

The Implication of Aberrant GM-CSF Expression in Decidual Cells in the Pathogenesis of Preeclampsia

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Preeclampsia is characterized by an exaggerated systemic inflammatory state as well as shallow placentation. In the decidual implantation site, preeclampsia is accompanied by an excessive number of both macrophages and dendritic cells as well as their recruiting chemokines, which have been implicated in the impairment of endovascular trophoblast invasion. Granulocyte-macrophage colony-stimulating factor is known to regulate the differentiation of both macrophages and dendritic cells, prompting both *in vivo* and *in vitro* evaluation of granulocyte-macrophage colony-stimulating factor expression in human decidua as well as in a mouse model of preeclampsia. This study revealed increased granulocyte-macrophage colony-stimulating factor expression levels in preeclamptic decidua. Moreover, both tumor necrosis factor- α and interleukin-1 β , cytokines that are implicated in the genesis of preeclampsia, markedly up-regulated granulocyte-macrophage colony-stimulating factor production in cultured first-trimester human decidual cells. The conditioned media of these cultures promoted the differentiation of both macrophages and dendritic cells from a monocyte precursor. Evaluation of a murine model of preeclampsia revealed that the decidua of af-

ected animals displayed higher levels of immunoreactive granulocyte-macrophage colony-stimulating factor as well as increased numbers of both macrophages and dendritic cells when compared to control animals. Because granulocyte-macrophage colony-stimulating factor is a potent inducer of differentiation and activation of both macrophages and dendritic cells, these findings suggest that this factor plays a crucial role in the pathogenesis of preeclampsia. (Am J Pathol 2010, 177:2472–2482; DOI: 10.2353/ajpath.2010.091247)

Implanting human blastocyst-derived extravillous cytotrophoblasts penetrate an underlying decidua comprised primarily of resident decidual cells (50%) and an assortment of immune cells. The extravillous cytotrophoblasts then invade the inner myometrium during the period of placentation. As they traverse the decidua, extravillous cytotrophoblasts enter and transform spiral arteries and arterioles into low-resistance, high-capacity vessels that increase utero-placental blood flow to the developing fetal-placental unit. In many cases of preeclampsia, a multisystem disorder that is the single leading cause of maternal and fetal morbidity and mortality worldwide,¹ shallow decidual invasion by extravillous cytotrophoblasts leads to incomplete vascular remodeling. The resulting decreased utero-placental blood flow causes the placenta to become relatively hypoxic² and secretes an array of antiangiogenic factors,² placental debris, reactive oxygen species, and proinflammatory cytokines into the maternal circulation. The resulting systemic endothelial cell activation and vascular damage leads to vaso-

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spasm, hypertension, increased platelet turnover, and a characteristic renal lesion with proteinuria.³

Decidual immune cells promote extravillous cytotrophoblast invasion and confer immune tolerance of the semiallogeneic embryo-fetal transplant.⁴ By contrast, preeclampsia-related impaired extravillous cytotrophoblast invasion is associated with local immune maladaptation reflecting in part excess infiltration and differentiation of leukocytes that mediate innate and adaptive immunity.^{3,5} In this regard, we⁶ and others⁷ observed that decidua from preeclamptic women contains an excess of macrophages, as well as immature and mature dendritic cells.⁸ In the implantation site of uncomplicated gestations, macrophages may modulate extravillous cytotrophoblast invasion secondary to phagocytosis of apoptotic cells and remodeling of the decidua.⁹ However, in preeclampsia, tumor necrosis factor- α (TNF- α) derived from excess decidual macrophages is implicated in impairing extravillous cytotrophoblast invasion by inducing extravillous cytotrophoblast apoptosis and by enhanced synthesis of plasminogen activator inhibitor-1, which inhibits urokinase plasminogen activator expressed at the leading edge of trophoblast invasion. Unlike the excess of macrophages, dendritic cell infiltration in the decidua from preeclamptic women does not appear to impede extravillous cytotrophoblast invasion directly.⁸ Alternatively, in view of the strong involvement of dendritic cells in inducing immune tolerance in other systems, the excess of dendritic cells in term decidua may account for the high incidence of subsequent immune tolerance of the allogeneic placenta with improved extravillous cytotrophoblast invasion and reduced recurrence and/or severity of preeclampsia in subsequent pregnancies.

Colony-stimulating factors (CSFs) are secreted by diverse cell types and mediate unique and pleiotropic functions.¹⁰ Among CSF family members, granulocyte-monocyte (GM)-CSF affects survival and growth of myeloid leukocytes. Monocytes migrate into tissues in response to inflammatory stimuli and then differentiate into either macrophages or immature dendritic cells.¹¹ *In vitro* studies indicate that incubation with macrophage (M)-CSF and GM-CSF transform monocytes to macrophages, whereas GM-CSF and interleukin (IL)-4 convert monocytes into immature dendritic cells.¹² Mature antigen-loaded dendritic cells migrate into peripheral lymph nodes and activate naïve T-lymphocytes. These activated T-lymphocytes are then recruited back to the peripheral site of inflammation to mediate adaptive immune response.

In the nonpregnant human endometrium, GM-CSF is synthesized primarily by luminal and glandular epithelial cells^{13–15} with a peak in GM-CSF mRNA levels observed during the “window of implantation.” In contrast, the mRNA for the GM-CSF receptor is localized to endothelial cells of spiral arteries.¹⁶ Moreover, GM-CSF is a growth factor for trophoblast and other placental cells.¹⁷

There are contradictory reports as to whether GM-CSF levels are elevated in the maternal serum and placenta of preeclamptic patients.^{18,19} Surprisingly, GM-CSF expression has not been assessed in decidua from preeclamptic women, the crucial site of impaired extravillous cy-

trophoblast invasion associated with local immune maladaptation. The critical contribution of macrophages and dendritic cells to preeclampsia-related immune maladaptation at the implantation site taken together with the importance of GM-CSF in determining the local macrophage and dendritic cell population emphasized the need to address placental bed GM-CSF involvement in the pathogenesis of preeclampsia. Major obstacles in carrying out the research into the pathogenesis of preeclampsia include: i) the late onset of clinical manifestations (usually in the third trimester), whereas the placental pathogenesis occurs early in pregnancy; and ii) ethical proscriptions regarding prospective obtaining of human tissues compounded by the absence of unambiguous early markers that predict those women who will go on to develop preeclampsia. Therefore, the current study approached this question by using an integrated three-tiered approach in which: i) *In situ* studies compared the cellular immunolocalization of GM-CSF in the major cell types of maternal-fetal interface in preeclamptic versus gestational age-matched control decidua; ii) *In vitro* studies assessed the effects of preeclampsia-related proinflammatory cytokines on the regulation of GM-CSF expression in monolayer leukocyte-free first trimester decidual cells,²⁰ the predominant cell type encountered by invading extravillous cytotrophoblasts; and iii) *in vivo* studies with a mouse model of preeclampsia was used to correlate decidual macrophage and dendritic cell infiltration with GM-CSF expression in decidual cells and the occurrence of preeclampsia-like symptoms.

