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Preeclampsia-related increase of interleukin-11 expression in human decidual cells

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Abstract

Preeclampsia is associated with increased systemic inflammation and superficial trophoblast invasion, which leads to insufficient uteroplacental blood flow. Interleukin (IL)-11 mediates pro- and anti-inflammatory processes and facilitates decidualization. To identify IL11 expression *in vivo* at the maternal–placental interface in preeclampsia and control specimens and to evaluate the regulatory effects of tumor necrosis factor-α (TNF) and IL1B, cytokines elevated in preeclampsia, on IL11 levels in first trimester decidual cells *in vitro*, placental sections were immunostained for IL11. Leukocyte-free first trimester decidual cells were incubated with estradiol (E_2)$\pm 10^{-7}$ mol/l medroxyprogesterone acetate±TNF or IL1B± inhibitors of the p38 MAP kinase (p38 MAPK), nuclear factor-κ B (NFKB), or protein kinase C (PKC) signaling pathways. An ELISA assessed secreted IL11 levels, and quantitative RT-PCR measured *IL11* mRNA. IL11 immunoreactivity in placental sections was significantly higher in the cytoplasm of preeclamptic decidual cells versus gestational age-matched controls. Compared to decidual cells, IL11 immunostaining in neighboring trophoblast is lower, perivascular, and not different between control and preeclamptic specimens. TNF and IL1B enhanced levels of *IL11* mRNA and secreted IL11 in cultured decidual cells. Specific inhibitors of the p38 MAPK and NFKB, but not PKC signaling pathways, reduced the stimulatory effect of IL1B. Expression of decidual IL11 is increased in preeclampsia and suggests a role for IL11 in the pathogenesis of preeclampsia.
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Introduction

The concerted actions of estradiol (E_2) and progesterone transform human endometrial stromal cells into decidual cells during the luteal phase of the menstrual cycle and maintain the decidua throughout gestation (Tabanelli *et al*. 1992). During implantation, blastocyst-derived extravillous trophoblasts (EVTs) invade the decidua and remodel spiral arteries into low-resistance, high-capacity vessels that markedly increase uteroplacental blood flow (Pijnenborg *et al*. 2006). The decidua normally constrains trophoblast invasion, which involves sequential attachment to and proteolysis of basement membrane proteins in the peri-decidual extracellular matrix (ECM; Damsky *et al*. 1994, Cohen *et al*. 2006). Shallow EVT invasion leads to incomplete vascular transformation and reduced blood flow to the developing fetal–placental unit (Caniggia *et al*. 2000, Pijnenborg *et al*. 2006). Impaired decidual invasion is the primary placental defect of preeclampsia, a leading cause of fetal and maternal morbidity and mortality and a primary contributor to preterm delivery (reviewed in Sibai *et al*. (2005)). Preeclampsia is associated with systemic inflammation (Sibai *et al*. 2005) and a decidual influx of macrophages (Reister *et al*. 2001, Abrahams *et al*. 2004, Lockwood *et al*. 2006) and dendritic cells (Huang *et al*. 2008) that promote immune maladaption at the implantation site.

Interleukin-11 (IL11) belongs to the IL6 family of cytokines that exert diverse biological effects by binding to surface receptor complexes comprised of a ligand-specific α chain with at least one subunit of the GP130 signal transducer (Heinrich *et al*. 2003). Initially identified as a hematopoiesis-promoting factor capable

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of enhancing growth of myeloid, erythroid, and megakaryocytic progenitor cells, IL11 was later found to mediate a complex array of pro- and anti-inflammatory effects (Trepicchio & Dorner 1998). In normal mice, uterine IL11 synthesis peaks during decidualization. Transgenic IL11 receptor α (*Il11ra*) gene knockout mice are infertile because of defective decidualization, which leads to dysregulated trophoblast invasion and proliferation and ends in necrotic loss of the fetus (Robb *et al*. 1998). Microarray results from control and pseudopregnant *Il11ra* knockout mice suggest that IL11 regulates changes in the uterine ECM required for decidualization (White *et al*. 2004). The decidua exhibits the most prominent immunostaining for IL11 and IL11RA at the implantation site of humans and other primates (Dimitriadis *et al*. 2003). In women, abnormal decidual and villous trophoblast IL11 expression leads to early pregnancy loss (Chen *et al*. 2002). Both IL11 and IL11RA mRNA and protein are localized in decidualized stromal cells during the luteal phase of cycling human endometrium (von Rango *et al*. 2004). In stromal cell monolayers from predecidualized human endometrium, IL11 has been shown to advance progestin-induced morphological and biochemical decidualization markers (Dimitriadis *et al*. 2002).

