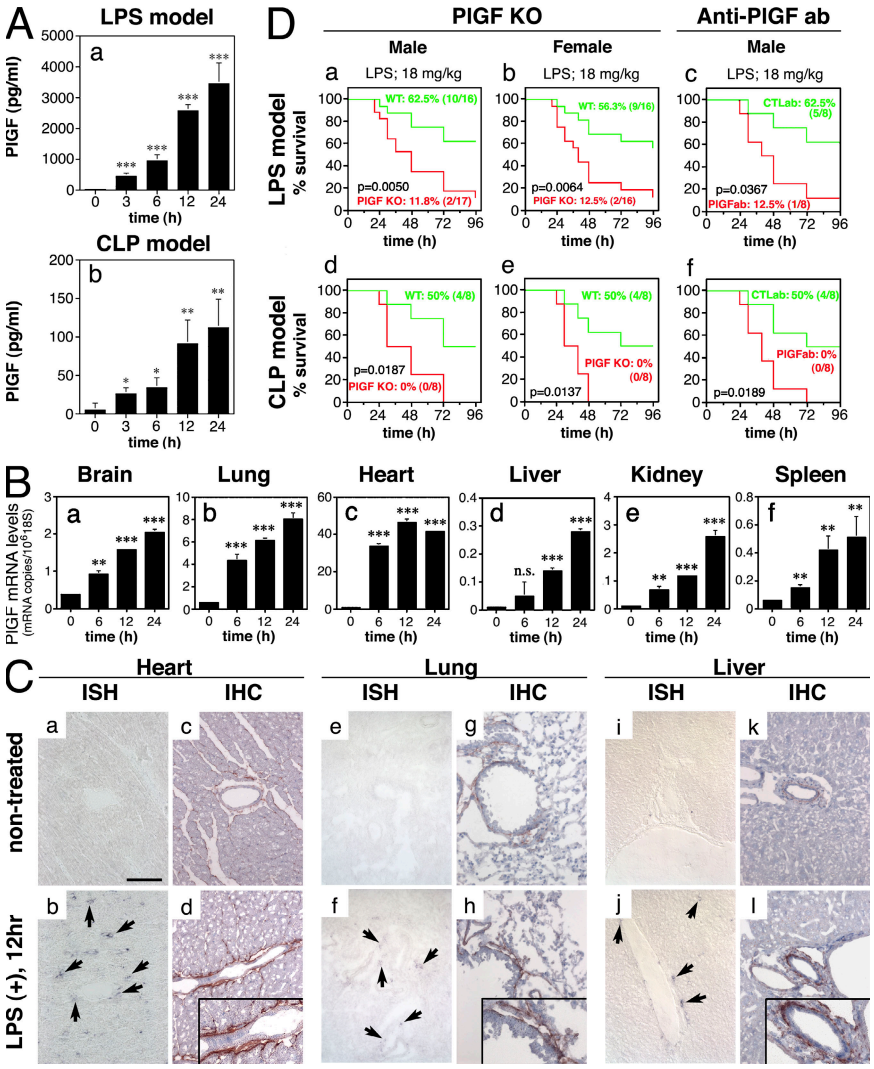

Figure 1. PlGF levels in sepsis and survival studies in PlGF-deficient mice. (A, a) Plasma levels of PlGF in wild-type male FVB mice injected i.p. with 18 mg/kg LPS at the time points indicated. (b) Same as in a but in a CLP mouse model. (B) Shown are results of quantitative real-time analyses (mRNA copy number per 10^6 copies of 18S) of PlGF in organs from male FVB mice at various time points after i.p. injection with 18 mg/kg LPS. (C) In situ hybridization (ISH) and immunohistochemistry (IHC) for PlGF in the heart, lung, and liver of male FVB mice treated in the absence (nontreated) or presence of 18 mg/kg LPS at 12 h. The insets in d, h, and i show a higher magnification of a representative area from each field. Arrows indicate PlGF signal. Bar: 50 µm; (insets) 25 µm. (D, a) Survival curves for male PLGF$^{+/+}$ (wild-type, WT) or PLGF$^{-/-}$ (knockout, KO) mice injected i.p. with 18 mg/kg LPS. (b) Survival curves for female PLGF$^{+/+}$ (WT) or PLGF$^{-/-}$ (KO) mice injected i.p. with 18 mg/kg LPS. (c) Survival curves for wild-type male mice pretreated with anti-PlGF antibody (PlGF ab) or IgG (CTL ab) and injected i.p. with 18 mg/kg LPS. (d) Survival curves for male PLGF$^{+/+}$ (WT) or PLGF$^{-/-}$ (KO) mice subjected to CLP. (e) Survival curves for female PLGF$^{+/+}$ (WT) or PLGF$^{-/-}$ (KO) mice subjected to CLP. (f) Survival curves for wild-type male mice pretreated with anti-PlGF antibody (PlGF ab) or IgG (CTL ab) and subjected to CLP. Data in A and B are expressed as means + SD of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.0001$ compared with no treatment (0 h time point).

including the brain, lung, heart, liver, kidney, and spleen (7). In real-time PCR assays, there was a time-dependent induction of PlGF transcripts at 24 h in the brain (5.3-fold), lung (14.3-fold), liver (28-fold), kidney (25.7-fold), and spleen (8.5-fold). PlGF mRNA levels in the heart peaked at 6 h (54.2-fold; Fig. 1 B). In situ hybridization and immunohistochemical studies revealed a PlGF signal in perivascular cells but not the endothelium (Fig. 1 C shows the heart, lung, and liver). Collectively, these findings suggest that sepsis is associated with the widespread induction of PlGF mRNA and protein expression.

PlGF deficiency is associated with increased sepsis mortality

We next wished to determine whether PlGF contributes to sepsis outcome. To that end, 10–12-wk-old PlGF$^{-/-}$ mice or wild-type littermates were administered 18 mg/kg LPS or subjected to CLP. PlGF deficiency was associated with significantly lower survival rate in both the endotoxemia model (wild-type male, 62.5% vs. PlGF$^{-/-}$ male, 11.8%; wild-type female, 56.3% vs. PlGF$^{-/-}$ female, 12.5%) and in CLP (wild-type male, 50% vs. PlGF$^{-/-}$ male, 0%; wild-type female, 50% vs.