Materials and Methods

Patients' Specimen Collections

Placental specimens were obtained from patients with preeclampsia defined as the occurrence of elevated blood pressure (>140/90 mm Hg) and proteinuria (>+1 on a urine dip stick) identified on two occasions six hours apart after 20 weeks of gestation²¹ in the absence of signs and symptoms of chorioamnionitis or chronic villitis. All cases (37–40 weeks) were delivered by Cesarean section without labor and specimens obtained under Institutional Review Board from the Mackay Memorial Hospital, Taipei, Taiwan and the University of Siena, Siena, Italy. In Mackay Memorial Hospital, decidual tissues from placental bed biopsies were obtained by sharp dissection of the decidua basalis about 1 to 2 cm in diameter from the maternal surface of placenta around cord insertion area following Cesarean delivery. Microscopic examination after hematoxylin and eosin stain confirmed the identity of each issue before use. The incidence of preeclampsia accompanied with intrauterine growth restriction in our study population was 25% (2/8). Gestational age-matched controls without labor and signs of preeclampsia were also obtained after Cesarean delivery. In Siena University, multiple full-thickness placental blocks were taken and histologically examined. For each specimen, a block that best represented the maternal decidua was selected for immunohistochem-

istry. Tissues were snap-frozen in liquid nitrogen or fixed in 10% buffered neutral formalin and embedded in paraffin.

Mouse Model of Preeclampsia

Female BALB/c mice and C57BL/6 male were obtained from BgVV or Harlan Winkelmann, Germany and maintained in a barrier animal facility with food and water *ad libitum* and 12-hour light cycles. Animal care and experimental procedures were approved by the German ministry and followed according to the institutional guidelines (LaGeSo, Berlin, Germany; Reg 039/03). C57/BL6-mated BALB/c females received either 3×10^7 Th1-like splenocytes or PBS on gestational days 10 and 12 [preeclampsia ($n = 9$) and control ($n = 13$)].^{22,23} Th1-like cell transfer predisposed fetal rejection (Supplemental Figure 1A at <http://ajp.amjpathol.org>). Blood pressure was measured by a tail cuff method as previously described.^{22,23} On gestational day 14, the mice were sacrificed. Placentae were prepared in paraffin blocks for immunohistochemistry and in Trizol for total RNA isolation.

Immunohistochemistry

Human Tissues

Sections (4 μm) of formalin-fixed paraffin-embedded placenta and attached fetal membranes were cut, deparaffinized, rehydrated, and washed in Tris-buffered saline [TBS: 20 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 7.6)]. TBS was used for all subsequent washes and for antibody. Antigen retrieval was carried out by incubating sections in Target Retrieval Solution High pH (DAKO, Copenhagen, Denmark), in a microwave oven at 750 Watts for 15 minutes. Sections were subsequently rinsed in 3% hydrogen peroxide to block endogenous peroxidase and incubated overnight at 4°C with monoclonal antibodies against GM-CSF (R&D Systems, Minneapolis, MN), vimentin, and cytokeratin (DAKO) diluted in TBS. Slides were then washed three times with TBS for 5 minutes and incubated with the universal labeled streptavidin biotin (LSAB) Kit (DAKO). After washing three times for 5 minutes in TBS, sections were stained with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO) as chromogen substrate and counterstained with Harris hematoxylin (Sigma-Aldrich). The reaction was stopped by washing the sections in distilled water, and slides were mounted and observed under a light microscope. Staining specificity was tested by substituting monoclonal IgG isotype of anti-GM-CSF antibody (R&D Systems) for the primary antibody. In preeclampsia-associated and control placentae, positive immunostaining for GM-CSF was detected in virtually all of the decidual cells. This observation mandated immunostaining for GM-CSF to be quantified semiquantitatively on the basis of staining intensity. This was carried out by a single observer blinded to the specimens using a three-grade scale, ranging from the most intense staining (++) to the weaker (moderate staining: +; weak staining: 0/+).

For each slide, the whole decidua was assessed. Areas were chosen at $\times 25$ magnification and each of them was examined in-deep for semiquantitative evaluation at $\times 200$ magnification. A minimum of twelve fields were examined for each slide.

Mouse Tissues

Immunohistochemistry was performed as previously described²⁴ using rat monoclonal anti-GM-CSF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-GRP94 (Imgenex, San Diego, CA), and rat monoclonal antibody to macrophages (Abcam Inc. Cambridge, MA). Briefly, 5 μm formalin-fixed paraffin-embedded sections were deparaffinized in xylene and rehydrated through a descending series of ethanol. Antigen retrieval was then performed by boiling the slides in citrate buffer (10 mmol/L; pH 6.0) for 15 minutes for GRP94 and macrophage surface marker, and by incubating with trypsin for 10 minutes for GM-CSF. The staining was performed according to the manufacturer's instruction for Consolidate Immunohistochemistry DAB Kit (BioGenex, San Ramon, CA) for GM-CSF and macrophages. For GRP94 antibody, Super Sensitive Immunohistochemistry Detection System for Automation Kit (BioGenex, San Ramon, CA) and Rabbit Link HRP/DAB were used. After antigen retrieval, slides were incubated with peroxide for 5 minutes at room temperature to quench endogenous staining according to the manufacturer's instructions. The slides were then incubated with Power Block Reagent for 5 minutes at room temperature in a humidified chamber. Excess serum was then removed, and serial sections were incubated with anti-GM-CSF at 1:50 dilution in TBS, anti-GRP94 at 1:50 dilution in TBS, and rat monoclonal antibody to macrophages at 1:150 dilution in TBS overnight in a humidified chamber at 4°C. Nonspecific rabbit IgG polyclonal and rat IgG isotype antibodies were used at the same concentrations as the primary antibodies for negative controls. The slides were washed $\times 3$ in TBS for 5 minutes and then incubated with secondary antibody for 20 minutes at room temperature. After washing in TBS, slides were incubated with streptavidin-horseradish peroxidase for 20 minutes at room temperature to detect the antigen-antibody complex using 3,3'-diaminobenzidine tetrahydrochloride dehydrate as chromogen. Slides were then counterstained with hematoxylin and mounted with Permount (Fisher Chemicals, Springfield, NJ).

The intensity of immunostaining was semiquantitatively evaluated using HSCORE analysis. The intensity of immunostaining was categorized into the following scores: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (intense staining). An HSCORE value was derived for each specimen by calculating the sum of the percentage of cells that stained at each intensity category multiplied by its respective intensity score, using the formula $\text{HSCORE} = \sum P_i (i + 1)$, where i represents the intensity score and P_i is the corresponding percentage of cells.²⁵ For each slide, five different fields were evaluated at $\times 400$ magnification. HSCORE evaluation was performed

independently by two investigators blinded to the source of the samples; the average score of both was then used.

