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PIGF enhances TLR-dependent inflammatory responses in human mononuclear phagocytes

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Abstract

Problem—Levels of placental growth factor (PIGF) peak during third trimester of pregnancy, a time when women are at increased risk of virus-induced morbidity. We hypothesized PIGF might contribute to an exaggerated inflammatory response to toll-like receptor (TLR)-activation.

Method of Study—Primary human adult and cord blood CD14⁺ cells were cultured in the presence of TLR-ligands and/or PIGF.

Results—PIGF significantly enhanced the magnitude and duration of TNF mRNA and protein production by TLR-7/8 activated monocytes, and increased subsequent production of TNF-independent inflammatory cytokines. This PIGF/TLR effect involved multiple inflammatory cytokines/chemokines and was seen with the majority of TLR-agonists. PIGF enhanced phosphorylation of IkappaB kinase (IKK) in monocytes stimulated with the TLR-7/8 agonist R848, and IKK inhibition completely suppressed the PIGF effect.

Conclusion—PIGF enhances TLR-signaling upstream of IKK and contributes to an exaggerated pathologic pro-inflammatory state in response to activation of maternal and fetal mononuclear phagocytes by specific TLR-agonists.

Keywords

Innate immunity; mononuclear phagocytes; placental growth factor; pregnancy; toll-like receptors; viral infections

Introduction

During pregnancy, the maternal immune system balances the development of tolerance to foreign (paternal) antigens with maintaining immune surveillance to pathogenic stimuli. However, pregnant women are often at an increased risk of infectious complications leading to potentially devastating effects on both maternal and fetal morbidity and mortality.^{1,2} For example, during the 2009 H1N1 influenza pandemic, pregnancy was associated with an 8-fold increased risk of death.³ While bacterial complications are known to occur in the setting of severe influenza infections, in the reported series of early deaths among pregnant women with influenza, there was a lack of evidence of secondary bacterial pneumonia and rather an increased prevalence of inflammatory complications such as acute respiratory distress syndrome (ARDS),⁴⁻⁶ suggesting that the response to the viral infection per se contributed to overall morbidity and mortality. The highest incidence of death occurred in pregnant women during their third trimester,⁷⁻⁹ a time point at which levels of a number of pregnancy-associated factors peak, including placental growth factor (PIGF),¹⁰ a member of the vascular endothelial growth factor (VEGF) family.¹¹

Responses of the innate immune system to viral (and other) infections are controlled in large part by toll-like receptor (TLR) signaling pathways in mononuclear phagocytes. The recognition of conserved microbial signatures, and endogenous molecules produced as a result of tissue injury and/or inflammation, by TLRs plays a critical role in inducing the expression of pro-inflammatory cytokine genes, thereby activating both the innate and adaptive immune responses.¹²⁻¹⁵ These processes must be tightly regulated, because without feedback mechanisms designed to suppress such responses in a timely fashion, the resulting unrestrained inflammation can result in life-threatening complications.¹⁶⁻¹⁹ Indeed, inappropriate immune responses play an important role in regulation of pregnancy outcomes.^{1,2} Evidence of enhanced activation of the innate immune system has been demonstrated in pregnancy, including the expression of monocyte phenotypic activation markers,²⁰⁻²³ and increased monocyte production of intracellular reactive oxygen species.²⁰ Others have observed increased cytokine production by mononuclear phagocytes of pregnant women.^{22,24,25} The factors contributing to viral-induced morbidity and mortality in pregnancy may include a variety of angiogenic stimuli.²⁶⁻²⁹ PIGF is one such factor. Specifically, pathologic increases in PIGF have been described in diverse clinical settings characterized by an increased risk of inflammatory complications, including the hemoglobinopathies, inflammatory arthritis, inflammatory bowel disease, and allogeneic hematopoietic stem cell transplantation.³⁰⁻³⁴ However, studies linking PIGF to tumor necrosis factor-alpha (TNF) production have reported the effect of PIGF on *TNF* transcript abundance but have not included studies on TNF protein levels.^{30,31} In light of the clear tie between TNF levels and H1N1-induced mortality,³⁵ we sought to determine whether PIGF influenced TNF gene expression under conditions that mimic viral infection of mononuclear phagocytes.

We focused largely on TLR-7/8 activation, as these specific TLRs are known to be required for the recognition of single-stranded viruses such as influenza.³⁶ Using primary mononuclear phagocytes, we determined that PIGF alone did not influence TNF secretion by normal human monocytes, and that PIGF has a minimal effect on *TNF* mRNA production on

its own, but that PIGF enhances both cytokine gene transcription and cytokine secretion induced by TLR-pathway activators in those same cells. The PIGF-enhanced TLR-7/8 response involved multiple inflammatory cytokines and chemokines, was most prominently observed with specific TLRs, and was also seen in fetal (cord blood) CD14⁺ cells. These findings support the hypothesis that PIGF enhances TLR-pathway mediated inflammatory responses in human mononuclear phagocytes, and provide a potential mechanism for viral-mediated morbidity and mortality in pregnancy.

Materials and Methods

Reagents

R848 (ALX-420-038, TLR-7/8) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). PIGF-1 was purchased from PeproTech (Rocky Hill, NJ, USA) and ProSpec (Ness-Ziona, Israel). PIGF-2, PIGF-3, and VEGF-A(151) were purchased from PeproTech. Human TLR 1-9 agonist kit [Pam3CSK4 (TLR-1/2), HKLM (TLR-2), polyinosinic-polycytidylic acid [poly(I:C)] high and low molecular weight (TLR-3), LPS (TLR-4), Flagellin (TLR-5), FSL-1 (TLR-6/2), Imiquimod (TLR-7), ssRNA40 (TLR-8), and ODN2006 (TLR-9)] was purchased from InvivoGen (San Diego, CA, USA). Recombinant human TNF RI/TNFRSF1A Fc chimera protein was obtained from R&D Systems (Minneapolis, MN, USA); the effective concentration of TNF RI/TNFRSF1A Fc chimera protein used in blocking experiments was first determined as that concentration required to abrogate exogenous TNF-mediated IL-8 protein secretion (for >1ng/ml of TNF protein). Concentrations of > 0.1 µg/ml sTNFR1 completely abrogated all TNF-mediated IL-8 production; subsequent blocking experiments were performed using 1 µg/ml sTNFR1. BIRB 796 (doramapimod) was obtained from Axon Medchem (Groningen, The Netherlands). LY2228820, PI103, CAL-101, GDC0941, GSK1120212, AZD6244, PD184352, and SCH772984 were purchased from Selleck Chemicals (Houston, TX, USA). BMS-345541 was obtained from Sigma-Aldrich. Anti-IKK-alpha, anti-IKK-beta, and anti-phospho-IKK-alpha/beta antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-GAPDH antibody was purchased from Abcam (Cambridge, MA, USA).