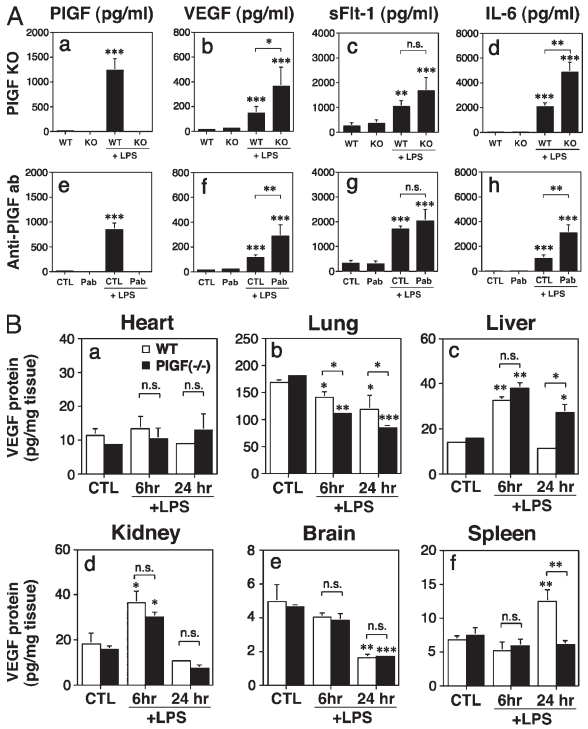

Figure 2. Effect of PlGF deficiency on tissue and/or circulating levels of PlGF, VEGF, sFlt-1, and IL-6. (A, a–d) PLGF$^{+/+}$ (WT) or PLGF$^{-/-}$ (KO) male mice were injected i.p. with or without 16 mg/kg LPS. Blood samples were taken 24 h later and assayed for plasma levels of free PlGF, free VEGF, sFlt-1, and IL-6. (e–h) Same as in a–d, except that wild-type mice were pretreated with anti-PlGF antibody (Pab) or IgG (CTL) and injected i.p. with or without 16 mg/kg LPS. (B) PLGF$^{+/+}$ (WT) or PLGF$^{-/-}$ male mice were injected i.p. with or without 16 mg/kg LPS. Mouse organs were assayed for VEGF protein levels by ELISA at the time points indicated. Data are expressed as means + SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.0001$ compared with the respective untreated controls (and where indicated between LPS-treated PlGF-deficient and wild-type mice).

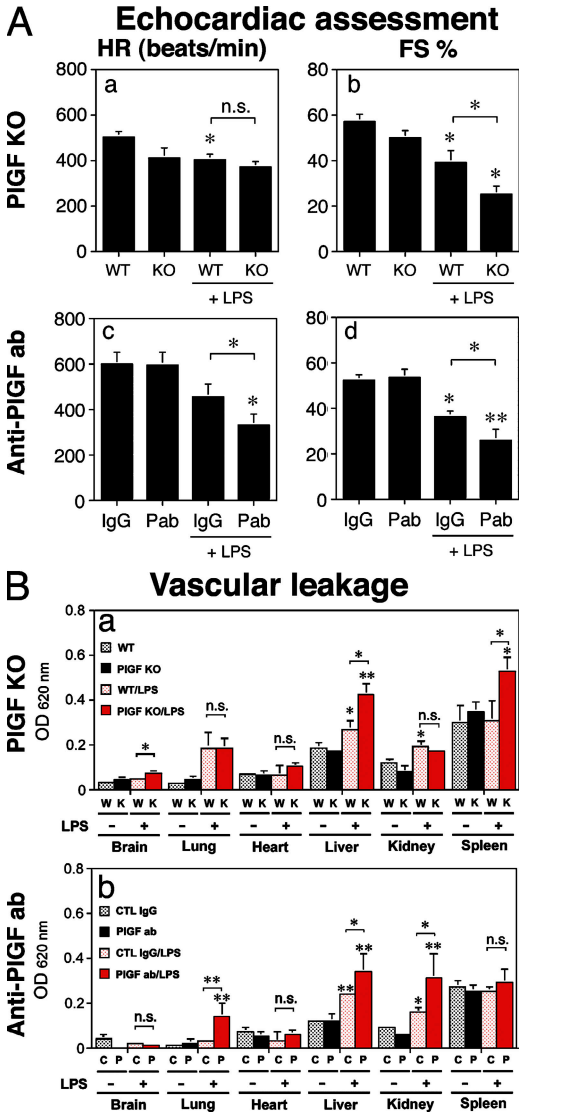

Figure 3. Effect of PlGF deficiency on cardiac function and vascular permeability in a mouse model of endotoxemia. (A, a and b) PLGF$^{+/+}$ (WT) or PLGF$^{-/-}$ (KO) male mice were injected i.p. with or without 16 mg/kg LPS and were subjected to echocardiogram and electrocardiogram 24 h later. Shown are quantitative analyses for heart rate (HR) and fractional shortening (FS). (c and d) Same as in a and b except that wild-type mice were pretreated with anti-PlGF antibody (Pab) or IgG (CTL) and injected i.p. with or without 16 mg/kg LPS. (B, a) PLGF$^{+/+}$ (WT, W) or PLGF$^{-/-}$ (KO, K) male mice were injected i.p. with or without 16 mg/kg LPS. 24 h later, the animals were injected i.v. with 0.1 ml 1% Evans blue dye. After 40 min, the mice were perfused, and the brain, lung, heart, liver, kidney, and spleen were harvested and incubated in formamide for 3 d to elute Evans blue dye. Shown is the quantitation of Evans blue extravasation (OD = 620 nm). (b) Same as in a except that wild-type mice were pretreated with anti-PlGF antibody (PlGF ab, P) or IgG (CTL IgG, C) and injected i.p. with or without 16 mg/kg LPS. Data are expressed as means + SD of three independent experiments. *, $P < 0.05$; and **, $P < 0.01$ compared with the respective untreated controls (and where indicated between PlGF-deficient and wild-type mice).