Immunofluorescent Staining

Decidual tissues from placental bed biopsies were collected under Institutional Review Board approval and snap frozen in liquid nitrogen for cryosections (5 μm in thickness). Tissue was confirmed by hematoxylin and eosin stain. Sections were air-dried and fixed in ice-cold acetone for 10 minutes, then rehydrated with PBS for 5 minutes, blocked with protein block (DAKO) for 20 minutes, and incubated in mouse anti-human GM-CSF antibody diluted to 1:20 in PBS (R&D Systems) for 1 hour at room temperature. Staining specificity was tested by substituting mouse monoclonal IgG isotype of the anti-human GM-CSF antibody (R&D Systems) for the primary antibody. After three washes in PBS, sections were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (1:100; Millipore; Temecula, CA) or rabbit anti-mouse antibody (1:100; DAKO) for 1 hour. The sections were then washed three times and incubated in goat anti-human vimentin antibody (1:100; Sigma-Aldrich) or rabbit anti-human CD45 antibody (1:200; AbD Serotec, Raleigh, NC) for 1 hour at room temperature. After three washes in PBS, sections were incubated with rhodamine-conjugated rabbit anti-goat antibody (1:400; Sigma-Aldrich) or goat anti-rabbit antibody (1:100; Millipore; Temecula, CA) for 1 hour. Finally, the sections were washed quickly in PBS three times, stained with 4',6'-diamidino-2-phenylindole (DAPI) diluted 1:5 $\times 10^5$ in ddH₂O (Sigma-Aldrich), and mounted in a non-fade mounting medium (Life Sciences International, Basingstoke, UK). Immunofluorescence was examined under a Zeiss microscope (Müchen-Hallbergmoos, Germany) equipped with a cooled charge-coupled device camera (AxioCam HRm, Zeiss). No cross-reaction was found between these antibodies.

Cell Cultures

Decidual specimens from elective terminations between 6 and 12 weeks of gestation were collected under Yale University Human Investigation Committee approval. The villous cytotrophoblasts were dissected from the decidua by forceps. Pulverized tissues were digested in 0.1% collagenase type IV and 0.01% DNase in RPMI 1640 containing 20 $\mu\text{g}/\text{ml}$ penicillin/streptomycin, 1 $\mu\text{l}/\text{ml}$ fungizone (Invitrogen Inc., Grand Island, NY) in a 37°C shaking water bath for 30 minutes. After washing with sterile 1 \times PBS, the digestate was washed $\times 3$ and subjected to consecutive filtration through 100 μm , 70 μm , and 40 μm Millipore filters, respectively. Cells were resuspended in RPMI 1640 and then seeded on polystyrene tissue culture plates. The presence of leukocytes after each passage was monitored by flow cytometric analysis with anti-CD45 and anti-CD14 mAbs (BD Pharmingen, San Diego, CA). Cell cultures were found to be leukocyte-free (<1%) after 3–4 passages. Cultured decidual cells were found to be vimentin-positive and cy-

tokerin-negative and displayed decidualization-related morphological and biochemical changes during incubation with a progestin. The latter include enhanced expression of prolactin and plasminogen activator inhibitor-1 and inhibited expression of interstitial collagenase and stromelysin-1 and as we recently demonstrated prolonged up-regulation of tissue factor mRNA and protein expression.²⁰ Cell aliquots after harvest using trypsin/EDTA were then frozen in fetal calf serum/DMSO (9:1) (Sigma-Aldrich) and stored in liquid nitrogen.

Thawed first trimester decidual cells (5×10^5 cells per ml) were suspended in basal medium, a phenol red-free 1:1 (vol/vol) mix of DMEM (Invitrogen, Inc.) and Ham's F-12 (Flow Laboratories, Rockville, MD) containing 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.25 $\mu\text{g}/\text{ml}$ fungizone supplemented with 10% charcoal-stripped calf serum. The decidual cells were seeded onto polystyrene tissue culture flasks coated with 2% type B gelatin (Sigma-Aldrich), grown to confluence in a standard 95% air:5% CO₂ incubator at 37°C, and passaged $\times 6$ then grown to confluence. Confluent decidual cells were incubated for 7 days in basal medium + charcoal-stripped calf serum containing 10^{-8} mol/L estradiol plus 10^{-7} mol/L medroxyprogesterone acetate (Sigma-Aldrich) with one change of medium. The cultures were washed twice with HBSS to remove residual serum components and switched to a serum-free defined medium consisting of basal medium plus ITS+ (BD Biosciences, Bedford, MA), 5 μm FeSO₄, 50 μm ZnSO₄, 1 nm CuSO₄, 20 nm Na₂SeO₃, trace elements (Invitrogen, Inc.), 50 $\mu\text{g}/\text{ml}$ ascorbic acid (Sigma-Aldrich), and 50 ng/ml epidermal growth factor (BD Biosciences, Bedford, MA). Subsequently, the cells were incubated with steroids with or without 1 ng/ml IL-1 β or TNF- α for 6 hours (total RNA purification) or 24 hours (protein extraction).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Human Decidual Cells

The primer pairs were synthesized and gel purified at the Yale DNA Synthesis Laboratory (Critical Technologies). Reverse transcription was initially carried out using Omniscript kit (Qiagen, Valencia, CA) in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). Each RT reaction contained 2 μg of total RNA, 2 μl 1 \times buffer RT, 0.5 mmol/L dNTPs, 1 $\mu\text{mol}/\text{L}$ T7-(dT)₂₄ oligo-primer and 4 units of Omniscript reverse transcriptase. This 20- μl reaction was immediately incubated at 37°C for 1 hour. Specific primer sets were designed accordingly to each gene for quantitative PCR. The sequences of these primer sets are shown in Table 1. A quantitative standard curve was then created using a range of RT products. The curve was created with the Roche Light Cycler (Roche, Indianapolis, IN) by monitoring the increasing fluorescence of PCR products during amplification. Once the standard curve was established, quantification of unknowns was determined with the Roche Light Cycler and adjusted to the quantitative expression of β -actin from these same unknowns. Melting curve analysis was conducted to

Table 1. Sequences of Primer Sets for First-Trimester Decidual Cell Cultures

Gene	Forward primers	Reverse primers
<i>β-Actin</i>	5'-CGTACCCTGGCATCGTGAT-3'	5'-GTGTTGGCGTACAGGTCTTTG-3'
<i>GM-CSF</i>	5'-AACTTCCTGTGCAACCCAGATTAT-3'	5'-ATGCCTGTATCAGGGTCAGTGT-3'

determine the specificity of the amplified products and the absence of primer-dimer formation.