Given the complex involvement of IL11 expression with inflammation, decidualization, and trophoblast invasion, we posited an association between decidual IL11 expression and preeclampsia. To test this hypothesis, IL11 immunohistochemical levels were compared in the decidua of preeclamptic versus gestational age-matched normal placentas. Tumor necrosis factor-α (TNF) and IL1B have been implicated in the early pathogenesis of preeclampsia (Rinehart *et al*. 1999, Hefler *et al*. 2001, Bauer *et al*. 2004, Lockwood *et al*. 2006), and the previous studies have implicated that the major sources of TNF and IL1B are secreted from macrophages in preeclamptic decidua (as paracrine interaction) (Rinehart *et al*. 1999, Hefler *et al*. 2001, Reister *et al*. 2001, Bauer *et al*. 2004).

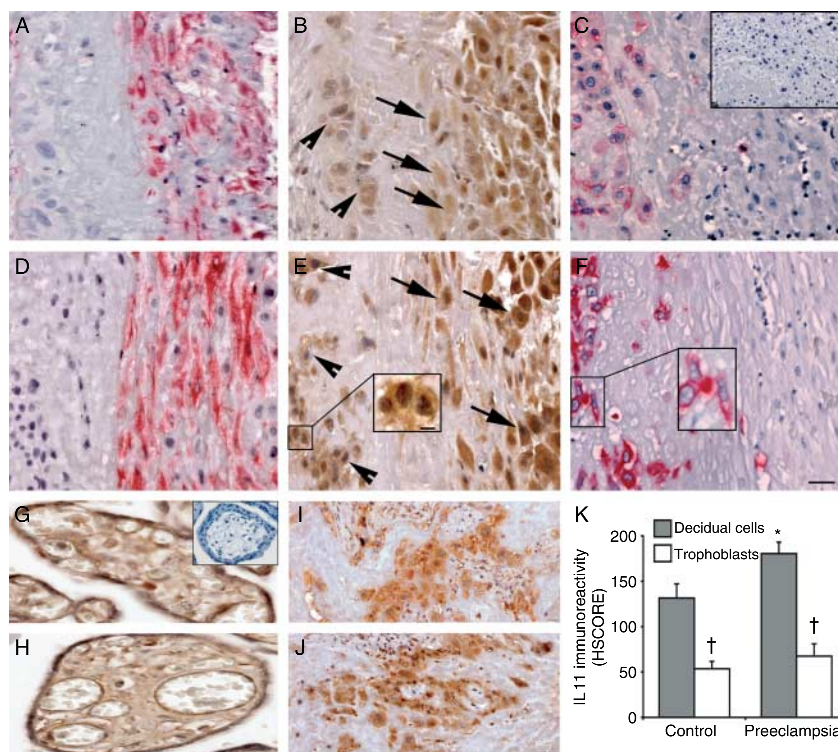

Figure 1 Immunohistochemical analysis of IL11 expression in control and preeclamptic decidua. Representative micrographs of serial sections from control (A–C) and preeclamptic (D–F) specimens are shown. Vimentin immunostaining (red) identifies decidual cells in control (A) and preeclamptic (D) tissues. Cytokeratin immunostaining (red) identifies trophoblastic cells in control (C) and preeclamptic (F) tissues. IL11 immunostaining (brown) in decidual cells (arrows) was cytoplasmic and more intense than in interstitial trophoblast (arrowheads), where IL11 localization was mostly perimembranous (E inset). Between groups, IL11 immunostaining was greater in preeclamptic (E) versus control (B) decidual cells, while there was no significant difference among interstitial trophoblast. IL11 intensity HSCOREs in control and preeclamptic specimens (mean ± s.e.m.) are shown (K); *, versus control decidual cells; †, versus group-respective decidual cells; $P<0.05$. Additionally, no statistical significance for IL11 HSCOREs was observed between chorionic villi of preeclamptic and control tissues (G and H). On the other hand, no significant difference was found for the IL11R expression between preeclamptic (J) versus control interstitial cytotrophoblasts (I). Parallel staining with a mouse isotype was used as a negative control for IL11 MAB (C and G; inset). Note that IL11 immunoreactivity in interstitial trophoblast was mostly perimembranous, especially in preeclampsia specimens (E, inset). The scale bar in panel F represents 50 μm for all panels, except the panel E inset, where it represents 20 μm.