Primary cells

Primary human CD14⁺ cells were prepared from peripheral blood mononuclear leukocytes using Ficoll-Paque PLUS gradient separation (GE Healthcare, Piscataway, NJ, USA), and isolated using magnetic microbeads (EasySep human CD14 positive selection kit; StemCell Technologies, Vancouver, BC, Canada). For enzyme-linked immunosorbent assays (ELISAs), CD14⁺ cells were cultured at a concentration of 50,000 CD14⁺ cells/ml for 24-hours in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Thermo Scientific Hyclone, Logan, UT, USA), and in the presence of R848 3 µM and/or PIGF-1 250 ng/ml, unless otherwise specified. For mRNA, CD14⁺ cells were cultured at a concentration of 500,000 CD14⁺ cells/ml for the indicated culture times. Blood from healthy donor volunteers was collected after informed consent, in accordance with research studies approved by the Institutional Review Board at Oregon Health and Science University (OHSU). Human umbilical cord blood cells were obtained from healthy,

full-term deliveries as part of an IRB approved protocol at OHSU. CD14⁺ cord blood mononuclear cells were isolated by Ficoll-Paque PLUS gradient separation and magnetic microbeads.

Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was prepared from CD14⁺ cells at the indicated times, using the RNeasy Micro kit (Qiagen, Valencia, CA, USA) followed by DNase 1 treatment (Qiagen). Complementary DNA synthesis and quantitative real-time PCR were performed as described previously.^{37,38} Primer and probe sets for human *TNF* (Hs01113624_g1), *IL-8* (Hs00174103_m1), *IL-1 β* (Hs01555410_m1), *IL-6* (Hs00985639_m1), *MIP-1 α* (*CCL3*, Hs00234142_m1), *MIP-1 β* (*CCL4*, Hs00237011_m1), and human 18S ribosomal RNA (rRNA; 4319413E) were purchased as TaqMan Gene Expression Assays (Life Technologies). Primers and TaqMan probes for unspliced human *TNF* were designed using Primer Express v3.0 software (Thermo Fisher) and were as follows: forward primer AIN1GBD_F CTCGATGTTAACCATTCTCCTTCTC; reverse primer AIN1GBD_R GGGCCAGAGGGCTGATTAG; probe AIN1GBD_M CCAACAGTTCCCCAGGG. All were purchased from Thermo Fisher.

Quantification of mRNA decay

Actinomycin D (5 μ g/ml, Sigma-Aldrich) was added to cells pretreated for 4- or 6-hours with 3 μ M R848, with or without 250 ng/ml PIGF-1. Total RNA was prepared at regular time intervals thereafter as described. *TNF* mRNA was quantified using real-time qRT-PCR.

Immunoassays

Conditioned medium was harvested from CD14⁺ cells for TNF- α and IL-8 quantification using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions, with triplicate wells for each sample. To permit calculations of fold-changes of TNF protein for the treatment condition PIGF-1 plus TLR-agonist versus TLR-agonist alone, ELISA results with negative or zero values were set at the minimum detectable dose (MDD) of 0.5 pg/mL, per the product datasheet. Luminex multiplex cytokine arrays were performed using Cytokine Human Magnetic 30-Plex panels obtained from Life Technologies (Carlsbad, CA, USA).

Immunoblotting

Immunoblot analyses were performed as described previously.^{37,39}

Statistical analysis

Statistical analyses were performed using a 2-tailed Student *t* test. A *p* value of < 0.05 was considered statistically significant. Unless otherwise specified, data are presented as mean values, with error bars representing the standard error of the mean (SEM) of these values.

Results

PIGF-1 enhances TNF expression in TLR-7/8 activated CD14⁺ cells

We first cultured primary normal donor peripheral blood CD14⁺ cells with PIGF-1 alone, and found that PIGF-1 was not capable of inducing TNF secretion on its own. Given the critical role of the endosomal TLR-7 and TLR-8 ligands in the recognition of ssRNA viruses such as influenza,³⁶ we next cultured CD14⁺ cells with the combination of PIGF-1 and the TLR-7/8 ligand R848. CD14⁺ cells exposed to both PIGF-1 and R848 produced significantly more TNF protein than cells exposed to R848 alone (Figure 1A), with an average 2.35-fold increase in secreted TNF with the combination of PIGF-1 and R848 versus R848 treatment alone ($p < 0.01$, Supplementary Figure 1A). To better understand the mechanism of control responsible for the PIGF/TLR effect, we next quantified *TNF* mRNA in R848-stimulated CD14⁺ cells with and without exposure to PIGF-1. Similar to its effect on TNF secretion (Figure 1A), PIGF-1 significantly enhanced R848-induced *TNF* mRNA production compared to R848 alone (Figure 1B), with an average 2.46-fold increase in *TNF* mRNA with combination PIGF-1 and R848 treatment compared to R848 alone ($p < 0.01$, Supplementary Figure 1B). As expected, there was variability among donors in the responses of mononuclear phagocytes to TLR-agonists, with or without PIGF-1; however, for each individual donor, the response to R848 plus PIGF-1 was always greater than the response to R848 alone, for both TNF protein and mRNA (Supplementary Figure 1C-1D). Importantly, in each experiment, the fractional increase in TNF protein released matched the fractional increase in *TNF* mRNA.