Murine Samples

Total RNA (2 μg) was transcribed to cDNA using dNTPs (2.5 mmol/L, Amersham, Freiburg, Germany), RB (Moloney mouse leukemia virus), RT-buffer, DNase (2 U/μl, Stratagene, Amstelveen, Netherland), and RNase inhibitor (40 U/μl, Promega, Mannheim, Germany) at 37°C for 30 minutes, then heated at 75°C for 5 minutes. Reverse transcriptase (200 U/μl) and RNase inhibitor were added and incubated at 42°C for 1 hour, then 95°C for 5 minutes and cooling on ice. Reactions were carried out with 1 μl cDNA, 6.5 μl Sybr-Green-Mastermix (Stratagene), 2 μl RNase-free water, 3 μl primers, and 0.5 μl Fluorescein (25 nmol/L, Fermentas, St. Leon-Rot, Germany) with results normalized to *β-actin*. The sequences of these primer sets are shown in Table 2.

ELISA

Placental Bed Biopsies

Frozen decidual tissues were homogenized in RIPA lysis buffer (NP-40 2%, SDS 0.02%, sodium deoxycholate 1%, 1× PBS) with protease inhibitor cocktail (Sigma-Aldrich). Total protein (25 μg/ml) was used to detect the GM-CSF levels by ELISA kit as manufacturer's protocol (R&D Systems). The GM-CSF levels are expressed as pg/ml.

First Trimester Decidual Cell Cultures

Whole cell lysates prepared from cultured decidual cells were used to measure total cell protein levels by the Bio-Rad Assay (Bio-Rad Laboratories, Inc. Hercules, CA). A commercial ELISA kit measured immuno-reactive levels of GM-CSF in conditioned media according to instructions provided by the manufacturer (R&D Systems). The ELISA has a sensitivity of <10 pg/ml. The intra-assay and interassay coefficients of variation vary from 2.3 to 2.6 and from 5.0 to 6.5, respectively.

Cell Differentiation Assay

Peripheral blood was collected from adult nonpregnant healthy reproductive age female donors. The CD14+

monocytes isolated from buffy coats²⁶ using CD14 MicroBeads (Miltenyi Biotec, Auburn, CA) were incubated with conditioned media from TNF-α or IL-1β-treated or -untreated first trimester decidual cells with or without anti-GM-CSF neutralizing antibody (R&D Systems) for 5 days. For dendritic cell differentiation, 20 ng/ml of human recombinant IL-4 (R&D Systems) was added. The identity of macrophages and dendritic cells was confirmed by flow cytometry using anti-CD68-PE and anti-CD11c-fluorescein isothiocyanate antibodies (R&D Systems), respectively.

Statistical Analysis

The Kolmogorov-Smirnov one-sample test was used to distinguish normally from nonnormally distributed results to apply the appropriate parametric or nonparametric tests. Tissue and cell ELISAs, immunohistochemistry, qRT-PCR as well as cell differentiation assay results are presented as mean ± SEM. All parameters are expressed as mean ± SEM. The differences were assessed by Wilcoxon Signed Ranks test or the Student's *t*-test, when appropriate. A *P* value less than 0.05 was considered significant.

Results

GM-CSF Expression Is Increased in Decidua from Preeclamptic Women

For immunostaining of formalin-fixed paraffin-embedded serial sections, GM-CSF was significantly greater in the cytoplasm of decidual cells from preeclamptic (Figure 1B) compared with control specimens (Figure 1A). Decidual cells are identified by the positive vimentin staining in control (Figure 1C) and preeclamptic specimens (Figure 1D). Co-immunostaining of the pan-leukocyte maker, CD45 (Figure 1, E and F), or vimentin (Figure 1, G and H), and GM-CSF in serial frozen sections showed that changes in GM-CSF expression were found primarily in decidual cells, which are large vimentin-positive CD45-negative cells. These observations were confirmed by demonstrating greater prominence of yellow immunofluorescence denoting colocalization of GM-CSF (green) and vimentin (red) in a preeclamptic (Figure 1H) versus gestational age-matched control specimen (Figure 1G). Table 3 sum-

Table 2. Sequences of Primer Sets for Mouse Placenta

Gene	Forward primers	Reverse primers
<i>β-Actin</i>	5'-GCTTCTTTGACGCTCCTTCGTT-3'	5'-GTTGTGACGACCCAGCGC-3'
<i>GM-CSF</i>	5'-TGCGTAATGAGCCAGGAAC-3'	5'-TCTCGTTTGTCTCCGCTTGT-3'