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Therefore, the effects of these classic pro-inflammatory cytokines were assessed on IL11 mRNA and protein expression in first trimester decidual cells, the predominant cell type encountered by invading EVTs at the implantation site (Dunn *et al*. 2003). The integral role that progesterone plays in inducing and maintaining decidualization (Tabanelli *et al*. 1992, Dunn *et al*. 2003) prompted evaluation of effects of a progestin with and without TNF or IL1B on IL11 expression in the cultured decidual cells. Incubations were extended to include specific inhibitors of the nuclear factor-κ B (NFKB), p38 MAP kinase (p38 MAPK), or protein kinase C (PKC) pathways since each has been shown to act as intracellular mediators of cytokine effects on IL11 expression in many cell types (Lacroix *et al*. 1998, Bamba *et al*. 2003, Scicchitano *et al*. 2008).

Results

Immunostaining of IL11 and IL11R in decidua of control and preeclamptic specimens

Control and preeclamptic decidua were immunostained for IL11, vimentin, and cytokeratin (Fig. 1). Decidual cells and interstitial cytotrophoblasts were identified by positive (red) vimentin and cytokeratin staining respectively in control (Fig. 1A and C) and preeclamptic (Fig. 1D and F) specimens. HSCORE analysis revealed that immunoreactivity for IL11 (brown) was significantly greater in the cytoplasm of decidual cells from preeclamptic tissues (Fig. 1E) compared with control (Fig. 1B) specimens (mean ± s.e.m. HSCORE: 186 ± 13 versus 121 ± 15 respectively, $P<0.05$; Fig. 1E). However, IL11 HSCOREs in the cytoplasm of interstitial trophoblasts were not significantly different between preeclamptic (Fig. 1E) and control (Fig. 1B) tissues (71 ± 15 versus 52 ± 10 respectively; Fig. 1K). Additionally, no statistical significance for IL11 HSCOREs was observed between chorionic villi of preeclamptic and control tissues (Fig. 1G and H). Within both groups, decidual cells had significantly greater IL11 HSCOREs than interstitial trophoblasts (186 ± 13 versus 71 ± 15 in preeclamptic tissues (Fig. 1E) and 121 ± 15 versus 52 ± 10 in control tissues (Fig. 1B) respectively; $P<0.05$). Notably, while IL11 immunostaining in vimentin-positive decidual cells was homogenous and cytoplasmic, the immunoreactivity in interstitial trophoblasts (Fig. 1E, inset) was mostly perimembranous.

Normal and preeclamptic decidual tissues were immunostained for IL11R expression, which is predominantly found in the interstitial cytotrophoblasts, while decidual cells have a weak IL11R immunoreactivity. No significant difference was found for the IL11R expression between preeclamptic (Fig. 1J) versus control interstitial cytotrophoblasts (Fig. 1I).

IL11 protein secretion in cultured decidual cells

Individual and interactive effects of medroxyprogesterone acetate (MPA) and cytokines were assessed on levels of IL11 secreted by leukocyte-free first trimester decidual cells. Figure 2 indicates that secreted basal IL11 levels were not significantly different between incubations with E_2 (0.38 ± 0.20 pg/ml per μg protein) and E_2 + MPA (0.17 ± 0.07 pg/ml per μg protein). In the E_2-treated cells, TNF and IL1B significantly increased IL11 output by 5.6 ± 1.6- and 385.4 ± 191.6-fold respectively ($P<0.05$). The addition of MPA reduced the response to each cytokine by half for both TNF and IL1B ($P<0.05$). This inhibition by MPA virtually eliminated the response to TNF as secreted IL11 levels did not differ significantly between parallel incubations with E_2 + MPA and E_2 + MPA + TNF. However, the IL1B-mediated increase in IL11 output in incubations with E_2 + MPA was still greater than baseline E_2 + MPA ($P<0.05$) and was reduced compared with E_2 + IL1B. Both IL1B- and TNF-enhanced IL11 expression was dose dependent over a concentration range of 0.01–10.0 ng/ml (Fig. 3).

IL11 mRNA expression in cultured decidual cells

The effects of IL1B and TNF on steady-state *IL11* mRNA levels in first trimester decidual cell monolayers were determined by quantitative real-time RT-PCR (Fig. 4). In E_2 cultures, TNF and IL1B enhanced *IL11* mRNA levels by 1.55 ± 0.65- and 18.8 ± 5.1-fold respectively, although only the effects of IL1B were statistically significant. A similar IL1B-stimulated enhancement of *IL11* mRNA levels was noted in incubations with E_2 + MPA, with an increase in *IL11* mRNA levels by 7.6 ± 1.3-fold ($P<0.05$). Consistent with the IL11 protein results presented above, IL1B was more effective