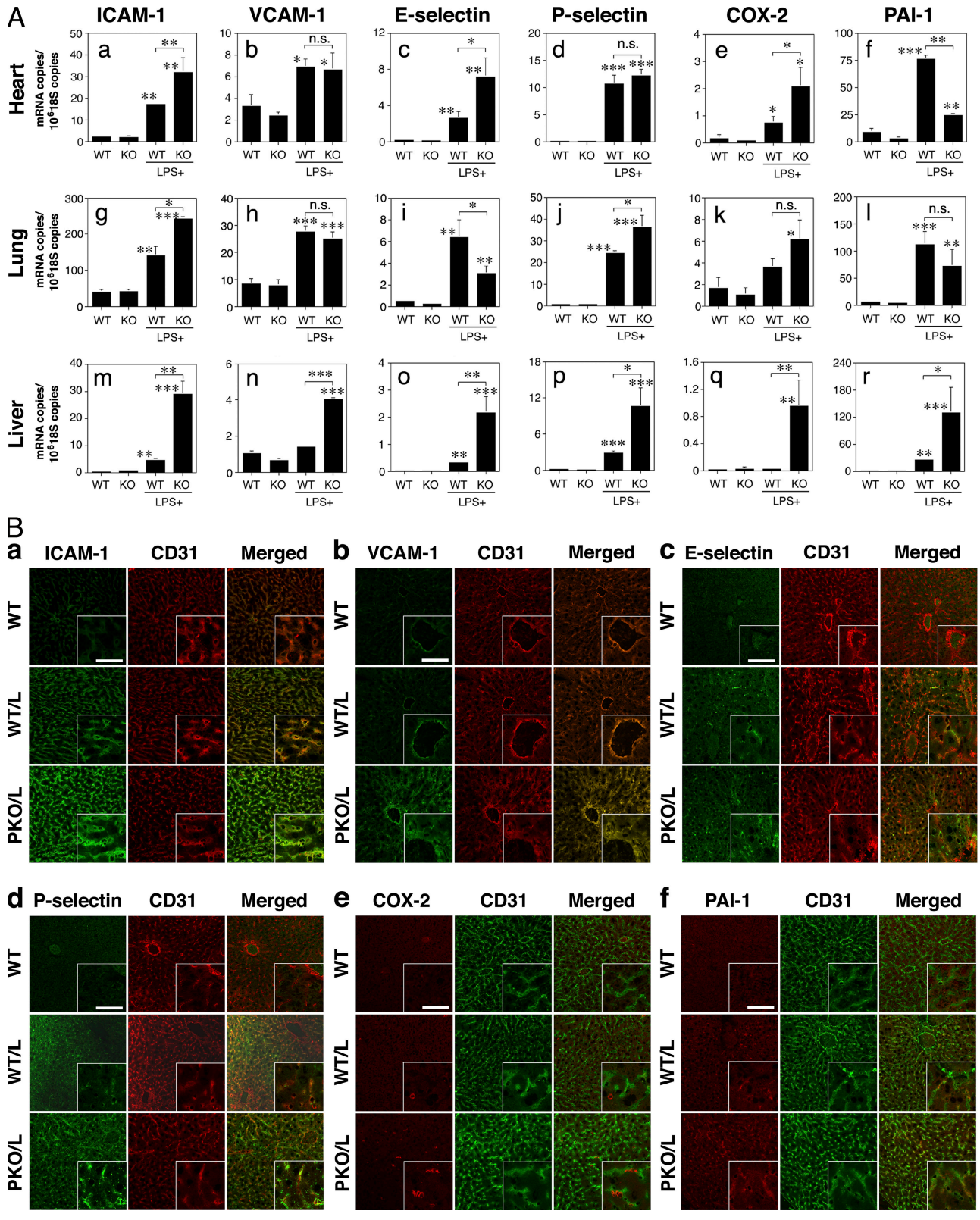

Figure 4. Effect of PlGF deficiency on tissue mRNA/protein levels of inflammatory and hemostatic markers in a mouse model of endotoxemia. PLGF$^{+/+}$ (WT) or PLGF$^{-/-}$ (KO) male mice were injected i.p. with or without 16 mg/kg LPS. (A) Shown are the results of quantitative real-time PCR analyses (mRNA copy number per 10^6 copies of 18S) of ICAM-1, VCAM-1, E-selectin, P-selectin, COX-2, and PAI-1 in the heart, lung and liver at 24 h. Data are expressed as means + SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.0001$ compared with untreated controls

$PlGF^{-/-}$ female, 0%; Fig. 1 D). The differences in survival between wild-type male and female FVB mice were not statistically significant. Interestingly, the increased mortality associated with the genetic deficiency of PlGF was less pronounced in younger mice (6 wk old; unpublished data). To test for the effect of acute PlGF down-regulation in the adult animal, FVB mice were administered 1 mg of neutralizing monoclonal anti-PlGF antibody (clone 5D11D4) i.p. 20 h before initiation of sepsis. In these experiments, PlGF levels in the plasma were undetectable (see Fig. 2 A). In 10–12-wk-old male mice, anti-PlGF antibody–mediated depletion of PlGF resulted in reduced sepsis survival in LPS-treated mice (control IgG, 62.5% vs. PlGF antibody, 12.5%) and mice subjected to CLP (control IgG, 50% vs. PlGF antibody, 0%; Fig. 1 D). Similar results were obtained in female mice and in younger animals (6 wk old; unpublished data). Thus, PlGF has a protective role in sepsis.

PlGF deficiency is associated with increased VEGF levels in sepsis

We previously demonstrated that endotoxemia in mice is associated with increased circulating levels of VEGF, sFlt-1, and the inflammatory marker IL-6 (7). In this study, there were no differences in baseline plasma levels of VEGF, sFlt-1, or IL-6 in PlGF-deficient mice versus wild-type controls (Fig. 2). As expected, PlGF levels were undetectable under all conditions in $PlGF^{-/-}$ mice and antibody-treated animals (Fig. 2 A, a and e). In response to 16 mg/kg LPS, $PlGF^{-/-}$ mice demonstrated significantly higher plasma levels of VEGF (2.6-fold) and IL-6 (2.4-fold) compared with wild-type controls (Fig. 2 A, b and d). The dose of 16 mg/kg LPS was chosen for these and subsequent morbidity experiments because 18 mg/kg LPS led to nearly 100% mortality (Fig. 1). A similar effect was observed in PlGF antibody–treated animals compared with IgG–injected controls (VEGF, 3-fold; IL-6, 1.7-fold; Fig. 2 A, f and h). In contrast, PlGF deficiency did not influence endotoxemia-mediated induction of sFlt-1 (Fig. 2 A, c and g). In wild-type mice, endotoxemia was associated with reduced VEGF protein levels in the lung at 6 h, and in the lung and brain at 24 h, and with increased VEGF protein levels in the liver and kidney at 6 h and in the spleen at 24 h (Fig. 2 B). Compared with wild-type mice, $PlGF^{-/-}$ mice demonstrated significantly higher levels of VEGF protein and mRNA in the liver at 24 h. Thus, PlGF deficiency results in increased circulating levels of VEGF and sustained induction of VEGF expression in the liver.