The PIGF isoforms and VEGFR-1 ligands differentially enhance R848-mediated TNF secretion

Due to alternative mRNA splicing, different human PIGF isoforms have been identified which vary in size, heparin-binding affinity, VEGF receptor binding, and tissue expression patterns.⁴⁰⁻⁴² We cultured primary CD14⁺ cells with the PIGF isoforms, in the presence and absence of R848, in order to determine the effect of the isoforms on TLR-7/8 activation. None of the isoforms alone were capable of inducing TNF secretion; however, in contrast to PIGF-1 (Figure 1A, Supplementary Figure 1A), in TLR-7/8 activated cells neither PIGF-2 nor PIGF-3 had any enhancing effect on TNF protein production relative to R848 alone (Supplementary Figure 2A). Thus, the PIGF-1 isoform selectively enhances TLR-7/8 induced TNF secretion.

PIGF-1 is known to bind and activate the VEGF receptor-1 (VEGFR-1),⁴³ and in contrast to VEGFR-2, VEGFR-1 is expressed on the cell surface of monocytes.^{44,45} As VEGF-A is also reported to activate VEGFR-1,⁴³ we tested the ability of VEGF-A to enhance R848-mediated TNF secretion in primary CD14⁺ cells. There was no effect of VEGF-A on R848-mediated TNF secretion (Supplementary Figure 2B). Therefore, there is differential enhancement of R848-mediated TNF production by the VEGFR-1 ligands.

PIGF-1 does not influence responsiveness to all TLR ligands

We next determined whether PIGF-1 could also enhance the production of TNF mediated by other TLR agonists, and whether this 'PIGF/TLR' effect was specific to the endosomally

located TLRs (TLR-7, -8, -9). CD14⁺ primary cells were cultured with PIGF-1 and the human TLR ligands Pam3CSK4 (TLR-1/2), HKLM (TLR-2), poly(I:C) high and low molecular weight (TLR-3), LPS (TLR-4), Flagellin (TLR-5), FSL-1 (TLR-6/2), Imiquimod (TLR-7), ssRNA40 (TLR-8), and ODN2006 (TLR-9)], Figure 2. While LPS (TLR-4) alone was able to induce TNF secretion by CD14⁺ cells, there was no enhancement of LPS-induced TNF production in the presence of PIGF-1. Pam3CSK4 (TLR-1/2), Flagellin (TLR-5), FSL-1 (TLR-6/2), and ssRNA40 (TLR-8) induced TNF production in a dose-dependent manner, which was further enhanced by PIGF-1. In contrast, HKLM (TLR-2), Imiquimod (TLR-7), and ODN2006 (TLR-9) were alone unable to induce TNF secretion, but detectable TNF was produced in the presence of PIGF-1. We did not quantify any secreted TNF in response to low or high molecular weight poly(I:C) (TLR-3), with or without PIGF-1. While the combination of PIGF-1 and R848 led to the highest absolute levels of TNF protein (Figure 2), the fold change dimension was greatest in CD14⁺ primary cells treated with PIGF-1 and the TLR-2 ligand (HKLM) (Supplementary Figure 3). Thus, PIGF-1 modulates TLR-responsiveness to both endosomal and cell surface TLR ligands, but does not influence the responsiveness of all TLR ligands.

PIGF-1 enhances TNF production by cord blood CD14⁺ cells in response to TLR-7/8 but not TLR-4 agonists

In addition to the increased risk of maternal morbidity, the 2009 H1N1 pandemic was also associated with an increased risk of fetal morbidity and mortality.^{8,46} Consequently, we sought to determine whether PIGF-1 could also modulate the TLR-responsiveness of fetal monocytes, using cord blood CD14⁺ cells obtained from term deliveries as a surrogate, and focused on TLR-7/8 and TLR-4 ligands given the differential responses to these ligands seen in adult CD14⁺ cells. While absolute levels of TNF protein secreted by cord blood mononuclear phagocytes were reduced, compared to adult CD14⁺ cells (Supplementary Figure 4), cord blood CD14⁺ cells exposed to PIGF-1 and R848 produced significantly more TNF than they did after exposure to R848 alone (Figure 3). Additionally, as was the case with adult monocytes, there was either no or minimal enhancement in LPS-induced TNF production by PIGF-1 in cord blood CD14⁺ cells.

PIGF-1 enhances the magnitude and duration of TLR-7/8 mediated TNF gene expression

Seeking to determine molecular mechanisms involved in the PIGF effect, we next sought to define the differential kinetics of the induced TNF response, and to identify the degree to which transcriptional and post-transcriptional mechanisms were involved. In kinetic studies of primary cells treated with PIGF-1 and/or R848, we determined that PIGF-1 enhances both the magnitude and duration of the TNF response, with equivalent increases in unspliced (Figure 4A) and spliced (Figure 4B) *TNF* mRNA, and TNF protein (Figure 4C). PIGF-1 also prolonged the duration of TNF gene expression compared to CD14⁺ cells treated with R848 alone (Supplementary Figure 5A-B). While this increase in total *TNF* mRNA could reflect enhanced transcription or mRNA stability, the increase in unspliced pre-*TNF* mRNA supports our hypothesis that the PIGF effect is due to increased gene transcription. To ensure that PIGF-1 did not function to stabilize *TNF* mRNA, we performed actinomycin D chase experiments. Primary cells were treated with PIGF-1 in combination with R848, or in some experiments the addition of PIGF-1 and R848 was staggered so that one factor was added 2

hours before the other, followed by actinomycin D treatment. The results of these experiments were very similar regardless of the order of addition of PlGF-1 and R848. PlGF-1 did not increase the *TNF* mRNA half-life (Figure 4D), compatible with a transcriptional effect of PlGF-1. These results demonstrate that the PlGF-1 pathway increases *TNF* mRNA transcriptionally.