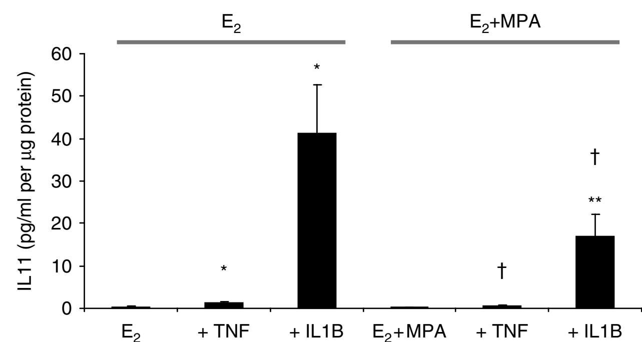

Figure 2 Effects of E_2, MPA, TNF, and IL1B on IL11 output by decidual cell monolayers. Confluent, leukocyte-free first trimester decidual cells were incubated for 7 days in 10^{-8} M E_2 or $E_2 + 10^{-7}$ M MPA and then switched to defined medium (DM) with corresponding steroid(s) ± 1 ng/ml of TNF or IL1B for 24 h. IL11 levels were measured by ELISA in conditioned DM and normalized to total protein levels. Bars represent mean ± s.e.m. of IL11 levels as pg/ml per μg cell protein ($n=14$ separate patients' decidual specimens). * versus E_2, ** versus E_2 + MPA, † versus corresponding E_2 + TNF or IL1B ($P<0.05$).

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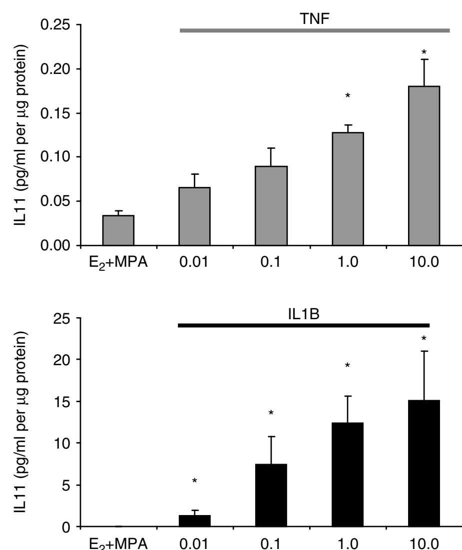

Figure 3 Concentration-dependent effects of IL1B and TNF on IL11 output by decidual cell monolayers maintained in E_2 + MPA. Confluent, leukocyte-free first trimester decidual cells were incubated for 7 days in 10^{-8} M $E_2 + 10^{-7}$ M MPA, and then switched to DM with the steroids ± the indicated amount of IL1B (0.01–10.0 ng/ml) or TNF (0.01–10.0 ng/ml). IL11 levels were measured by ELISA in conditioned DM and normalized to cell protein. Bars represent mean ± S.E.M. of IL11 levels as pg/ml per μg cell protein (n=3 separate patients' decidual specimens, mean ± S.E.M.). * versus E_2 + MPA; $P<0.05$.

than TNF in upregulating steady-state *IL11* mRNA levels, and the cytokine effects were blunted by the addition of MPA.

Intracellular mediators of IL1B-enhanced IL11 secretion in cultured decidual cells

NFKB activation inhibitor III at 10^{-5} M, SB203580 at 10^{-5} M (p38 MAPK inhibitor), and Calphostin C at 10^{-7} M (PKC inhibitor) were used to infer the intracellular signaling pathways involved in IL1B-enhanced IL11 expression. Both the p38 MAPK and NFKB inhibitors virtually eliminated the effects of IL1B on IL11 output (Fig. 5). Specifically, the IL1B-induced increase in IL11 output was reduced from 60.0 ± 30.9 to 2.62 ± 1.27 pg/ml per μg by the p38 MAPK inhibitor and to 1.04 ± 0.29 pg/ml per μg by the NFKB inhibitor ($P<0.05$; Fig. 5). By contrast, the addition of the PKC inhibitor did not significantly reduce IL1B-enhanced IL11 output (Fig. 5).

Discussion

Shallow EVT invasion of the basement membrane (BM) protein-enriched decidualized ECM is the primary placental insult leading to preeclampsia (Sibai *et al.* 2005). In view of abundant evidence linking local IL11 expression to decidualization of human endometrium (Dimitriadis *et al.* 2002, von Rango *et al.* 2004), IL11 immunostaining was compared in the decidua of preeclamptic versus gestational age-matched preterm controls. Immunoreactive IL11 in decidual cells was primarily localized in the cytoplasm, which suggests *in vivo* production of IL11 in decidual cells. Significant increases in intensity and numbers of IL11-positive decidual cells as indicated by HSCOREs in preeclamptic versus control women suggest a role for IL11 in the inflammatory process of preeclampsia. Although IL11 immunostaining in adjacent interstitial cytotrophoblasts was not significantly greater in the preeclamptic specimens, this staining was much less prominent than that observed in the decidual cells and was primarily perimembranous. This differential localization of IL11 between the two cell types suggests a potential, previously undisclosed, paracrine interaction in which IL11 is synthesized and secreted by decidual cells, and then bound to receptors in adjacent interstitial trophoblasts.