PlGF deficiency is associated with increased endotoxemia-mediated cardiac dysfunction and barrier dysfunction

Consistent with our previous results (7), systemic administration of LPS or CLP in mice resulted in marked depression of cardiac function, as indicated by reduced fractional shortening and heart rate on echocardiography (Fig. 3 A). The effect of endotoxin on fractional shortening was accentuated in $PlGF^{-/-}$ mice (Fig. 3 A, b) and in wild-type mice administered PlGF antibody (Fig. 3 A, d). Endotoxemic-associated bradycardia was accentuated by PlGF antibody (Fig. 3 A, c). PlGF deficiency was also associated with an accentuation of barrier dysfunction. Compared with wild-type mice, LPS administration in $PlGF^{-/-}$ mice resulted in increased extravasation of Evans blue dye in the liver and spleen (Fig. 3 B, a). Antibody-mediated depletion of PlGF resulted in increased extravasation occurring in the lung, liver, and kidney (Fig. 3 B, b). Collectively, these findings suggest that PlGF deficiency is associated with impaired cardiac function and organ-specific accentuation of vascular permeability during sepsis.

PlGF deficiency results in altered endotoxemia-mediated expression of inflammatory and coagulation mediators

Compared with wild-type controls, endotoxemic $PlGF^{-/-}$ mice demonstrated significant vascular bed–specific changes in the expression of inflammatory and procoagulant molecules (Fig. 4; and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20080398/DC1). Specifically, PlGF deficiency resulted in increased expression of intercellular adhesion molecule (ICAM)–1 in the heart (1.9-fold), lung (1.7-fold), liver (6.5-fold), kidney (2.7-fold), and spleen (1.4-fold); increased expression of vascular cell adhesion molecule (VCAM)–1 in the liver (2.9-fold); increased E-selectin expression in the heart (2.8-fold) and liver (6.9-fold) but decreased E-selectin expression in the lung (0.5-fold); increased P-selectin expression in the lung (1.5-fold) and liver (3.7-fold); increased cyclooxygenase (COX)-2 expression in the heart (2.9-fold), liver (47.5-fold), kidney (5.7-fold), and spleen (4.5-fold); and increased plasminogen activator inhibitor (PAI)–1 in the liver (5.3-fold) and decreased PAI-1 in the heart (0.3-fold), brain (0.3-fold), kidney (0.6-fold), and spleen (0.3-fold; Fig. 4 A and Fig. S1).

Immunofluorescent studies were used to determine the localization of the mediators described in the previous paragraph in the liver, heart, and lung. In the wild-type liver, LPS resulted in increased expression of ICAM-1 and P-selectin in endothelial cells of the sinusoids and veins (Fig. 4 B, a and d), and PAI-1 in sinusoidal endothelial cells alone (Fig. 4 B, f). LPS appeared to result in a slight induction of VCAM-1 in venular endothelial cells (Fig. 4 B, b) and of E-selectin in sinusoidal and venular endothelial cells (Fig. 4 B, c). COX-2 was induced in occasional CD31-negative cells (Fig. 4 B, e). LPS injection of $PlGF^{-/-}$ mice resulted in the further induction of ICAM-1 and P-selectin in sinusoidal and venular endothelium (Fig. 4 B, a and d), increased expression of VCAM-1 in both endothelial cell populations (Fig. 4 B, b), increased expression of E-selectin and PAI-1,

(and where indicated between PlGF-deficient and wild-type mice). (B) Double immunofluorescence staining for activation markers and CD31 in the liver of wild-type mice treated in the absence (WT) or presence of 16 mg/kg LPS (WT/L) and $PlGF^{-/-}$ mice treated with 16 mg/kg LPS (PKO/L) at 24 h. (a) ICAM-1 (green) and CD31 (red). (b) VCAM-1 (green) and CD31 (red). (c) E-selectin (green) and CD31 (red). (d) P-selectin (green) and CD31 (red). (e) COX-2 (red) and CD31 (green). (f) PAI-1 (red) and CD31 (green). Bars: 132 μm; (insets) 42 μm.

particularly in sinusoidal endothelial cells (Fig. 4 B, c and f), and increased expression of COX-2 in CD31-negative cells (Fig. 4 B, e). Larger images of the liver data are shown in Fig. S2 (A and B; available at http://www.jem.org/cgi/content/full/jem.20080398/DC1).

In the heart of LPS-treated wild-type mice, ICAM-1, VCAM-1, E-selectin, and P-selectin were induced in capillary and venular endothelium, whereas COX-2 and PAI-1 were slightly increased in CD31-negative cells (Fig. S2, C and D). In PlGF$^{-/-}$ mice, LPS administration resulted in increased endothelial expression of ICAM-1 and E-selectin but not VCAM-1 or P-selectin. Endotoxemic PlGF$^{-/-}$ mice demonstrated increased expression of COX-2 and decreased expression of PAI-1 in CD31-negative endothelial cells.

In the lung, LPS injection of wild-type mice resulted in increased expression of ICAM-1 and P-selectin in venular and capillary endothelium and possibly in parenchymal cells (Fig. S2, E and F). VCAM-1 and E-selectin were increased primarily in venular endothelium. COX-2 was increased in CD31-negative cells on the luminal side of venular endothelium. PAI-1 was slightly induced, primarily in venous endothelial cells. In PlGF$^{-/-}$ mice, LPS resulted in a further increase in the expression of ICAM-1 and P-selectin but no demonstrable change in VCAM-1, E-selectin, or PAI-1. In addition, COX-2 was up-regulated in CD31-negative cells in the lumen of veins. Together with the real-time PCR data, these findings demonstrate that PlGF deficiency results in vascular bed–specific changes in the expression of inflammatory and coagulation markers, with the most widespread changes occurring in the liver.

PlGF deficiency results in liver dysfunction and leukocyte infiltration

The results described in the previous three sections suggest that PlGF deficiency is associated with disproportionate endotoxemia-mediated changes in the liver, including sustained elevation of VEGF, increased permeability, and higher expression of activation markers. Based on these findings, we wished to further explore the effect of PlGF deficiency on the liver phenotype. Alanine aminotransferase, a measure of liver damage, was increased in PlGF$^{-/-}$ versus wild-type controls (wild-type, 63.3 ± 35.1 U/liter; PlGF$^{-/-}$, 223.3 ± 92.9 U/liter; Fig. 5 A). Moreover, endotoxemia resulted in significantly greater myeloperoxidase (MPO) activity in the liver of PlGF$^{-/-}$ versus wild-type controls (Fig. 5 B). Consistent with these latter findings, CD45 staining demonstrated increased leukocyte infiltration in the liver of PlGF$^{-/-}$ compared with wild-type endotoxemic mice (Fig. 5 C). In contrast to the findings in the liver, MPO activity in the lung did not differ between endotoxemia wild-type and PlGF$^{-/-}$ mice (unpublished data).