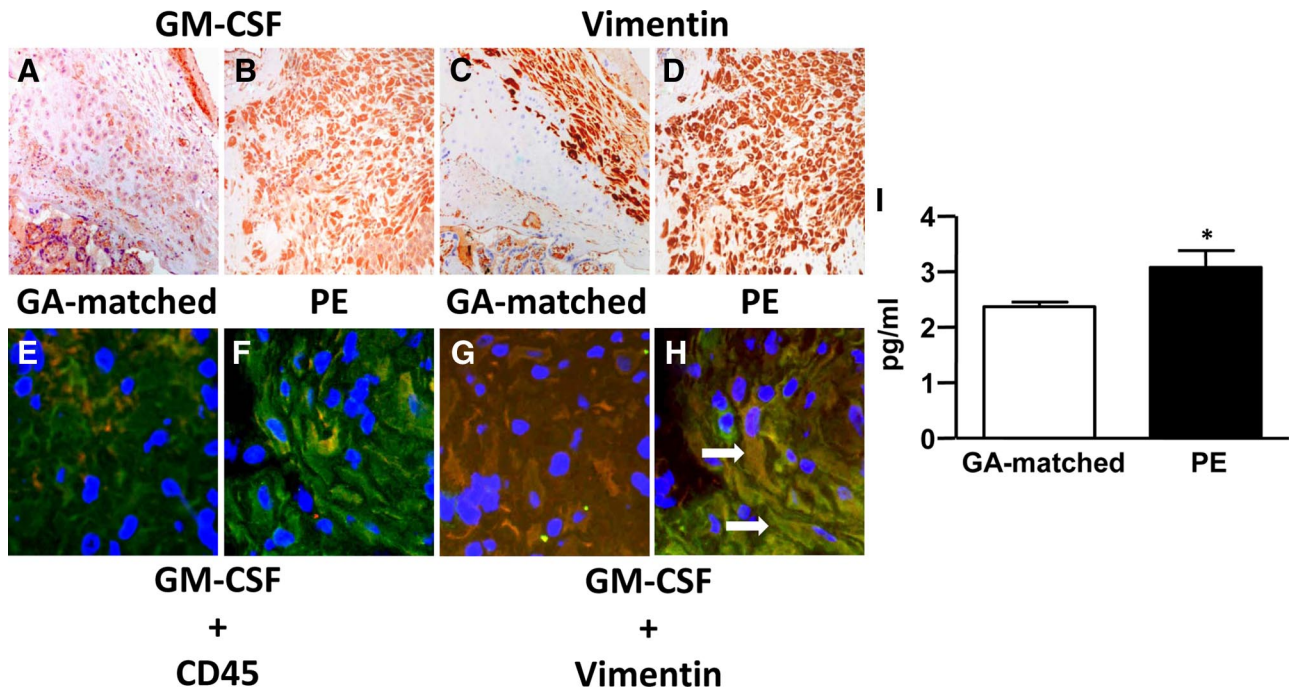


Figure 1. Expression of GM-CSF is increased in decidual cells from preeclamptic women. Representative serial sections for GM-CSF immunostaining of (A, $\times 200$) gestational age (GA)-matched control and (B, $\times 200$) preeclamptic paraffin-embedded decidual tissues visualized by DAB staining. (C and D, $\times 200$) Serial sections from corresponding tissue were stained for the decidual cell marker, vimentin. The expression of GM-CSF in decidua was also assessed by colocalization of immunofluorescence staining of frozen sections. Yellow staining indicates colocalization of individual staining of GM-CSF (green) and (E and F, $\times 400$) CD45 (red) or (G and H, $\times 400$) vimentin (red). The **arrows** indicate the large GM-CSF and vimentin double-positive cells. **I:** ELISA of GM-CSF expression in extracts of decidual tissues from GA-matched and preeclamptic patients ($n = 8$, $*P < 0.05$).

marizes the immunostaining results for the formalin-fixed paraffin-embedded sections. It indicates that compared with control decidual sections, decidua from preeclamptic women more frequently displayed prominent immunostaining. Consistent with the results of immunostaining, extracts of decidual tissues from preeclamptic patients contain significantly elevated levels of immuno-reactive GM-CSF (Figure 1I) ($P < 0.05$).

GM-CSF Expression Is Induced by IL-1 β and TNF- α in Cultured First Trimester Decidual Cells

Figure 2A uses a log scale and indicates that TNF- α and IL-1 β up-regulated steady state GM-CSF mRNA expression in estradiol and medroxyprogesterone acetate primed leukocyte-free first trimester decidual cells by 20-fold ($n = 3$, $P < 0.05$) and 180-fold ($n = 5$, $P < 0.005$) (Figure 2A), respectively. In parallel incubations, corresponding effects were observed on secreted levels of GM-CSF measured by a specific ELISA (Figure 2B). Spe-

cifically, GM-CSF expression was found to be significantly up-regulated by TNF- α and IL-1 β by threefold ($n = 6$, $P < 0.05$) and 123-fold ($n = 6$, $P < 0.005$), respectively.

Inflammatory Cytokine-Enhanced GM-CSF Expression by First Trimester Decidual Cells Mediates Differentiation of Monocytes to Macrophages and Dendritic Cells

To elucidate potential mechanism(s) by which augmented GM-CSF expression by decidual cells may mediate the characteristic excess of macrophages and dendritic cells found in decidua from preeclamptic women,^{6,8}

Table 3. Immunohistochemical Analysis of Decidual GM-CSF Expression in Preeclampsia and Control Specimens

	GM-CSF immunostaining		
	++	+	0/+
Preeclampsia	5	3	0
Controls	0	5	3

The following scoring system was used: ++, marked staining; +, moderate staining; and 0/+, weak staining.

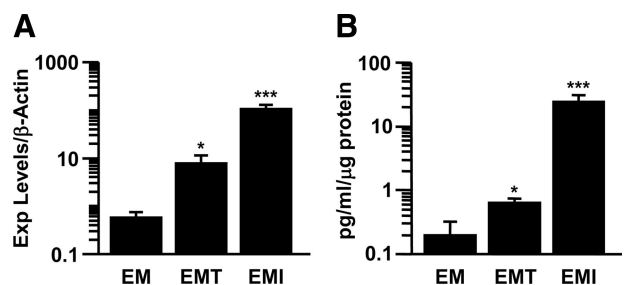


Figure 2. TNF- α and IL-1 β induce GM-CSF expression in leukocyte-free first-trimester decidual cells. **A:** qRT-PCR of GM-CSF in first-trimester decidual cells treated with or without TNF- α (EMT) or IL-1 β (EMI). The results were normalized to β -actin. **B:** ELISA of GM-CSF in the conditioned media of first-trimester decidual cells treated with or without TNF- α or IL-1 β ; the results were normalized to total cell protein ($n = 3-6$, $*P < 0.05$, $***P < 0.005$). The plots are shown in log scale.