PlGF-1 enhances TLR-7/8 mediated inflammatory cytokine gene expression in primary CD14⁺ cells

To identify other inflammatory cytokines influenced by the PlGF/TLR effect, we performed Luminex multiplex cytokine arrays of conditioned culture media generated from CD14⁺ cells cultured for 6 and 24-hours with PlGF-1 and R848. In two separate experiments, PlGF-1 enhanced R848-induced protein levels of pro-inflammatory cytokines including TNF, interleukin (IL)-6, IL-8, IL-1 β , macrophage inflammatory protein (MIP)-1 α , MIP-1 β , monocyte chemoattractant protein (MCP)-1, interferon- α , granulocyte-colony stimulating factor (G-CSF), and monocyte interferon gamma inducing factor (MIG) (Figure 5A-5B). While TNF protein levels peaked after 6-hours of exposure to R848 with and without PlGF-1, protein levels of cytokines including IL-8, IL-6, MIP-1 α , MIP-1 β , and MCP-1 continued to increase in a '2nd wave' at 24-hours compared to 6-hours (Figure 5C). We confirmed these results at the transcript level, and determined that R848-induced expression of *IL-8* (average 1.51-fold, 4 independent experiments), *IL-1 β* (average 1.31-fold, 3 independent experiments), *IL-6* (average 1.70-fold, 2 independent experiments), *MIP-1 α* (average 1.25-fold, 2 independent experiments), and *MIP-1 β* (average 1.58-fold, 2 independent experiments, $p=0.0969$ for second replicate) mRNA was also significantly enhanced by PlGF-1 (Figure 5D, 5E). These results provide evidence that the PlGF/TLR interaction enhances the production of a broad array of inflammatory cytokines, and that this 2nd wave of inflammatory cytokines continues to increase at 24-hours.

Enhancement of TLR-7/8 mediated inflammatory cytokine production by PlGF-1 is independent of autocrine TNF production

Given the known role of TNF as a 'master-regulator' of inflammatory cytokine production,⁴⁷ we tested the idea that the production of this '2nd wave' of inflammatory cytokines (all of which can be induced by TNF) was, in fact dependent upon autocrine TNF activity. CD14⁺ cells were cultured with R848 and/or PlGF-1, in the presence and absence of soluble TNF receptor 1 (sTNFR1), to inhibit the interaction of free TNF with its membrane-bound receptor. Complete inhibition of signal transduction through the TNF receptor did not abrogate the effect of PlGF-1 on R848-mediated production of other inflammatory cytokines (e.g. IL-8, IL-1 β) (Figure 6A-6B, Supplementary Figure 6A-6B). Thus the second wave of cytokine production is independent of autocrine TNF.

PlGF-1 enhances TLR-pathway receptivity independently of p38 MAPK, PI3K, MEK-1/2, and ERK-1/2 signaling

Prior studies of PlGF-mediated chemokine expression (independent of TLR-pathway activation) demonstrated that chemokine levels were dependent upon activation of phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase kinase (MEK)-1/2, and extracellular signal-related kinase (ERK)-1/2.³¹ Regulation of TNF expression is known

to be dependent on the activity of the p38 mitogen-activated protein kinase (MAPK) pathway.^{38,48} We thus tested whether the PlGF/TLR effect could be abrogated by inhibitors of PI3K, MEK-1/2, and/or ERK-1/2. TNF gene expression was not inhibited by small molecule inhibitors of PI3K, MEK-1/2, or ERK-1/2 (Supplementary Figure 7A-7C). While the p38 MAPK inhibitors (BIRB 796, LY2228820) markedly suppressed R848-induced TNF secretion (Figure 7A, Supplementary Figure 8A), they did not suppress the effect of PlGF-1 on R848-induced *TNF* gene transcription (Figure 7B, Supplementary Figure 8B).

Activation of IKK-alpha/beta is required for the PlGF/TLR effect

As TLR pathways activate the IkappaB kinase (IKK) complex, which controls the nuclear localization and activation of nuclear factor-kappaB (NF- κ B),^{49,50} we reasoned that PlGF-1 might enhance the activation state of that complex, in which case inhibition of IKK might fully abrogate the effect of PlGF-1 on TLR-agonist induced TNF expression. Treatment of primary cells with an IKK inhibitor (BMS-345541; 0.5, 1.5, 5 μ M) did, indeed, inhibit the enhancing effect of PlGF-1 on R848-induced TNF protein (Figure 8A, Supplementary Figure 9A) and mRNA production (Figure 8B, Supplementary Figure 9B). Immunoblots of whole cell lysates of CD14⁺ cells cultured for 6-hours with R848 and PlGF-1 demonstrated a marked increase in phosphorylation of IKK (Figure 8C). Moreover, inhibition of IKK with BMS-345541 attenuated the phosphorylation of IKK-alpha/beta (Figure 8D). Therefore, we hypothesize that PlGF-1 enhances the receptivity of the canonical TLR-pathway resulting in hyper-activation of IKK.

Discussion

Elevated circulating levels of PlGF are associated with normal pregnancies, but have also been described in other clinical settings including sickle cell disease, sickle/beta-thalassemia, allogeneic stem cell transplantation, and the inflammatory arthropathies.^{30-34,51,52} These disparate conditions do share in common a higher than normal risk of morbidity from viral-mediated inflammatory infections. PlGF is highly expressed in the human placenta throughout gestation, and may serve two key functions: controlling the growth and differentiation of trophoblasts and serving as a paracrine factor for placental angiogenesis.⁵³⁻⁵⁵ While some studies suggest that PlGF is not required for normal vascular development (knockout mice exhibit normal development and viability without an aberrant phenotype),⁵⁶ it does seem to be required to execute a normal angiogenic response during pathologic conditions such as ischemia.^{56,57} In addition, PlGF is also known to stimulate the growth, migration, and survival of endothelial cells,^{56,58} and is a chemoattractant for macrophages.^{59,60}