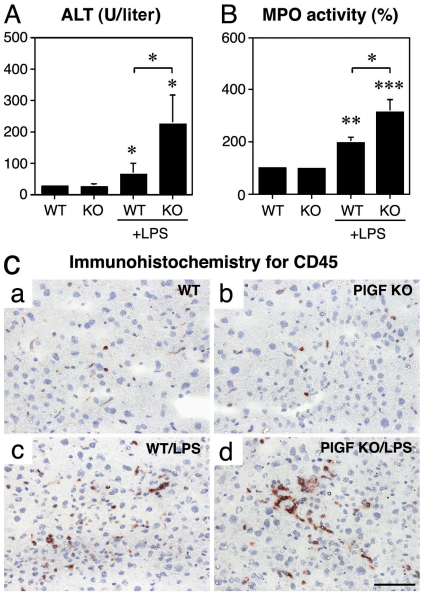

Figure 5. Effect of PlGF deficiency on liver phenotype in a mouse model of endotoxemia. PLGF$^{+/+}$ (WT) or PLGF$^{-/-}$ (KO) male mice were injected i.p. with or without 16 mg/kg LPS. (A) Blood samples were taken at 24 h and assayed for plasma levels of alanine aminotransferase (ALT). (B) The liver was removed at 24 h and assayed for MPO activity. (C) The liver was removed at 24 h and stained for CD45. Data in A and B are expressed as means + SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.0001$ compared with untreated controls (and where indicated between PlGF-deficient and wild-type mice. Bar, 50 µm.

Excess mortality in endotoxemic PlGF-deficient mice is accentuated by overexpression of VEGF and rescued by overexpression of sFlt-1

The increased mortality in endotoxemic PlGF$^{-/-}$ mice was rescued by overexpression of PlGF (Fig. 6 A). However, the

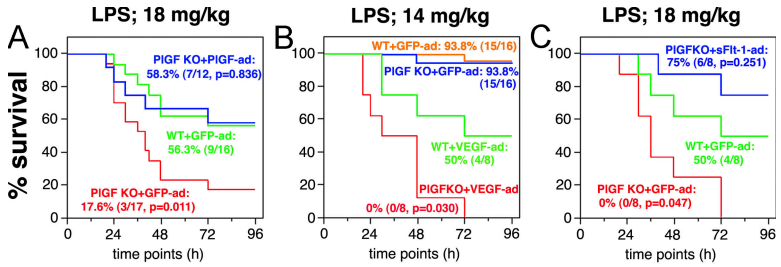

Figure 6. Additional survival studies in mouse models of sepsis. Male PLGF$^{+/+}$ (WT) or PLGF$^{-/-}$ mice were injected with Ad overexpressing GFP (GFP-ad) or PlGF (PlGF-ad; A), VEGF (VEGF-ad; B), or sFlt-1 (sFlt-ad; C). 3 d later, the animals were administered saline (control) or LPS i.p. and monitored for survival.

elevated circulating levels of PlGF in the PlGF-adenovirus (Ad)–treated mice (5,823.4 ± 833.6 pg/ml) did not confer a survival advantage over GFP-Ad (control)–injected wild-type animals. To determine whether PlGF deficiency rendered animals more sensitive to the effects of VEGF, mice were injected with control virus or VEGF-Ad and administered small doses of LPS (14 mg/kg). At the latter dose, 93.8% of wild-type and $PlGF^{-/-}$ mice survived (Fig. 6 B). However, overexpression of VEGF (plasma levels, 4,204.5 ± 901.8 pg/ml) resulted in a marked increased in mortality in $PlGF^{-/-}$ compared with wild-type animals (wild-type, 50% vs. $PlGF^{-/-}$, 100%) (Fig. 6 B). Finally, the increased mortality associated with PlGF deficiency was reversed by overexpression of sFlt-1 (Fig. 6 C). Collectively, these results suggest that elevated VEGF levels are both sufficient and necessary to promote sepsis mortality in PlGF-deficient mice.

DISCUSSION

Compared with our understanding of VEGF, little is known about the function of PlGF. PlGF is thought to bind to VEGFR1/Flt-1 and the coreceptors neuropilin-1 and -2. The interaction between PlGF and VEGFR1/Flt-1 has been shown both to trigger downstream signaling and to enhance VEGF signaling via VEGFR2/Flk-1 (for review see reference 35). Although PlGF is not required for developmental angiogenesis, it appears to play an important role in postnatal angiogenesis, arteriogenesis, and vasculogenesis (21). In addition, increasing evidence points to a proinflammatory effect of PlGF. Most notably, PlGF-mediated induction of monocyte migration and activation has been shown to contribute to tumor growth (26), atherosclerosis (27), sickle cell disease (25), and wound healing (26). In addition, PlGF has been causally linked to vascular permeability and plasma extravasation (21, 23, 28–31).

From a mechanistic standpoint, it has been proposed that PlGF displaces VEGF from VEGFR1/Flt-1, thus promoting VEGF–VEGFR2/Flk-1 interactions (36). Moreover, PlGF-mediated activation of VEGFR1/Flt-1 may lead to the transactivation of VEGFR2/Flk-1, further enhancing the response to VEGF (37). Previous studies have revealed that PlGF upregulates the expression of VEGF by periendothelial fibroblasts, smooth muscle cells, or inflammatory cells in wound or tumor stroma (38, 39). Together with our recent observations that sepsis is associated with elevated levels of VEGF and PlGF, and that VEGF plays a pathogenic role in sepsis (7), these data led us to hypothesize that elevated levels of PlGF would accentuate sepsis morbidity and mortality. On the contrary, we found that PlGF plays a net protective role in mouse models of sepsis.

A clue to the mechanism by which PlGF deficiency causes increased morbidity and mortality was found in our discovery of elevated circulating VEGF levels in septic PlGF-deficient mice. Moreover, Ad-mediated expression of sFlt-1 reversed the excess mortality in LPS-treated $PlGF^{-/-}$ mice. Thus, the increased mortality associated with PlGF deficiency is attributed, at least in part, to exaggerated VEGF levels. Previous studies have demonstrated that PlGF and VEGF, when produced by the same cell, may form PlGF/VEGF heterodimers (40, 41). PlGF/VEGF heterodimers, in turn, have been shown to inhibit VEGF signaling (41). In response to LPS, we found the majority of PlGF up-regulation in perivascular cells. Perivascular cells have also been shown to express VEGF (42). Thus, it is tempting to speculate that the loss of PlGF yields not only elevated levels of free VEGF but also increased homodimerization and, thus, bioactivity of VEGF.