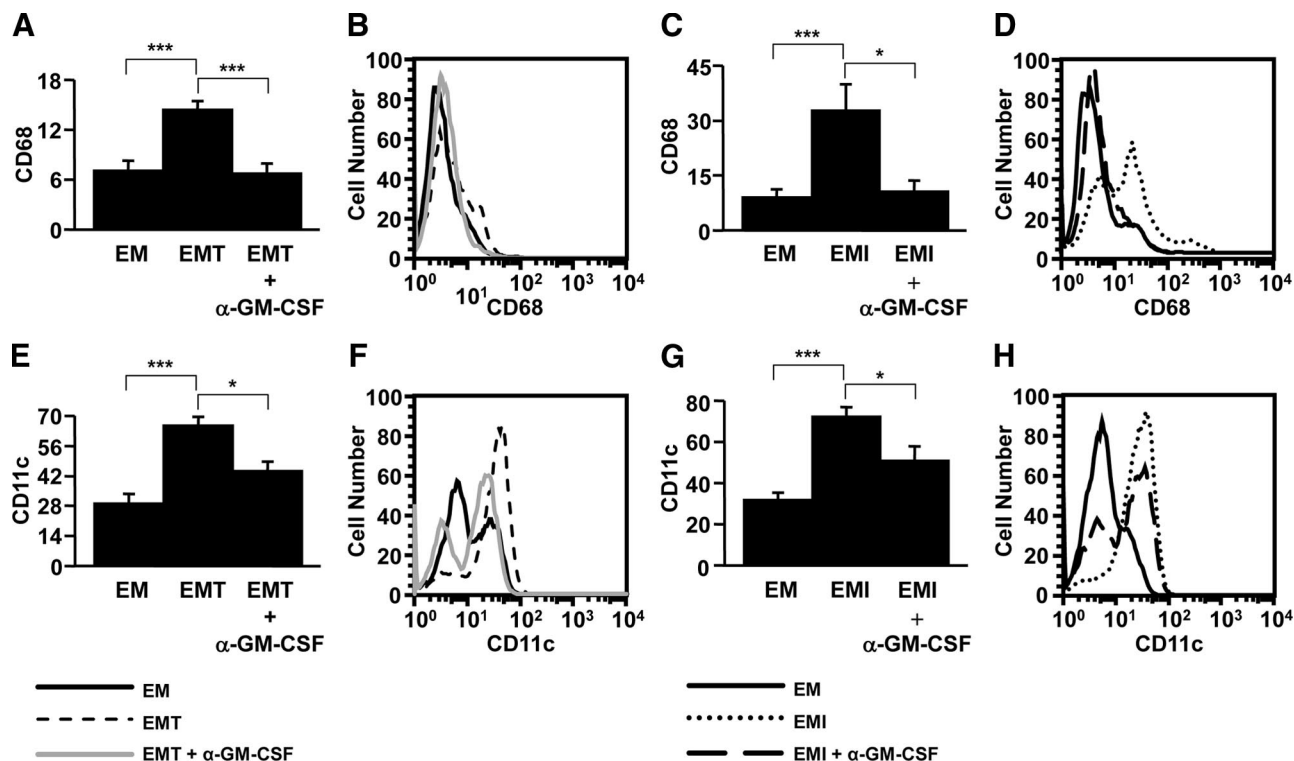


Figure 3. The development of macrophages and dendritic cells was enhanced by TNF- α - or IL-1 β -treated first-trimester decidual cell-derived GM-CSF. Monocytes isolated from peripheral blood were incubated with conditioned media from leukocyte-free first-trimester decidual cells treated with estradiol + medroxyprogesterone acetate \pm TNF- α (EMT) or IL-1 β (EMI) in the presence or absence of anti-human GM-CSF-neutralizing antibody (α -GM-CSF). Expression of (A–D) the macrophage (CD68) or (E–H) dendritic cell (CD11c) surface marker was examined by flow cytometric analysis ($n = 5-9$, * $P < 0.05$, *** $P < 0.005$). Histograms of flow cytometric analysis for surface marker expression are from representative experiments. Black line: EM; Short dash line: EMT; Gray line: EMT + α -GM-CSF; Dot line: EMI; Long dash line: EMI + α -GM-CSF.

the functional role of enhanced GM-CSF expression in first trimester decidual cells was evaluated on the differentiation of monocytes to macrophages or dendritic cells. Conditioned medium from TNF- α - or IL-1 β -treated first trimester decidual cells enhanced the differentiation of monocytes isolated from peripheral blood to either macrophages (CD68) (Figure 3, A–D; $n = 5-7$, $P < 0.005$) or dendritic cells (CD11c) (Figure 3, E–H; $n = 5-9$, $P < 0.005$) by 51–73%. Exposure to GM-CSF neutralizing antibody inhibited TNF- α - or IL-1 β -treated first trimester decidual cell-induced monocyte differentiation to macrophages or dendritic cells by 51–66% (Figure 3, A–D; $n = 5-7$, $P < 0.005$ for macrophages and $P < 0.05$ for dendritic cells) and 30–32% (Figure 3, E–H; $n = 5-9$, $P < 0.05$), respectively. These results are consistent with a key role played by decidual cell-derived GM-CSF in the pathogenesis of preeclampsia in view of the marked excess of macrophages and dendritic cells in decidua from preeclamptic women.

Murine Preeclampsia Is Associated with Increased Decidual GM-CSF Expression as Well as the Infiltration of Macrophages and Dendritic Cells

Consistent with the maternal syndrome of preeclampsia in humans, transfer of activated Th1-like splenocytes to

recipient mice elicited an immediate increase in the systolic blood pressure of recipient mice measured on gestational day 14 (124 ± 8 mmHg for PBS treated controls versus 146 ± 20 mmHg for preeclamptic mice, $P < 0.001$) and induced proteinuria (>1 g/L) in preeclamptic mice (Supplemental Figure 1B at <http://ajp.amjpathol.org>). Hematoxylin and eosin staining demonstrated extensive edema, hemorrhage, and tissue damage in the decidual and spongiotrophoblast layer of utero-placental units from pregnant activated Th1-like cell recipients (preeclamptic mice) (Figure 4, B and D) compared to controls (Figure 4, A and C). As expected, staining of kidney sections revealed glomeruli without histopathological change in control pregnant mice (Figure 4E); however, renal hemorrhage and abnormal glomerular cell infiltration were observed in pregnant cell recipients (Figure 4F).

Further links between elevated GM-CSF expression in the placenta and the onset of preeclamptic symptoms were established by demonstrating that, compared with control mice, recipients of activated Th1-like splenocytes displayed augmented levels of GM-CSF mRNA (Figure 5A) and increased expression of immuno-reactive GM-CSF (Figure 5, B, C, and H). Consistent with an increase of macrophages and dendritic cells in the human decidua from preeclamptic women, the numbers of macrophages (Figure 5, D, E, and I) and dendritic cells (Figure 5, F, G, and J) were also increased in the decidua of activated Th1-like cell recipient mice.

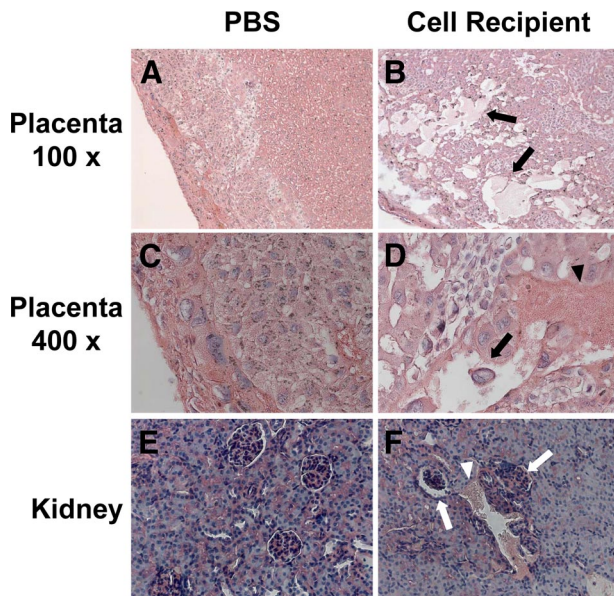


Figure 4. Histopathological changes in the utero-placental units and kidneys in preeclamptic mice. Eight-week-old female BALB/c mice mated with male C57BL/6 mice were injected with either PBS or 3×10^7 activated Th1-like splenocytes isolated from spleen on gestational days 10 and 12. Samples were collected at gestational day 14. The paraffin-embedded utero-placental units and kidneys were stained with hematoxylin and eosin. **A** ($\times 100$) and **C** ($\times 400$): The tissue was intact in placenta from PBS-injected mice. **B** ($\times 100$) and **D** ($\times 400$): Extensive edema (**black arrows**) and hemorrhage (**black arrowhead**) were found in the spongiosotrophoblast layer of placenta from activated Th1-like cell recipient. **E** ($\times 200$): No histopathological change was found in the kidney from PBS-injected mice. **F** ($\times 200$): Hemorrhage (**white arrowhead**) and increase of cell infiltration (**white arrows**) in glomeruli were shown in the kidney from an activated Th1-like cell recipient.