Pathologically low PlGF levels have been shown to associate with, and predict for, the development of preeclampsia.^{10,61,62} While pre-eclampsia has also been described as an inflammatory syndrome, including evidence for aberrant activation of mononuclear phagocytes, the exact mechanisms responsible for this pathologically enhanced state of innate immune activation remain unclear.²⁶ And while specific infections have been linked with an increased potential risk for pre-eclampsia, this risk does not apply to all infectious stimuli.⁶³ Consequently, whether pre-eclampsia is directly linked to infections and TLR-

pathway activation is also unknown. Here we have determined that PlGF contributes to the activation state of primary human mononuclear phagocytes but that PlGF's effect requires the simultaneous activation of specific TLR-pathways. Because PlGF is produced by a variety of non-placental cells, we speculate that this model may also pertain to disease states such as inflammatory arthritis, hemoglobinopathies, and allogeneic hematopoietic stem cell transplantation in which PlGF levels are known to increase, and may account at least in part to the known increased risks of infectious complications seen in these patients.

Angiogenic stimuli and inflammation

Increasing evidence supports a functional link between angiogenic stimuli and inflammatory responses, via the production of chemokines, cytokines, and growth factors, and particularly occurring in pathologic conditions.^{28,29,64,65} One of these factors, PlGF, is known to enhance IL-1 β , IL-8, and MCP-1 transcript numbers in peripheral blood mononuclear cells (PBMCs) of healthy subjects; these PlGF-inducible genes were also expressed at high levels in PBMCs from subjects with sickle cell disease.^{30,31} However, these studies only reported effects of PlGF on *TNF* mRNA, not TNF protein. And while PlGF has also been shown to stimulate the production of TNF and IL-6 protein from rheumatoid arthritis patient-derived synovial fluid mononuclear cells, normal healthy donor PBMCs responded weakly to PlGF,³² in agreement with our findings. Specifically, we find that the effect of PlGF on cytokine production by monocytes from normal peripheral blood is trivial. Instead, PlGF influences cytokine production in a major way *only* in TLR-pathway activated monocytes. PlGF did not stabilize *TNF* mRNA but did enhance the amount of both spliced and unspliced *TNF* mRNA. Moreover, the fractional increase in protein production matched the increase in transcripts, confirming that the control point for the PlGF effect was largely transcriptional. That PlGF did not stabilize *TNF* mRNA is congruent with prior work by Selvaraj et al.,³¹ though their studies were done in the context of PlGF alone, and not in combination with activators of the TLR pathway.

TLR-pathways influenced by PlGF

The innate immune response involves a diversity of TLR receptors and pathogen-associated molecular patterns (PAMPs) recognized by these receptors. We determined that PlGF enhanced TLR-mediated TNF production, or induced TNF production in combination with TLR-agonists (where there was no TNF production with TLR-agonists alone), for the majority of human TLR receptors. We found the highest levels of secreted TNF in response to PlGF-1 and TLR-7/8 stimulation of mononuclear phagocytes. However, while the absolute TNF values were greatest with TLR-7/8, we found the greatest fold change in TNF protein, comparing PlGF-1 with TLR-agonist versus TLR-agonist alone, in response to TLR-2 stimulation. The TLR-2 receptor recognizes a wide variety of ligands, including cell-wall components, lipoproteins and lipopeptides of gram-positive bacterial and mycobacterial origin, and yeast zymosan.⁶⁶ It can bind to these PAMPs in concert with TLR-1 or TLR-6. Interestingly, while there was also evidence of a PlGF effect with the TLR-1/2 agonist (PAM3cSK4) and the TLR-6/2 agonist (FSL-1), the greatest TNF response was seen with the TLR-2 agonist HKLM. Whether this differential TLR-2 effect is due to specific properties of the ligand is unknown. For example, the more robust response to the gram-positive bacterium *Listeria monocytogenes* (HKLM), than to the diacylated (FSL-1)⁶⁷ or

triacylated (PAM3cSK4)⁶⁸ lipopeptides, might be the result of PIGF's capacity to influence the structure or function of TLR-2. Alternatively, PIGF might influence the interaction of TLR-2 with cooperative factors (e.g. TLR-1 or TLR-6).

We also observed that neither of the specific TLR-7 or TLR-8 ligands were as potent as the TLR-7/8 ligand R848. We hypothesize this may be due to synergistic activity of both TLRs, because when Imiquimod (TLR-7) was combined with ssRNA40 (TLR-8), there was a dose-dependent enhancement in TNF production by CD14⁺ cells (not shown). A variety of differential responses have been described in antigen presenting cells stimulated with more than one TLR-ligand. They include synergy, cross-tolerance, and even suppression of cytokine production, depending on the immune cell activated, the TLR-ligand and concentration tested, as well as the duration of TLR-stimulation and the quantified cytokine of interest.^{69,70} It remains to be determined whether PIGF influences TLR cooperativity or differentially influences the binding affinity of select PAMPs to their specific TLRs, but our results do suggest that the PIGF/TLR effect involves multiple ligands of the TLR-pathway.

Notable exceptions to this PIGF/TLR effect were TLR-3 [poly(I:C)] and TLR-4 (LPS). That is, PIGF did not enhance poly(I:C) or LPS-induced TNF secretion. While we saw no TNF production by monocytes treated with poly(I:C) with/without PIGF, this observation may be an artifact reflecting our method of using freshly isolated CD14⁺ peripheral blood cells, treated directly with poly(I:C) without transfection. Others have reported an effect in monocytes when poly(I:C) was transfected into the cells in culture,⁷¹ or with poly(I:C) treatment of cells first cultured in vitro and then selected based on adherence.^{72,73} Thus, our failure to observe a PIGF effect for TLR-3 may be simply due to a lack of sufficiently robust TLR-3 agonism.