The prevailing view in the literature is that PlGF functions as a proinflammatory and permeability-enhancing cytokine. However, the results of the present study indicate that these effects of PlGF are highly context dependent. For example, although PlGF induces vascular permeability in several skin models (30, 31), it promotes barrier function in sepsis. PlGF has been shown to promote monocyte infiltration in ischemic tissues (43), tumors (26), atherosclerotic plaques (27), and bone fractures (24). However, as we have demonstrated in this paper, PlGF inhibits leukocyte accumulation in the liver of septic animals. Previous studies have shown that PlGF up-regulates VEGF expression in wound or tumor stroma (38, 39). Moreover, neutralizing anti-PlGF antibody failed to induce VEGF levels in the plasma in tumor-bearing mice (26). In contrast, PlGF deficiency is clearly associated with increased VEGF levels in sepsis. Additional studies will be needed to determine the precise mechanism by which PlGF signaling limits VEGF release and signaling under these conditions.

In summary, our findings suggest that the up-regulation of PlGF represents an adaptive host response to infection. As anti-PlGF therapies become more widespread for the treatment of such conditions as cancer and atherosclerosis, it will be important to carefully monitor patients for the development of infections and systemic inflammatory response syndrome.

MATERIALS AND METHODS

Mice. Mouse models of endotoxemia and CLP; measurement of IL-6, VEGF, sFlt-1, and PlGF in plasma; Ad-mediated overexpression of sFlt-1, VEGF, and PlGF; cardiac physiological studies; permeability assays; tissue RNA isolation; and real-time PCR were performed as previously described (7). Unless otherwise indicated in the figures, all experiments were performed using 10–12-wk-old FVB mice. All animal studies were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Survival studies. Survival studies were performed using endotoxemia and CLP models. Survival was assessed at 24, 48, 72, and 96 h after 14–18 mg/kg body weight LPS injection or CLP. In studies involving adenoviral administration, the virus was injected 3 d before LPS administration. The same lot of LPS was used throughout the study. An LPS dose-response curve is shown in Fig. S3 (available at http://www.jem.org/cgi/content/full/jem.20080398/DC1).

Antibody administration. 20 h before LPS or CLP administration, mice were injected i.p. with 1 mg anti–mouse PlGF-2 antibody (PL5D11D4) or the same dose of control antibody (1C8). The efficacy of this antibody has been previously reported (26).

Immunohistochemistry. Immunohistochemistry was performed using the following primary antibodies: hamster monoclonal anti–mouse ICAM-1 (Serotec), rat monoclonal anti–mouse VCAM-1 antibody (BD Biosciences), rat monoclonal anti–mouse E-selectin antibody (BD Biosciences), rat monoclonal anti–mouse P-selectin antibody (Millipore), rabbit polyclonal anti–mouse COX-2 antibody (Cayman Chemical), and rabbit polyclonal anti–mouse

PAI-1 antibody (Innovative Research Inc.). For double immunofluorescence, ICAM-1, COX-2, and PAI-1 antibodies were combined with rat monoclonal anti–mouse CD31 (BD Biosciences); VCAM-1, E-selectin, and P-selectin were combined with goat polyclonal anti–mouse CD31 antibody (Santa Cruz Biotechnology, Inc.. Secondary antibodies included anti–hamster IgG-FITC (Serotec), anti–rat IgG-Cy3 (Invitrogen), anti–rat IgG-FITC (Jackson ImmunoResearch Laboratories), anti–goat IgG-Cy3 (Jackson ImmunoResearch Laboratories), and anti–rabbit IgG-Cy3 (Invitrogen). The combinations of primary and secondary antibodies are detailed in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20080398/DC1). As a negative control, slides were incubated with secondary antibody alone. Representative images were captured by using a confocal microscope (LSM 510 Meta; Carl Zeiss, Inc.).

Immunohistochemistry for mouse PlGF-2 was performed on 5-μm fixed frozen sections using a goat anti–mouse PlGF antibody (Santa Cruz Biotechnology, Inc.). In brief, after tissue fixation with 4% paraformaldehyde for 1 h, tissues were incubated in PBS for 1 h, followed by 20% sucrose for 4 h before embedding in optimal cutting temperature compound. Cryosections were pretreated with heat (antigen retrieval procedure) in 10 mM citrate buffer (pH 6.4) and incubated with primary antibody. Biotinylated anti–goat IgG and the Elite vectastain ABC kit (Vector Laboratories) were used to detect PlGF protein expression. Biotin blocker (Dako) was used to block endogenous biotin.

In situ hybridization for PlGF. In situ hybridization was performed as previously described (44). For in situ hybridization probes, mouse PlGF-2 cDNA (472 bp, nucleotides 17–489; available from GenBank/EMBL/DDBJ under accession no. X80171) was amplified by RT-PCR of mouse heart RNA and subcloned into the pcDNA3 (+) vector (Invitrogen). Digoxigenin-labeled antisense and sense RNA probes (Roche) were prepared by in vitro transcription with T7 RNA polymerase, using linearized mouse PlGF-2 pcDNA3 (+) as a template.

MPO activity. Snap-frozen liver samples were homogenized in 20 mM phosphate buffer (pH 7.4) and centrifuged at 10,000 *g* for 10 min at 4°C, and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich). The suspension was resubjected to four cycles of freezing and thawing and disrupted further by sonication (40 s). The sample was then centrifuged at 10,000 *g* for 5 min at 4°C, and the supernatant was used for the MPO activity assay. The MPO assay was performed using the NWLSS Myeloperoxidase Activity Assay kit (Northwest), as instructed by the manufacturer. The absorbance was corrected for the DNA content of the tissue sample and expressed as the percent increase over those of wild-type liver tissue.

Statistical analyses. A two-way analysis of variance followed by a Fisher's exact test was used to compare morbidity measurements, including cytokine levels, vascular leakage, cardiac physiology, MPO activity, and gene expression in wild-type and PlGF knockout mice, and in control IgG or PlGF neutralizing antibody–treated mice with or without LPS administration. The Wilcoxon log-rank test was used for survival studies.

Online supplemental material. Fig. S1 shows the effect of PlGF deficiency on mRNA levels of inflammatory and hemostatic markers in the brain (a–f), kidney (g–l), and spleen (m–r) of control and endotoxemic mice, as measured by real-time PCR. Fig. S2 shows the effect of PlGF deficiency on protein expression of inflammatory and hemostatic markers in the liver (A and B), heart (C and D), and lung (E and F) of control and endotoxemia mice, as measured by double immunofluorescence. The images of the liver represent larger versions of those shown in Fig. 4 B. Fig. S3 shows an LPS dose-response survival study in wild-type mice. Table S1 shows antibodies used in immunohistochemistry. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080398/DC1.