By contrast, IL11 attenuates macrophage-associated inflammation by inhibiting NFKB-mediated effector function, as reflected in reduced expression of IL12, IL6, IL1B, and TNF as well as nitric oxide production from activated macrophages (Trepicchio & Dorner 1998). Therefore, unlike the chronic inflammatory action of IL6, augmented IL11 expression in decidual cells in response to IL1B or TNF appears to exert an anti-inflammatory effect. Not only is the action of IL11 inconsistent with macrophage-impaired trophoblast invasion of the decidua, but also IL11 has now been shown to enhance migration of primary EVTs by a mechanism that involves STAT signaling (Paiva *et al.* 2007). Such increase in EVT migration was observed even at very high IL11 concentrations (100 ng/ml), arguing strongly against a direct role for IL11 in impaired EVT invasion that leads to preeclampsia.

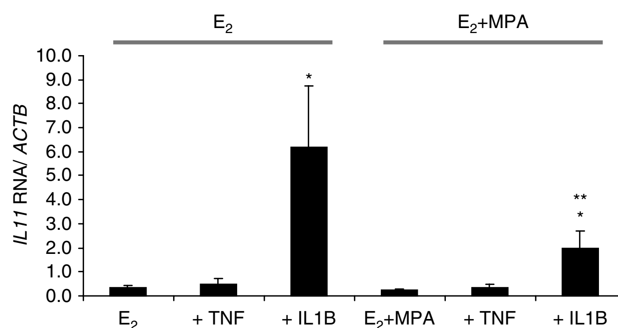

Figure 4 Effects of E_2, MPA, TNF, and IL1B on *IL11* mRNA levels in decidual cell monolayers. Confluent leukocyte-free first trimester decidual cells were incubated for 7 days in 10^{-8} E_2 or 10^{-8} M $E_2 + 10^{-7}$ M MPA, and then switched to DM with corresponding steroid(s) ±1 ng/ml of TNF or IL1B for 6 h. Aliquots of extracted total RNA were used to measure *IL11* mRNA levels by quantitative real-time RT-PCR. Bars represent mean ± S.E.M. of IL11 mRNA/*ACTB* mRNA levels (n=5 separate patients' decidual specimens). * versus E_2 or E_2 + MPA; ** versus E_2 + IL1B; $P<0.05$.

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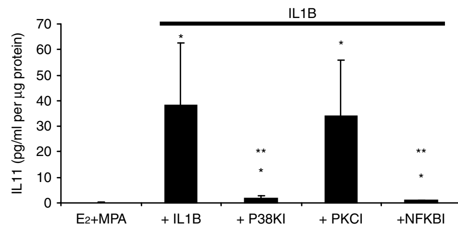

Figure 5 Involvement of NFKB, p38 MAPK, and PKC signaling in IL1B-enhanced IL11 output by decidual cells maintained in E_2+MPA. Confluent, leukocyte-free first trimester decidual cells were incubated for 7 days in 10^{-8} M E_2+10^{-7} M MPA, and then switched to DM with the steroids alone ± 1 ng/ml IL1B with and without an NFKB inhibitor (activation inhibitor III, NFKBI) at 10^{-5} M, or a p38 MAP kinase inhibitor (SB203580; p38KI) at 10^{-5} M, or a PKC inhibitor (Calphostin C, PKCI) at 10^{-7} M. Bars represent mean$\pm$s.e.m. of IL11 levels as pg/ml per µg cell protein as measured by ELISA in conditioned DM and normalized to cell protein ($n=4$ separate patients' decidual specimens). * versus E_2+MPA; $P<0.05$. **versus E_2+1L1B; $P<0.05$.