TLR-4 activation is not influenced by PIGF

Our finding that PIGF does not enhance TLR-4 activation is more certain, and intriguing. We believe that this observation will aid in future studies seeking to identify specific signaling pathways that permit PIGF to enhance inflammatory cytokine production. For example, LPS is known to activate both canonical MyD88/IRAK pathways and non-canonical (MyD88-independent) pathways.⁷⁴ One possibility is that the non-canonical pathway (activated by LPS but not by R848) might function to suppress the signaling pathway by which PIGF enhances canonical TLR-signaling. Conversely, PIGF may heighten the activation state of molecules typically functioning to constrain TLR-4 responses (e.g. SOCS1, IRAK-M, ST2).⁷⁴ Whether PIGF is activating non-canonical or alternate inflammatory signaling pathways, or instead functioning to inhibit the inhibitors of TLR-mediated inflammatory responses, remains to be determined. Nonetheless, taken together our results suggest that the effect of PIGF on priming of the innate immune system may be broader than we had initially anticipated (e.g. beyond TLR-7/8), and may involve maternal immune responses to diverse infectious stimuli.

PIGF isoforms and VEGFR-1 ligands

We also determined that of the different VEGFR-1 ligands, only the PIGF-1 isoform was able to enhance TLR-mediated TNF expression, although the mechanism responsible for

this isoform specificity is unclear. In pregnancy, expression of the PIGF isoform transcripts has been demonstrated in normal human trophoblasts and umbilical vein endothelial cells.⁷⁵ While there are few studies focused on circulating levels of the different PIGF isoforms, maternal levels of serum PIGF-1 and PIGF-2 are highly correlated. That is, both increase with gestational age and have similar predictive value for late pre-eclampsia and delivery of small for gestational age offspring.⁷⁶ Similarly, in conditions characterized by aberrant expression of PIGF, such as rheumatoid arthritis, both the PIGF-1 and PIGF-2 isoforms increased survival, migration, and invasiveness of rheumatoid fibroblast-like synoviocytes,⁷⁷ though differences in PIGF-1 and PIGF-2 expression have been described in the setting of malignancy (e.g. breast cancer).⁷⁸

The role of the VEGFR-1 ligand VEGF-A in inflammatory events is less clear. In pregnancy, while circulating VEGF-A levels normally increase,⁷⁹ maternal VEGF levels are decreased in pre-eclampsia.⁸⁰ We found no evidence of an enhancing effect of VEGF-A on TLR-7/8-mediated TNF production; instead, we found higher levels of VEGF-A may contribute to decreased TNF secretion in TLR-7/8-stimulated monocytes. Whether the comparative differential activities we describe result from differences in receptor-binding affinity remains to be determined.

PIGF influences multiple cytokines and chemokines

PIGF influences TLR-agonist dependent production of a broad array of inflammatory cytokines, including the expression of IL-1 β , IL-6, IL-8, MIP-1 alpha, MIP-1 beta, and MCP-1, at both the mRNA and protein levels. Interestingly, a major effect of PIGF involved the duration of the production of these pro-inflammatory cytokines, which in contrast to TNF, continued to increase at 24-hours, suggesting that PIGF enhances the production, as well as prolongs the duration of activation, of numerous inflammatory cytokines. It was reasonable to consider that TNF might trigger an autocrine pathway responsible for the prolonged and durable production of inflammatory cytokines under these conditions, we found that it did not. That is, blocking TNF signaling did not alter the production of other inflammatory cytokines (e.g. IL-8, IL-1 β). These results suggest a broader effect of PIGF on inflammatory cytokine production, including an early enhancement of TNF production, and a subsequent, more durable TNF-independent enhancement of inflammatory cytokine expression.

PIGF/TLR signaling

Studies on PIGF signaling pathways have been reported in sickle cell disease,^{30,31,52} wherein the effects of PIGF (as a single agent) were shown to be dependent on activation of PI3K, MEK-1/2, and ERK-1/2.³¹ When we combined PIGF with TLR-agonists, TNF gene expression was not inhibited by small molecule inhibitors of PI3K, MEK-1/2, or ERK-1/2, suggesting that PIGF signaling is differentially controlled in the context of TLR-pathway activation. While p38 MAPK inhibitors partially suppressed PIGF-enhanced R848-mediated TNF secretion, there was no impact of p38 MAPK inhibition on the enhancing effect of PIGF on R848-induced *TNF* transcription, compatible with prior work, including our own,³⁸ indicating that the effect of p38 MAPK on TNF secretion was regulated post-transcriptionally. It is also possible that the p38 MAPK inhibitors may influence membrane-

bound TNF release. After translation, human TNF is first expressed as a membrane-bound precursor protein, followed by proteolytic cleavage by a metalloprotease TNF- α -converting enzyme (TACE) resulting in the release of soluble TNF.⁴⁷ There is evidence that TACE activation might be at least in part dependent on the p38 MAPK.⁸¹ Whether the p38 MAPK pathway modulates post-transcriptional control of TNF expression, or influences membrane-bound TNF release, is unclear, but p38 MAPK activation on its own is clearly not sufficient to explain the PIGF/TLR effect on TNF gene expression.

TLR-pathway activation results in downstream activation of the IKK complex,^{49,50} so it was reasonable to quantify the activation state and function of that complex. We determined not only that PIGF markedly enhanced R848-induced phosphorylation of IKK but also that treatment of primary cells with an IKK inhibitor (BMS-345541) suppressed the enhancing effect of PIGF on R848-induced *TNF* mRNA expression and TNF protein production. While the lack of a PIGF effect with IKK inhibition could be solely because the TLR effect was blocked, it is also possible that IKK inhibition affected the TNF-enhancing effect of PIGF itself. To address this, we performed immunoblotting experiments of primary cells treated with and without R848, PIGF-1, and the IKK inhibitor BMS-345541. We found that the combination of R848 with PIGF-1 synergistically enhanced IKK activation. Moreover, the IKK inhibitor also attenuated the enhancing effect of PIGF on R848-mediated phosphorylation of IKK- α /beta. Consequently, our results suggest that the PIGF effect itself requires its enhancement of IKK activation.