We thank Pat D'Amore and Richard Mulligan for help with the generation of Ad's expressing PlGF, VEGF-A (isoform 120), and sFlt-1, respectively.

Supported in part by National Institutes of Health grants HL076540, HL077348, and HL082927 (to W.C. Aird). S.A. Karumanchi is an investigator of the Howard Hughes Medical Institute.

P. Carmeliet declares to be named as inventor on patent EP1297016 and pending foreign counterparts, claiming subject matter that partially covers the results described in this paper. This patent is outlicensed and entitles P. Carmeliet to receive royalty payments. The authors have no additional competing financial interests.

Submitted: 26 February 2008
Accepted: 18 September 2008

REFERENCES

1. Claesson-Welsh, L. 2003. Signal transduction by vascular endothelial growth factor receptors. *Biochem. Soc. Trans.* 31:20–24.
2. Senger, D.R., S.J. Galli, A.M. Dvorak, C.A. Perruzzi, V.S. Harvey, and H.F. Dvorak. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science.* 219:983–985.
3. Leung, D.W., G. Cachianes, W.J. Kuang, D.V. Goeddel, and N. Ferrara. 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science.* 246:1306–1309.
4. Kuenen, B.C., M. Levi, J.C. Meijers, A.K. Kakkar, V.W. van Hinsbergh, P.J. Kostense, H.M. Pinedo, and K. Hoekman. 2002. Analysis of coagulation cascade and endothelial cell activation during inhibition of vascular endothelial growth factor/vascular endothelial growth factor receptor pathway in cancer patients. *Arterioscler. Thromb. Vasc. Biol.* 22:1500–1505.
5. Harada, M., K. Mitsuyama, H. Yoshida, S. Sakisaka, E. Taniguchi, T. Kawaguchi, M. Ariyoshi, T. Saiki, M. Sakamoto, K. Nagata, et al. 1998. Vascular endothelial growth factor in patients with rheumatoid arthritis. *Scand. J. Rheumatol.* 27:377–380.
6. Taha, Y., Y. Raab, A. Larsson, M. Carlson, L. Loof, B. Gerdin, and M. Thorn. 2004. Vascular endothelial growth factor (VEGF)–a possible mediator of inflammation and mucosal permeability in patients with collagenous colitis. *Dig. Dis. Sci.* 49:109–115.
7. Yano, K., P.C. Liaw, J.M. Mullington, S.C. Shih, H. Okada, N. Bodyak, P.M. Kang, L. Toltl, B. Belikoff, J. Buras, et al. 2006. Vascular endothelial growth factor is an important determinant of sepsis morbidity and mortality. *J. Exp. Med.* 203:1447–1458.
8. van der Flier, M., H.J. van Leeuwen, K.P. van Kessel, J.L. Kimpen, A.I. Hoepelman, and S.P. Geelen. 2005. Plasma vascular endothelial growth factor in severe sepsis. *Shock.* 23:35–38.
9. Pickkers, P., T. Sprong, L. Eijk, H. Hoeven, P. Smits, and M. Deuren. 2005. Vascular endothelial growth factor is increased during the first 48 hours of human septic shock and correlates with vascular permeability. *Shock.* 24:508–512.
10. Shapiro, N.I., K. Yano, H. Okada, C. Fischer, M. Howell, K.C. Spokes, L. Ngo, D.C. Angus, and W.C. Aird. 2008. A prospective, observational study of soluble Flt-1 and vascular endothelial growth factor in sepsis. *Shock.* 29:452–457.
11. Tsao, P.N., F.T. Chan, S.C. Wei, W.S. Hsieh, H.C. Chou, Y.N. Su, C.Y. Chen, W.M. Hsu, F.J. Hsieh, and S.M. Hsu. 2007. Soluble vascular endothelial growth factor receptor-1 protects mice in sepsis. *Crit. Care Med.* 35:1955–1960.
12. DiPalma, T., M. Tucci, G. Russo, D. Maglione, C.T. Lago, A. Romano, S. Saccone, G. Della Valle, L. De Gregorio, T.A. Dragani, et al. 1996. The placenta growth factor gene of the mouse. *Mamm. Genome.* 7:6–12.
13. Maglione, D., V. Guerriero, G. Viglietto, P. Delli-Bovi, and M.G. Persico. 1991. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc. Natl. Acad. Sci. USA.* 88:9267–9271.
14. Maglione, D., V. Guerriero, G. Viglietto, M.G. Ferraro, O. Aprelikova, K. Alitalo, S. Del Vecchio, K.J. Lei, J.Y. Chou, and M.G. Persico. 1993. Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. *Oncogene.* 8:925–931.
15. Cao, Y., W.R. Ji, P. Qi, A. Rosin, and Y. Cao. 1997. Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing. *Biochem. Biophys. Res. Commun.* 235:493–498.