In humans and mice, the expression of IL11 and its receptor IL11RA is tightly coupled to the induction and maintenance of decidualization (Robb *et al*. 1998, Dimitriadis *et al*. 2002, von Rango *et al*. 2004, White *et al*. 2004). The integral role that decidualization plays in restraining the intrinsically invasive EVTs is evident in the uncontrolled invasion with often life-threatening consequences, which stems from implantation at sites where the decidua is absent as in ectopic pregnancies including cesarean scar pregnancies or deficient as in placenta accreta (Norwitz 2006, Rosen 2008). Invasion of the decidua involves sequential attachment of EVT-expressed adhesion molecules, particularly integrins, which recognize basement membrane-type proteins in the decidual ECM, followed by their degradation (Damsky *et al*. 1994, Cohen *et al*. 2006, Pijnenborg *et al*. 2006). Several studies have evaluated the role of trophoblast-expressed matrix metalloproteinases (MMPs 2 and 9), which preferentially degrade basement membrane proteins, in promoting EVT invasion of the decidua (Cohen *et al*. 2006). Recently, we found that IL1B and TNF markedly enhanced MMP9 expression in leukocyte-free first trimester decidual cell cultures by about 100- and 1000-fold respectively, and that decidual cells in placental sections from cases of preeclampsia displayed significantly higher immunohistochemical MMP9 levels compared with controls (Lockwood *et al*. 2008*a*). Preferential degradation of basement membrane proteins in the decidual ECM by this aberrantly large increase in MMP9 expression in decidual cells is expected to dysregulate the sequential invasion of the decidua by EVTs and interfere with remodeling of the spiral arteries and arterioles required to increase uteroplacental blood flow to the developing fetal–placental unit (Damsky *et al*. 1994, Pijnenborg *et al*. 2006).

The absence of a direct inhibitory effect on EVT migration (Paiva *et al*. 2007), taken together with the crucial role that IL11 plays in decidualization, suggests that excess IL11 indirectly impedes EVT invasion by augmenting decidual cell-expressed basement membrane protein synthesis and/or degradation or by alternative as yet unidentified mechanism(s). One study by Paiva *et al*. (2009) reported that IL11 inhibits the EVT invasion resulting in shallow placentation, which supports our hypothesis that IL11 may be involved in pathogenesis of preeclampsia by regulating trophoblast invasion. Preeclampsia is associated with placental oxidative stress and activation of NFKB and p38 MAPK pathways in placental villi. The observations in this study that both the NFKB and p38 MAPK signaling pathways are involved in inflammatory cytokine-enhanced IL11 expression in first trimester human decidual cells suggest that one or both pathways may be involved in mediating preeclampsia-related pathogenic changes in decidual cells *in vivo*.

In conclusion, our observations that decidual expression of IL11 is increased in preeclampsia *in vivo* and that IL11 is regulated by inflammatory cytokines IL1B and TNF in cultured decidual cells implicate IL11 in the pathophysiology of preeclampsia. One possibility is that elevated IL11 expression may be resultant of the earlier insults leading to preeclampsia and not causative of them. Further studies investigating the mechanistic role of IL11 in normal and preeclamptic decidual cells are needed to explore these possibilities and to hopefully shed new light on our understanding of the pathogenesis of preeclampsia.

Materials and Methods

Patients and tissue

For immunohistochemistry, placental biopsies from the decidual basalis were obtained from preeclamptic ($n=9$) and gestational age-matched idiopathic preterm labor control ($n=8$) placentas, under approval of the Yale University School of Medicine Human Investigation Committee. Table 1 provides relevant clinical details of the patients and controls. Preeclampsia was diagnosed according to standard criteria (ACOG Committee on Practice Bulletins–Obstetrics 2002) as systolic and diastolic blood pressure ≥ 140 and ≥ 90 mmHg respectively and proteinuria (≥ 0.3 g protein in a 24 h urine collection), occurring after 20 weeks of gestation in a woman who previously had normal blood pressure. Seven out of the nine preeclampsia patients had severe preeclampsia, defined as systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg on two occasions at least 6 h apart, with proteinuria >5 g in a 24 h urine collection. All preeclamptic placental specimens were obtained from cesarean delivery without labor. Control specimens were placentas from idiopathic preterm labor without clinical or histological evidence of chorioamnionitis or chronic villitis, and were obtained after either cesarean or vaginal delivery.

For cell cultures, first trimester decidual specimens from 14 uncomplicated, elective terminations between 8 and 12 weeks

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Table 1 Characteristics of the women who provided placental samples.

	Preterm control (n=8)	Preeclampsia (n=9)	P value
Age (years)[a]	29.3±3.0	29.4±3.8	NS
Nulliparity[b]	5 (50)	5 (50)	NS
Gestational age (weeks)[a]	30.5±1.0	29.7±1.2	NS
Systolic blood pressure (mmHg)[a]	117±6	172±7	<0.001
Diastolic blood pressure (mmHg)[a]	62±3	102±2	<0.001
Dipstick protein[c]	0 (0–0)	4 (2–4)	<0.001
24 h protein (g)	NA	5 (0.7–7.2)	NA
IUGR	0 (0)	3 (30)	NS
HELLP syndrome	0 (0)	3 (30)	NS
PPROM[b]	5 (50)	0 (0)	<0.05
Birth weight (g)[a]	1532±202	1077±139	NS
Cesarean delivery[b]	3 (30)	8 (80)	NS
Histological chorioamnionitis stages II–III[a]	0 (0)	0 (0)	NS