Discussion

In the United States, preeclampsia complicates 5 to 10% of pregnancies and accounts for 12 to 18% of all pregnancy-related maternal death.^{3,27} It is also a major cause of intrauterine growth restriction (10–25% of women with preeclampsia) and prematurity and is associated with various long-term neonatal morbidities.^{28,29} Delivery of the placenta remits the immediate clinical manifestations of preeclampsia and remains the only reliable treatment for preeclampsia. However, longitudinal studies indicate that the occurrence of preeclampsia whether treated or not, carries severe long-term maternal health risks such as accelerated cardiovascular death.³⁰

While the placental pathogenesis of most cases of preeclampsia appears to be initiated in early pregnancy (<18–20 weeks) as a consequence of shallow extravillous cytotrophoblast invasion of the decidua,³¹ the signs and symptoms of preeclampsia are generally evident much later, usually in the third trimester. The onset of clinical manifestations reflects endothelial cell activation, vasospasm, glomerular and hepatocyte damage, and central nervous syndrome changes. This temporal disconnection between placental pathology and maternal systemic manifestations has posed a major obstacle in attempts to elucidate the real-time placental pathogenesis of this disorder or developing earlier diagnoses or preventative therapies. Our laboratory has used a two-tiered approach in attempting to overcome this obstacle.

Initial observations revealed that *in situ* observations determine that numbers of macrophages as well as immature and mature dendritic cells are significantly increased in decidual cells of preeclamptic compared with gestational age-matched decidual sections. In correspondence with these increases in the two immune cell populations an array of monocyte/macrophage- and dendritic cell-recruiting chemokines were observed to be significantly elevated in decidual cells from preeclamptic women extracts as measured by ELISA and specifically in decidual cells by immunohistochemical localization.^{8,32} Subsequently, the second tier of our approach sought to determine by *in vitro* studies whether expression of these proteins is altered during incubation of first trimester leukocyte-free first trimester decidual cells with preeclampsia-associated proinflammatory cytokines, TNF- α and IL-1 β . These studies confirmed that TNF- α and IL-1 β each markedly increased expression of monocyte/macrophage- and dendritic cell-recruiting chemokines in first trimester decidual cells, the major resident cell type encountered by invading extravillous cytotrophoblasts. Previous studies from our laboratory reported parallel observations for IL-6 expression (ie, enhanced IL-6 levels were immunolocalized to decidual cells of preeclamptic versus control placental sections with inflammatory cytokines augmenting IL-6 mRNA and protein expression in cultured leukocyte-free first trimester decidual cells).^{32,33} In several tissues, IL-6 mediates the transition from acute to chronic inflammation by reciprocally inhibiting neutrophil trafficking and promoting monocyte differentiation away from dendritic cells toward macrophages.³³ Taken together, previous reports from our laboratory indicate a crucial role for decidual cells in determining the macrophage and dendritic cell population of the decidua from preeclamptic women.

The pivotal role played by GM-CSF in modulating differentiation of monocytes to macrophages and/or dendritic cells stimulated the current study to search for an association between decidual cell GM-CSF expression and preeclampsia-related trafficking of both immune cell types. Previously, decidual GM-CSF was shown to originate from T-lymphocytes responding to alloantigens,³⁴ whereas GM-CSF expression was not assessed in decidua from preeclamptic women. Seeking to fill in this gap in our knowledge, the current study found that: i) immuno-reactive GM-CSF levels are elevated in decidual cells of placental sections of patients with preeclampsia versus gestational-age matched controls; ii) GM-CSF mRNA and protein levels are enhanced in first trimester decidual cells during incubation with the preeclampsia-related inflammatory cytokines, TNF- α and IL-1 β . These observations, taken together with the well-documented roles of macrophages and dendritic cells in preeclampsia-related events as detailed above as well as those of GM-CSF in activating both macrophages and dendritic cells, strongly suggest that decidual cell-derived GM-CSF plays a regulatory role in the early pathogenesis of preeclampsia. Studies from our⁶ and other laboratories³⁵ observed an increase in macrophages in the decidua of women with preeclampsia. *In vitro* observations indicate that macrophage-derived TNF- α inhibits extravillous cy-