16. Khaliq, A., X.F. Li, M. Shams, P. Sisi, C.A. Acevedo, M.J. Whittle, H. Weich, and A. Ahmed. 1996. Localisation of placenta growth factor (PIGF) in human term placenta. *Growth Factors.* 13:243–250.
17. Autiero, M., A. Luttun, M. Tjwa, and P. Carmeliet. 2003. Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. *J. Thromb. Haemost.* 1:1356–1370.
18. Chen, C.N., F.J. Hsieh, Y.M. Cheng, W.F. Cheng, Y.N. Su, K.J. Chang, and P.H. Lee. 2004. The significance of placenta growth factor in angiogenesis and clinical outcome of human gastric cancer. *Cancer Lett.* 213:73–82.
19. Ho, M.C., C.N. Chen, H. Lee, F.J. Hsieh, C.T. Shun, C.L. Chang, Y.T. Lai, and P.H. Lee. 2007. Placenta growth factor not vascular endothelial growth factor A or C can predict the early recurrence after radical resection of hepatocellular carcinoma. *Cancer Lett.* 250:237–249.
20. Parr, C., G. Watkins, M. Boulton, J. Cai, and W.G. Jiang. 2005. Placenta growth factor is over-expressed and has prognostic value in human breast cancer. *Eur. J. Cancer.* 41:2819–2827.
21. Carmeliet, P., L. Moons, A. Luttun, V. Vincenti, V. Compernolle, M. De Mol, Y. Wu, F. Bono, L. Devy, H. Beck, et al. 2001. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat. Med.* 7:575–583.
22. Failla, C.M., T. Odorisio, F. Cianfarani, C. Schietroma, P. Puddu, and G. Zambruno. 2000. Placenta growth factor is induced in human keratinocytes during wound healing. *J. Invest. Dermatol.* 115:388–395.
23. Oura, H., J. Bertoncini, P. Velasco, L.F. Brown, P. Carmeliet, and M. Detmar. 2003. A critical role of placental growth factor in the induction of inflammation and edema formation. *Blood.* 101:560–567.
24. Maes, C., L. Coenegrachts, I. Stockmans, E. Daci, A. Luttun, A. Petryk, R. Gopalakrishnan, K. Moermans, N. Smets, C.M. Verfaillie, et al. 2006. Placental growth factor mediates mesenchymal cell development, cartilage turnover, and bone remodeling during fracture repair. *J. Clin. Invest.* 116:1230–1242.
25. Perelman, N., S.K. Selvaraj, S. Batra, L.R. Luck, A. Erdreich-Epstein, T.D. Coates, V.K. Kalra, and P. Malik. 2003. Placenta growth factor activates monocytes and correlates with sickle cell disease severity. *Blood.* 102:1506–1514.
26. Fischer, C., B. Jonckx, M. Mazzone, S. Zacchigna, S. Loges, L. Pattarini, E. Chorianopoulos, L. Liesenborghs, M. Koch, M. De Mol, et al. 2007. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell.* 131:463–475.
27. Khurana, R., L. Moons, S. Shafi, A. Luttun, D. Collen, J.F. Martin, P. Carmeliet, and I.C. Zachary. 2005. Placental growth factor promotes atherosclerotic intimal thickening and macrophage accumulation. *Circulation.* 111:2828–2836.
28. Monsky, W.L., D. Fukumura, T. Gohongi, M. Ancukiewcz, H.A. Weich, V.P. Torchilin, F. Yuan, and R.K. Jain. 1999. Augmentation of transvascular transport of macromolecules and nanoparticles in tumors using vascular endothelial growth factor. *Cancer Res.* 59:4129–4135.
29. Park, J.E., H.H. Chen, J. Winer, K.A. Houck, and N. Ferrara. 1994. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J. Biol. Chem.* 269:25646–25654.
30. Odorisio, T., C. Schietroma, M.L. Zaccaria, F. Cianfarani, C. Tiveron, L. Tatangelo, C.M. Failla, and G. Zambruno. 2002. Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. *J. Cell Sci.* 115:2559–2567.
31. Luttun, A., K. Brusselmans, H. Fukao, M. Tjwa, S. Ueshima, J.M. Herbert, O. Matsuo, D. Collen, P. Carmeliet, and L. Moons. 2002. Loss of placental growth factor protects mice against vascular permeability in pathological conditions. *Biochem. Biophys. Res. Commun.* 295:428–434.
32. Ito, H., N. Koide, F. Hassan, S. Islam, G. Tumurkhuu, I. Mori, T. Yoshida, S. Kakumu, H. Moriwaki, and T. Yokochi. 2006. Lethal endotoxic shock using alpha-galactosylceramide sensitization as a new experimental model of septic shock. *Lab. Invest.* 86:254–261.
33. De Maio, A., M.L. Mooney, L.E. Matesic, C.N. Paidas, and R.H. Reeves. 1998. Genetic component in the inflammatory response induced by bacterial lipopolysaccharide. *Shock.* 10:319–323.
34. Trentzsch, H., D. Stewart, and A. De Maio. 2003. Genetic background conditions the effect of sex steroids on the inflammatory response during endotoxic shock. *Crit. Care Med.* 31:232–236.
35. Luttun, A., M. Autiero, M. Tjwa, and P. Carmeliet. 2004. Genetic dissection of tumor angiogenesis: are PlGF and VEGFR-1 novel anticancer targets? *Biochim. Biophys. Acta.* 1654:79–94.
36. Hiratsuka, S., O. Minowa, J. Kuno, T. Noda, and M. Shibuya. 1998. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl. Acad. Sci. USA.* 95:9349–9354.
37. Autiero, M., J. Waltenberger, D. Communi, A. Kranz, L. Moons, D. Lambrechts, J. Kroll, S. Plaisance, M. De Mol, F. Bono, et al. 2003. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat. Med.* 9:936–943.
38. Bottomley, M.J., N.J. Webb, C.J. Watson, L. Holt, M. Bukhari, J. Denton, A.J. Freemont, and P.E. Brenchley. 2000. Placenta growth factor (PlGF) induces vascular endothelial growth factor (VEGF) secretion from mononuclear cells and is co-expressed with VEGF in synovial fluid. *Clin. Exp. Immunol.* 119:182–188.
39. Luttun, A., M. Tjwa, L. Moons, Y. Wu, A. Angelillo-Scherrer, F. Liao, J.A. Nagy, A. Hooper, J. Priller, B. De Klerck, et al. 2002. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat. Med.* 8:831–840.
40. Xu, L., D.M. Cochran, R.T. Tong, F. Winkler, S. Kashiwagi, R.K. Jain, and D. Fukumura. 2006. Placenta growth factor overexpression inhibits tumor growth, angiogenesis, and metastasis by depleting vascular endothelial growth factor homodimers in orthotopic mouse models. *Cancer Res.* 66:3971–3977.
41. Eriksson, A., R. Cao, R. Pawliuk, S.M. Berg, M. Tsang, D. Zhou, C. Fleet, K. Tritsaris, S. Dissing, P. Leboulch, and Y. Cao. 2002. Placenta growth factor-1 antagonizes VEGF-induced angiogenesis and tumor growth by the formation of functionally inactive PlGF-1/VEGF heterodimers. *Cancer Cell.* 1:99–108.
42. Reinmuth, N., W. Liu, Y.D. Jung, S.A. Ahmad, R.M. Shaheen, F. Fan, C.D. Bucana, G. McMahon, G.E. Gallick, and L.M. Ellis. 2001. Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival. *FASEB J.* 15:1239–1241.
43. Gigante, B., G. Morlino, M.T. Gentile, M.G. Persico, and S. De Falco. 2006. Plgf−/−eNos−/− mice show defective angiogenesis associated with increased oxidative stress in response to tissue ischemia. *FASEB J.* 20:970–972.
44. Berger, U.V., and M.A. Hediger. 2001. Differential distribution of the glutamate transporters GLT-1 and GLAST in tanycytes of the third ventricle. *J. Comp. Neurol.* 433:101–114.