PPROM, preterm premature rupture of the membranes; IUGR, intrauterine growth restriction; HELLP syndrome, syndrome of hemolysis, elevated liver enzymes, and low platelets.
[a]Data are represented as mean±S.E.M. and analyzed by Student's *t*-test.
[b]Data are represented as *n* (%) and analyzed by Fisher's exact test.
[c]Data are represented as median and analyzed by Mann–Whitney test.

of gestation were obtained under Institutional Review Board approval at Bellevue Hospital, New York, NY, USA. Decidual tissues were separated from the amnio-chorion, and a small portion of each was formalin-fixed and paraffin-embedded for histological examination for any signs of underlying acute or chronic inflammation. The remainder of each specimen was used for decidual cell isolation and culture.

All patients gave informed, written consent.

Immunohistochemistry

IL11 and IL11R immunostaining was performed as described previously (Kayisli *et al*. 2002). Briefly, 5 μm formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated through a descending ethanol series. Antigen retrieval was then performed by boiling the slides in citrate buffer (10 mM; pH 6.0) for 15 min, followed by endogenous peroxidase quenching with 3% hydrogen peroxide (in 50% methanol/50% distilled water) for 15 min. The slides were then incubated with 5% blocking horse serum (Vector Labs, Burlingame, CA, USA) in Tris-buffered saline (TBS) for 30 min at room temperature in a humidified chamber. Excess serum was then removed, and serial sections were incubated with either mouse monoclonal IL11 antibody (R&D Systems, Minneapolis, MN, USA) at 10 μg/ml in 1% blocking horse serum in TBS, or monoclonal IL11R antibody (R&D Systems) at 10 μg/ml in 1% blocking horse serum in TBS, or mouse monoclonal anti-vimentin antibody (DakoCytomation, Carpinteria, CA, USA) at 1:100 dilution in TBS, or anti-cytokeratin antibody (DakoCytomation) at 1:100 dilution in TBS overnight in a humidified chamber at +4 °C. Non-specific mouse IgG isotype antibody was used at the same concentrations as the primary antibodies for negative controls. After washing, the slides were incubated with biotinylated horse anti-mouse secondary antibody (Vector Labs) at 1:400 dilutions for 30 min at room temperature. After washing again in TBS, the antigen–antibody complex was detected with an avidin–biotin peroxidase or alkaline phosphatase kit (Vector Labs). Diaminobenzidine (3,3-diaminobenzidine tetrahydrochloride dihydrate) and Fast Red (Vector Labs) were used as the chromogens to detect IL11 and vimentin immunoreactivity respectively. Slides were then counterstained with hematoxylin and mounted.

The intensity of IL11 and IL11R immunostaining was semi-quantitatively evaluated using HSCORE analysis. Immunostaining intensity was categorized into the following scores: 0 (no staining), 1 (weak, but detectable, 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 HSCORE$=\sum_i i \times P_i$, where i represents the intensity category score; and P_i is the corresponding percentage of cells (Guzeloglu Kayisli *et al*. 2004). For each slide, five different fields were evaluated microscopically at ×200 magnification. HSCORE evaluation was performed independently by two investigators blinded to the source of the samples; the average score of both was then used.

Isolation of decidual cells

Decidual cell isolation was performed as described previously (Lockwood *et al*. 2008*b*). Briefly, minced tissues were digested with 0.1% collagenase type IV and 0.01% DNAse in RPMI containing 20 μg/ml penicillin/streptomycin and 1 μl/ml fungizone (Invitrogen) in a 37 °C shaking water bath for 30 min, and then washed with sterile PBS three times and subjected to consecutive filtration through 100, 70, and 40 μm Millipore filters. Cells were resuspended in RPMI and then grown to confluence on polystyrene tissue culture dishes. After harvesting with trypsin/EDTA, cells were analyzed by flow cytometric analysis with anti-CD45 and anti-CD14 MABs (BD Pharmingen, San Diego, CA, USA) to determine the presence of leukocytes after each passage. Cultures were found to be leukocyte free (<1%) after three to four passages. Cultured decidual cells were also found to be vimentin-positive and cytokeratin-negative, and displayed decidualization-related morphologic and biochemical changes during incubation with a progestin. Decidualization-related biochemical changes were detected as enhanced expression of prolactin and plasminogen activator inhibitor-1 and inhibited expression of interstitial collagenase and stromelysin-1 as found previously (Lockwood *et al*. 2008*a*, 2008*b*, Oner *et al*. 2008). Cell aliquots were frozen in FCS/DMSO (9:1; Sigma–Aldrich) and kept in liquid nitrogen for future uses.