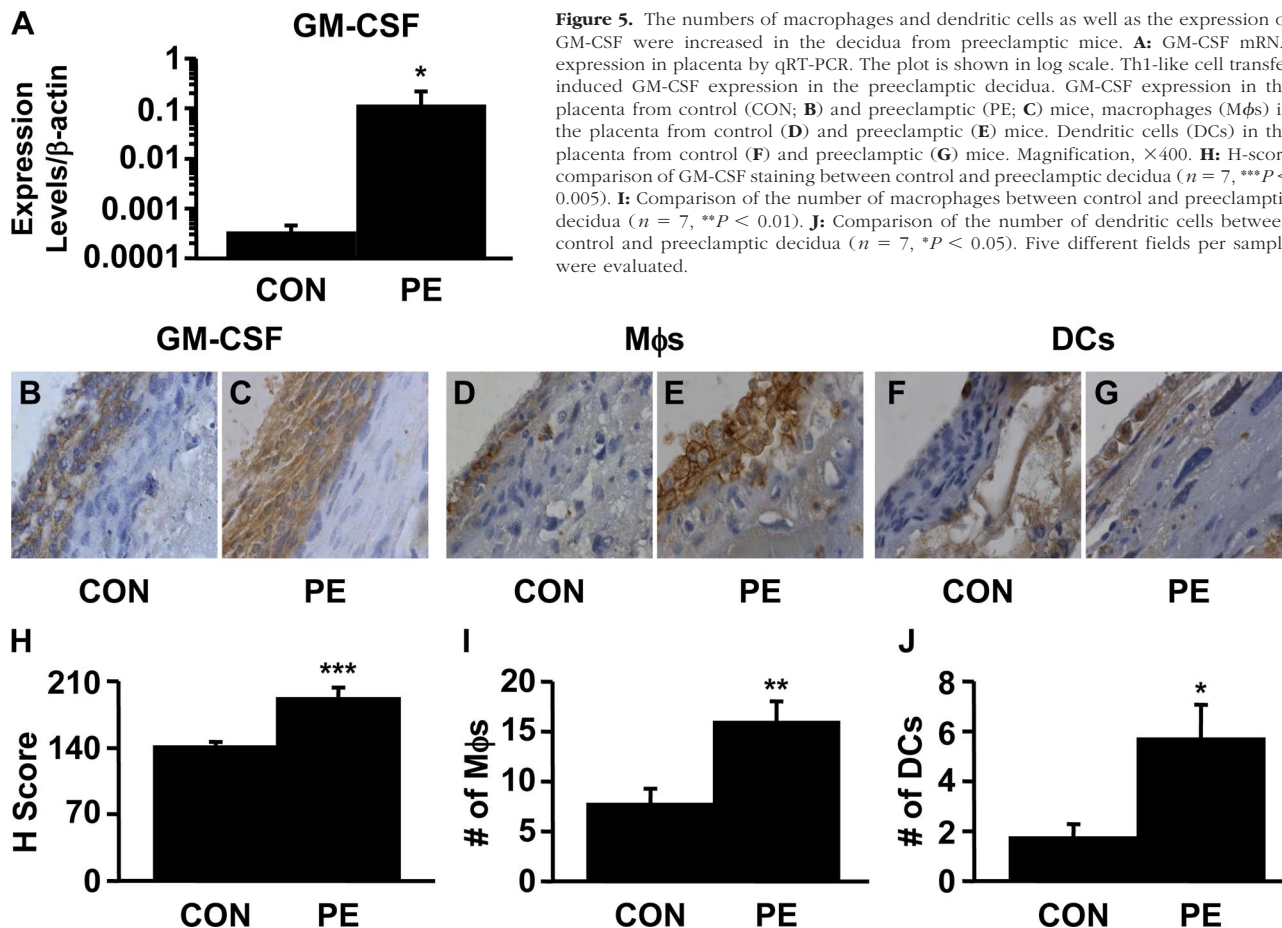


Figure 5. The numbers of macrophages and dendritic cells as well as the expression of GM-CSF were increased in the decidua from preeclamptic mice. **A:** GM-CSF mRNA expression in placenta by qRT-PCR. The plot is shown in log scale. Th1-like cell transfer induced GM-CSF expression in the preeclamptic decidua. GM-CSF expression in the placenta from control (CON; **B**) and preeclamptic (PE; **C**) mice, macrophages (M ϕ s) in the placenta from control (**D**) and preeclamptic (**E**) mice. Dendritic cells (DCs) in the placenta from control (**F**) and preeclamptic (**G**) mice. Magnification, $\times 400$. **H:** H-score comparison of GM-CSF staining between control and preeclamptic decidua ($n = 7$, *** $P < 0.005$). **I:** Comparison of the number of macrophages between control and preeclamptic decidua ($n = 7$, ** $P < 0.01$). **J:** Comparison of the number of dendritic cells between control and preeclamptic decidua ($n = 7$, * $P < 0.05$). Five different fields per sample were evaluated.

trophoblast invasion by inducing apoptosis in extravillous trophoblasts, thereby interfering with extravillous cytotrophoblast-mediated decidual vascular remodeling to produce the characteristic placental hypoxia and maternal symptoms of preeclampsia. This effect was blocked by an antibody against the TNF- α receptor,³⁵ whereas inhibition of extravillous cytotrophoblast invasiveness by lipopolysaccharide-activated macrophages was reversed by a TNF- α neutralizing antibody.³⁶ Mediating this process by decidual cells was indicated by our observations that macrophages incubated with conditioned medium from IL-1 β -treated first trimester human decidual cells inhibited extravillous cytotrophoblast invasion. A report from our laboratory was the first to demonstrate a preeclampsia-related increase in decidual dendritic cells.⁸ However, dendritic cells incubated with conditioned medium from IL-1 β -treated first trimester human decidual cells did not impede extravillous cytotrophoblast invasion. Ongoing work in our laboratory suggests that the preeclampsia-related infiltration of dendritic cells into the decidua may induce a compensatory immune tolerance consistent with the reduced incidence and severity of preeclampsia in subsequent pregnancies. *In vitro* experiments are needed to determine whether co-incubation with either macrophages or dendritic cells in fact induces apoptotic changes in extravillous cytotrophoblasts.

While use of the two-tiered approach to include GM-CSF in the current study has provided important insights into the pathogenesis of preeclampsia, we sought to extend this to a third tier by evaluating decidual expression of GM-CSF in a mouse model of preeclampsia. In particular, studies from the laboratory of Croy and associates have revealed similarities in implantation and placentation as well as the uterine immune cell population between the mouse and human.³⁷ A growing body of evidence shows that mouse models provide important clues that have aided investigators in identifying proteins that are required to coordinate embryo and maternal interactions during the peri-implantation period. The use of this specific mouse model allowed us to investigate whether dendritic cell and GM-CSF expressed in dendritic cell may be related to the preeclampsia-like symptoms observed exclusively in pregnant mice^{25,26} after transfer of activated Th1 cells. Such information has clear implications in elucidating the pathogenesis of preeclampsia.^{38,39} Specifically, the current study found that mice induced to exhibit preeclamptic symptoms (ie, proteinuria and hypertension) displayed elevated decidual GM-CSF expression together with significant macrophage and dendritic cell infiltration of the decidua.

Although several reports indicate that circulating IL-1 β and TNF- α levels are elevated in women with preeclampsia, the source of these increases is unclear. However,

these changes appear to represent exaggeration of the normal peri-implantational decidual macrophage infiltration. Compared with levels of IL-1 β , the follicular and peri-ovulatory phase human endometrium contains a 10- to 30-fold excess of IL-1 receptor antagonist (IL-1Ra), which blocks the IL-1 receptor. A decline in glandular IL-1Ra levels coincides with the "window of implantation."⁴⁰ Observations in our laboratory suggest that the resulting free IL-1 β enhances expression of several monocyte recruiting and activating chemokines in decidual cells that promote a physiological monocyte influx during early gestation but is normally transient because extravillous cytotrophoblasts release IL-1Ra.^{41,42} However, increased expression of IL-1 β in significant subsets of preeclampsia cases accompanying maternal infections, such as Chlamydia^{43,44} and viruses,^{45,46} and other inflammatory states may overwhelm this block of the IL-1R by IL-1Ra. In addition, TNF- α and IL-1 β polymorphisms are associated with impaired extravillous cytotrophoblast invasion, preeclampsia, and eclampsia.⁴⁷⁻⁵¹ Elevated levels of TNF- α or IL-1 β associated with inflammatory states or maternal genetic polymorphisms target decidual cells to enhance synthesis and secretion of monocyte/macrophage- and dendritic cell-recruiting chemokines and CSFs to enhance recruitment/differentiation/activation of macrophages and dendritic cells. Because macrophages are a rich source of TNF- α , the concerted actions of IL-1 β and macrophage-derived TNF- α may promote a feed-forward cycle to promote an excess of decidual macrophages in preeclampsia. The resulting impaired extravillous cytotrophoblast invasion and incomplete remodeling of the spiral arteries which decreases utero-placental blood flow to produce placental hypoxia. That hypoxia-reoxygenation enhances output of TNF- α in human placental tissue⁵² suggests that the placenta is an additional source of TNF- α .

In summary, the current use of a three-tier approach (ie, *in situ* and *in vivo* measurements in human decidual cells followed by *in vivo* confirmation) in the decidua of a mouse model for preeclampsia suggests that elevated GM-CSF expression plays an integral role in the pathogenesis of preeclampsia. Future studies that use GM-CSF null mice and extend the strategy used in the current study to include other preeclampsia-associated proteins offer great promise in making strides in the diagnosis and treatment of this major pathology of human pregnancy.

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