PIGF Responsiveness of Cord Blood Monocytes

In this study, we focused on adult peripheral blood CD14⁺ mononuclear phagocytes as a model of the innate immune response. Whether PIGF also modulates immune responses in diverse tissue types, including other cells of both the innate and adaptive immune system, as well as placental tissue and immune cells, remains to be determined and is the focus of ongoing investigation. In addition, the effect of endogenous PIGF exposure *in vivo* on the TLR-responsiveness of maternal and fetal mononuclear phagocytes is not yet known. An effect of PIGF on TLR responses might contribute to the enhanced immune activation previously reported in pregnancy.^{20–23} In our assays of cord blood mononuclear phagocytes obtained from full-term deliveries, we did find evidence of an (exogenous) PIGF effect in enhancing TLR-7/8 stimulated TNF production. If the term cord blood monocytes had been fully primed by endogenous PIGF exposure, we would have expected to see brisk responses to TLR-agonists alone, and less responsiveness to the addition of exogenous PIGF. However, we are aware that our CD14 cell selection process may have artifactually diminished the effect of endogenous PIGF priming, and also recognize that maternal levels of PIGF peak at approximately 30 weeks of pregnancy and decline thereafter,¹⁰ and so potentially any ‘priming’ effect may also have been diminished by the declining maternal PIGF levels. Therefore, whether PIGF contributes to a priming effect in pregnancy is not a settled issue.

In partial agreement with the findings of Levy et al.⁸² and Marchant et al.,⁸³ we found decreased absolute TNF protein levels produced by cord blood mononuclear phagocytes, compared to adult monocytes, at all dose levels of R848 and LPS tested, with or without PIGF. Despite these differences in total TNF secretion, in our assays we did identify

evidence of a PIGF effect. That is PIGF enhanced TLR-7/8 but not TLR-4 mediated TNF production in cord blood monocytes, similar our findings in adult mononuclear phagocytes.

During the H1N1 pandemic, not only was pregnancy associated with an increased risk of death, but mortality among H1N1 infected pregnant women was associated with an increased prevalence of inflammatory complications (e.g. ARDS).⁴⁻⁶ Work of Cardenas et al.⁸⁴ has demonstrated that viral infection of the placenta induces inflammatory responses in the fetus, without fetal transmission of the virus. Additionally, such viral infections can also lead to sensitization to bacterial products, implicating the viral-induced activation of the immune response in a process affecting both the mother and fetus. Thus, it is reasonable to speculate that for those women infected with H1N1 who did also develop concurrent bacterial infections, the activation state imposed by the viral infection itself would be an important element in the clinical outcome. We therefore hypothesize that the PIGF/TLR effect may contribute to an exaggerated pathologic inflammatory response to infectious stimuli in both maternal and fetal cells, accounting for both maternal and fetal morbidity in women challenged with specific viral infections during pregnancy (e.g. influenza).

Conclusions

Taken together, the results of our studies indicate that PIGF, levels of which peak during the third trimester of pregnancy and precisely the time at which pregnant women are particularly susceptible to viral-mediated morbidity, may contribute directly to an exaggerated pathologic pro-inflammatory state in response to activation of maternal and fetal mononuclear phagocytes by specific TLR-agonists. If our prospective *in vivo* studies currently underway confirm that PIGF effects can be identified in the monocytes of pregnant women, we would argue that mononuclear phagocytes and other auxiliary cells influenced by PIGF in this context likely contribute to maternal and fetal morbidity in the setting of infectious complications. How this pathway contributes to infectious processes *in vivo* (e.g. influenza, Herpes simplex, and Zika) is unknown and worthy of investigation. Preclinical models should be developed with the goal of identifying inhibitors of key PIGF pathways. Small molecules validated in this way may have a therapeutic role in the setting of pregnant women critically ill with viral infections. Naturally, in light of the known association between low PIGF levels and pre-eclampsia, careful attention would have to be paid to the potential risks of lowering PIGF activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
G-CSF	Granulocyte-colony stimulating factor
IKK	IkappaB kinase
IL	Interleukin
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MIG	Monocyte interferon gamma inducing factor
MIP	Macrophage inflammatory protein
NF-κB	Nuclear factor-kappaB
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PI3K	Phosphatidylinositol 3-kinase
PIGF	Placental growth factor
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
sTNFR1	Soluble TNF receptor 1
TLR	Toll-like receptor
TNF	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1
VEGFR-2	Vascular endothelial growth factor receptor-2

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Figure 1. PIGF-1 enhances TNF expression in TLR-7/8 activated CD14⁺ cells

(A) For ELISAs, CD14⁺ primary cells were plated at a concentration of 50,000/mL, and treated with 250 ng/ml PIGF-1, 3 μ M R848, or the combination for 24-hours. Secreted TNF was measured in the conditioned media by ELISA. Shown are the pooled data from 26 experiments, as the mean $\pm$ SEM. (B) For qRT-PCR, CD14⁺ primary cells were cultured at 500,000/mL with 250 ng/ml PIGF-1, 3 μ M R848, or the combination, followed by RNA isolation after 4–6 hours of culture. *TNF* mRNA was then measured by real-time qRT-PCR and normalized to 18S rRNA. Shown are the pooled data from 12 separate experiments, expressed as the mean *TNF* fold change (compared to untreated cells) $\pm$ SEM.

Figure 2. PIGF-1 does not influence responsiveness to all TLR ligands

CD14⁺ primary cells plated at 50,000/mL were cultured for 24-hours in the presence of the human TLR-agonists with or without 250 ng/ml PIGF-1. TNF secretion was quantified in the conditioned media by ELISA. Shown are the mean $\pm$ SEM, for 3 biologic replicates. * Denotes $p < 0.05$.