Experimental incubations

Thawed cells were seeded onto polystyrene culture-treated flasks and incubated in BMS, which consists of basal medium (a phenol red-free 1:1 v:v mix of DMEM (Invitrogen), and Ham's F-12 (Flow Labs, Rockville, MD, USA), with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone)

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supplemented with 10% charcoal-stripped calf serum. After two additional passages, confluent cultures were incubated in parallel in BMS containing either 10^{-8} mol/l E_2 with or without 10^{-7} mol/l MPA (Sigma–Aldrich). For these incubations, MPA was substituted for progesterone because of its greater stability in culture (Arici *et al.* 1999), whereas E_2 served as the control incubation for E_2 plus MPA. The latter was employed to mimic elevated circulating E_2 and progesterone levels during the first trimester of pregnancy. After 7 days, the cultures were washed twice with HBSS to remove residual serum elements. The cultures were switched to a defined medium (DM) consisting of basal medium plus ITS+ (Collaborative Research, Waltham, MA, USA), 5 μM $FeSO_4$, 50 μM $ZnSO_4$, 1 nM $CuSO_4$, 20 nM Na_2SeO_3, trace elements (Invitrogen), 50 μg/ml ascorbic acid (Sigma–Aldrich), and 50 ng/ml epidermal growth factor (Becton-Dickinson, Bedford, MA, USA) with either vehicle or steroids or 0.01–10 ng/ml of IL1B or TNF.

In selected experiments, after priming with E_2 plus MPA in BMS for 7 days, the cultures were switched to DM containing the steroids ±1 ng/ml of IL1B or TNF and ±a signaling pathway inhibitor added at the concentration recommended by the manufacturer. Specifically, these were NFKB inhibitor (activation inhibitor III) at 10^{-5} M, p38 MAPK inhibitor (SB203580) at 10^{-5} M, and PKC inhibitor (Calphostin C) at 10^{-7} M (all from EMD Biosciences Inc., Gibbstown, NJ, USA). After the test period, cells were harvested by scraping in ice-cold lysis buffer solution of TBS with 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride (Sigma), and complete protease inhibitor cocktail (Roche), and then briefly sonicated. Conditioned medium supernatants and cell lysates were stored at −70 °C. Total RNA was extracted from five separate, parallel incubations with TRIzol Reagent (Sigma–Aldrich).

ELISA

Total cell protein was assessed using a modified Lowry assay (Bio-Rad Laboratories Inc.). An ELISA kit (R&D Systems) was used to measure IL11 levels in the cell-conditioned DM according to the manufacturer's instructions. The sensitivity of the ELISA is 8 pg/ml, with intra-assay and inter-assay coefficients of variation of 2.4 and 6.9% respectively. According to the manufacturer, there is no significant cross-reactivity or interference by other cytokines in this assay.

Real-time quantitative RT-PCR

To verify that the *IL11* and β-actin (*ACTB*) probes yielded the correct bands, RNA was extracted from experimental cell incubations and subjected to semi-quantitative RT-PCR using a kit from Invitrogen, performing 35 cycles with the Eppendorf Mastercycler (Eppendorf, Westbury, NY, USA). For quantitative real-time RT-PCR, RT was carried out with AMV reverse transcriptase (Invitrogen). A quantitative standard curve was established (40 pg to 2.5 ng of cDNA) with a Roche Light Cycler (Roche) by monitoring increasing PCR product fluorescence during amplification. Quantitation of the unknowns was determined with the Roche Light Cycler and normalized to *ACTB* levels from the corresponding unknowns. Melting curve analysis confirmed the specificity of the amplified products and

Table 2 Primers for real-time quantitative RT-PCR.

Gene	Sense (5′–3′)	Antisense (5′–3′)	Size (bp)
ACTB	CGTACCACTGGCATCGTGAT	GTGTTGGCGTACAGGTCTTTG	452
IL11	ACAGTACCCGTATGGG	CCGGTCTCGAACTCTT	306

the absence of primer–dimer formation. All products generated the correct melting temperatures. Products were then run on a 1.2% agarose gel along with a 100 bp DNA ladder and visualized with ethidium bromide. The primers (Table 2) were synthesized and gel purified at the Yale DNA Synthesis Laboratory, Critical Technologies (New Haven, CT, USA).

Statistical analysis

Gestational ages of tissue specimens were normally distributed and analyzed by Student's *t*-test. Immunohistochemistry HSCOREs were also normally distributed (Kolmogorov–Smirnov test) and analyzed using one-way ANOVA, with *post hoc* Holm–Sidak testing. Control and treatment groups in culture conditions were compared by the Kruskal–Wallis ANOVA on ranks test followed by the Student–Newman–Keuls *post hoc* test. In all comparisons, statistical significance was defined as $P<0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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