Figure 3. PIGF-1 enhances TNF production by cord blood CD14⁺ cells in response to TLR-7/8 but not TLR-4 agonists

CD14⁺ cells were isolated from cord blood mononuclear cells, and cultured for 24-hours at 50,000/mL with R848 (1, 3, 10, 30 μ M) or LPS (0.1, 1, 10 ng/ml), with and without PIGF-1 (250 and 500 ng/ml). TNF secretion in conditioned culture media was quantified by ELISA. Shown is the mean fold change $\pm$ SEM in TNF protein relative to R848 or LPS, as specified in the figure, for 3 biologic replicates. * Denotes $p < 0.05$.

Figure 4. PIGF-1 enhances the magnitude and duration of TLR-7/8 mediated TNF gene expression

CD14⁺ primary cells plated at a concentration of 500,000/mL were cultured with 250 ng/ml PIGF-1, 3 μ M R848, or the combination. Total RNA was isolated at time 0, 2, 4, and 6-hours after treatment. (A) *Pre-TNF* (unspliced) mRNA and (B) *TNF* mRNA were measured using real-time qRT-PCR and normalized to levels of 18S rRNA. (C) 50,000 CD14⁺ primary cells/mL were treated with 3 μ M R848 and/or 250 ng/ml PIGF-1 for 2, 4, 6, and 24-hours. Secreted TNF in the conditioned media was measured by ELISA. (D) For actinomycin D experiments, 500,000 CD14⁺ cells/mL were cultured with 250 ng/ml PIGF-1, 3 μ M R848, or

the combination for 4 hours. In some experiments, addition of PIGF-1 and R848 was staggered so that one factor was added 2 hours before the other. After addition of the second factor, cultures were incubated an additional 2 hours. 5 µg/mL actinomycin D was then added to the cultures for the times indicated. Total RNA was prepared at 1, 2, and 4-hours after actinomycin D addition, and *TNF* mRNA was quantified using real-time qRT-PCR to determine *TNF*t_{1/2}. Results shown are representative of at least 3 separate experiments, expressed as the mean ± SEM. * Denotes p <0.05.

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Figure 5. PlGF enhances TLR-7/8 mediated inflammatory cytokine production in CD14⁺ primary cells

CD14⁺ cells plated at 50,000/mL were cultured in the presence of 3 μ M R848, 250 ng/ml PlGF-1, or the combination. For protein quantification, cells were cultured for 6-hours or 24-hours, and conditioned culture media was analyzed for inflammatory cytokine production by Luminex multiplex arrays. Results shown as the mean $\pm$ SEM for a representative experiment (A, C), and as the mean fold change $\pm$ SEM in TNF protein for R848 plus PlGF-1 relative to R848 alone, for all replicate experiments (B), n=2 biologic replicates. (D, E) For mRNA, cells were plated at 500,000 CD14⁺ cells/mL and cultured for 4-hours followed by RNA isolation. Cytokine transcript levels were measured using real-time qRT-PCR and normalized to levels of 18S rRNA. Shown is the mean $\pm$ SEM of a representative experiment (D), as well as the mean fold change $\pm$ SEM in mRNA for R848 plus PlGF-1 relative to R848 alone (E), for *IL-8* mRNA (4 biologic replicates), *IL-1 β* (3 biologic replicates), and *IL-6*, *MIP-1 alpha*, and *MIP-1 beta* mRNA (2 biologic replicates for each).

Figure 6. Enhancement of TLR-7/8 mediated inflammatory cytokine production by PIGF is independent of autocrine TNF production

500,000 CD14⁺ cells/mL were pre-treated for 1-hour with sTNFR1 0.1 or 1 µg/ml, followed by 4-hours of culture with 3 µM R848 +/- 250 ng/ml PIGF-1. Total RNA was isolated at time 0 and after 5-hours total culture. *IL-8* (A) and *IL-1β* (B) mRNA was measured using real-time qRT-PCR and normalized to levels of 18S rRNA. Shown is the mean ± SEM, representative of 2 replicate experiments.

Figure 7. PI3F-1 enhances TLR-pathway receptivity independently of p38 MAPK signaling
 CD14⁺ cells were cultured with 3 μ M R848 $\pm$ 250 ng/ml PI3F-1, with or without 500 nM BIRB 796 (BIRB) or LY2228820 (LY). (A) For ELISA, cells were cultured for 24-hours at 50,000/ml and secreted TNF was measured in the conditioned culture media. Shown is the mean $\pm$ SEM, representative of 3 independent experiments. (B) For RNA, 500,000 CD14⁺ cells/mL were cultured for 6-hours as above, followed by RNA isolation. *TNF* mRNA was measured using real-time RT-PCR and normalized to levels of 18S rRNA. Shown is the mean $\pm$ SEM, representative of 2 independent experiments. * Denotes $p < 0.05$.

D

Figure 8. Activation of IKK-alpha/beta is required for the PIGF/TLR effect

50,000 or 500,000 CD14⁺ cells/mL were cultured with 3 μ M R848, 250 ng/ml PIGF-1, and/or the IKK inhibitor BMS-345541 (BMS) (0.5, 1.5, or 5 μ M). (A) Secreted TNF in the conditioned media after 24-hours was quantified by ELISA. Results shown as the mean $\pm$ SEM, representative of 5 separate experiments. (B) 500,000 CD14⁺ cells/mL were cultured for 4-hours with 3 μ M R848, 250 ng/ml PIGF-1, and/or 5 μ M BMS-345541 followed by RNA isolation. *TNF* mRNA was measured using real-time qRT-PCR and normalized to levels of 18S rRNA. Shown is the mean $\pm$ SEM, representative of 2 separate experiments. (C-D) For western blotting, 500,000 CD14⁺ cells/mL were cultured with (C) 3 μ M R848 +/- 250 ng/ml PIGF-1. After 6-hours, whole cell lysates were isolated for immunoblotting with total IKK-alpha, total IKK-beta, and phospho-IKK-alpha/beta. Results shown are representative of 2 separate experiments. (D) Whole cell lysates were also prepared from 500,000 CD14⁺ cells/mL cultured for 6-hours with 3 μ M R848, 250 ng/ml PIGF-1, +/- 5 μ M BMS-345541, and immunoblotting performed as above. * Denotes $p < 0.05$.