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Interferon-α and Angiogenic Dysregulation in Pregnant Lupus Patients Who Develop Preeclampsia

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Objective. **To investigate whether an elevated interferon-α (IFNα) level early in pregnancy is associated with poor pregnancy outcomes and to examine the relationship of an elevated IFNα level to angiogenic imbalance.**

Methods. **Women were enrolled in a longitudinal case–control study of pregnant patients with lupus. Serum samples obtained monthly throughout pregnancy were assayed for IFNα and for the antiangiogenic factor soluble Flt-1 and the proangiogenic factor placenta growth factor (PlGF). Each of 28 patients with systemic lupus erythematosus (SLE) with a poor pregnancy outcome was matched to an SLE patient with an uncomplicated pregnancy and to a pregnant healthy control. The effects of IFNα and/or soluble Flt-1 on human endothelial cells and endothelial cell–trophoblast interactions were assessed.**

Results. **Compared to SLE patients with uncomplicated pregnancies, patients with preeclampsia had increased IFNα levels before clinical symptoms. Patients without autoimmune disease who developed preeclampsia did not have increased IFNα levels. In SLE patients with low IFNα levels, marked angiogenic imbalance (higher soluble Flt-1, lower PlGF, and higher soluble Flt-1:PlGF ratios) preceded maternal manifestations of preeclampsia, whereas in SLE patients with high IFNα levels, preeclampsia occurred without evidence of systemic angiogenic imbalance. Treatment of human endothelial cells with soluble Flt-1 induced expression of *sFLT1* messenger RNA, and IFNα dramatically amplified responses to soluble Flt-1. In a model of spiral artery transformation, only the combination of IFNα and soluble Flt-1 disrupted the ability of trophoblast cells to remodel endothelial tube structures.**

Conclusion. **Our findings identify a new mechanism by which IFNα induces an antiangiogenic milieu and increases the sensitivity of endothelial cells to soluble Flt-1, and suggest that elevated IFNα levels may contribute to the pathogenesis of preeclampsia in some pregnant patients with SLE.**

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Systemic lupus erythematosus (SLE), the prototypical systemic autoimmune disease, predominantly affects women and presents during the reproductive years. Pregnancy in patients with SLE is associated with an increased risk of maternal and fetal morbidity and mortality, including premature birth, miscarriage, fetal growth restriction, preeclampsia, and neonatal death (1–3). Placental dysfunction plays a major role in these complications.

Normal placental development requires coordinated expression of the angiogenic growth factors vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF), as well as expression of their receptors on invasive trophoblasts, VEGF receptor 1 (VEGFR-1; also known as Flt-1) and VEGFR-2 (4,5). Placental trophoblasts release a splice variant of VEGFR-1, soluble VEGFR-1 (sVEGFR-1; also known as soluble Flt-1) that sequesters circulating VEGF and PlGF, prevents their binding to trophoblast and endothelial cell receptors, and thus acts as a potent antiangiogenic growth factor (6). Adequate placental perfusion requires remodeling of uterine spiral arteries into dilated, flaccid vessels, a process that is dependent on trophoblast invasion and replacement of endothelium (7). Imbalance of angiogenic factors is associated with abnormal placental invasion and subsequent hypoperfusion and fetal growth restriction (8–10).

Circulating soluble Flt-1 levels normally increase slowly throughout pregnancy, but in women who develop preeclampsia, soluble Flt-1 levels are markedly increased in the blood and placenta and soluble Flt-1:PlGF ratios are increased, leading to the clinical manifestations of preeclampsia: widespread endothelial dysfunction, hypertension, and proteinuria (4,11,12). Inflammatory mediators, specifically tumor necrosis factor α (TNFα) and oxidants, have also been associated with placental insufficiency, fetal growth restriction, and renal structural alterations characteristic of preeclampsia (13). It is not known whether immune dysregulation associated with SLE contributes to a risk of poor pregnancy outcomes.

Type I interferons (IFNs), particularly IFNα, are thought to play a central role in the pathogenesis of SLE (14). Extensive data from patients with SLE demonstrate an association of IFN pathway activation, identified as an IFN signature in peripheral blood mononuclear cells, kidney, and skin tissue, with more severe disease and greater disease activity (15). Notably, IFNα is a potent antiangiogenic factor, contributing to downregulation of proangiogenic molecules such as VEGF, a decrease in hematopoietic progenitor cells involved in vascular remodeling, and impairment of vasculogenesis (16–20). Recent studies have linked type I IFNs to vascular damage and dysfunction in SLE, in part related to transcriptional repression of angiogenic factors (16,17). Given the requirement for VEGF by certain vascular beds, such as those in glomeruli (21), we considered the possibility that the endothelium of SLE patients exposed to elevated IFNα levels may be more vulnerable to the angiogenic imbalance induced by a dysfunctional placenta. We hypothesized that elevated IFNα levels contribute to an increased risk of preeclampsia and other complications in patients with SLE. Accordingly, we investigated whether pregnant SLE patients who had poor outcomes had increased IFNα activity early in pregnancy and examined the relationship between IFNα levels and angiogenic imbalance in the pathogenesis of pregnancy complications.

PATIENTS AND METHODS

Subjects. We performed a case–control study of SLE patients in the Predictors of Pregnancy Outcome: Biomarkers In Antiphospholipid Antibody Syndrome and Systemic Lupus Erythematosus (PROMISSE) study (22). Patients had the following characteristics: ages 18–45 years, live single intrauterine pregnancy confirmed by ultrasound, pregnancy of <12 weeks' gestation, hematocrit value >26%, and capacity to give informed consent. Detailed medical and obstetrical information and serial blood samples were obtained monthly beginning at 6 weeks of pregnancy.

Pregnant SLE patients met the American College of Rheumatology criteria for SLE (23). Exclusions for enrollment into the PROMISSE study at screening were: prednisone dosage of >20 mg/day, active renal disease (proteinuria >1 gm/24 hours or protein:creatinine ratio >1,000 mg protein/gm creatinine on spot urine, red blood cell casts, or serum creatinine level >1.2 mg/dl), diabetes mellitus (type 1 or type 2) antedating pregnancy, hypertension (blood pressure ≥140/90 mm Hg), and multiple fetal gestations. Disease activity was measured using the SLE Pregnancy Disease Activity Index (SLEPDAI), an instrument designed for pregnant SLE patients (24). Cases were SLE patients who had a poor pregnancy outcome, defined as one or more of the following: fetal death after 12 weeks' gestation (n = 7); preterm delivery before 36 weeks' gestation due to placental insufficiency, gestational hypertension, or preeclampsia (n = 16); or severe growth restriction (birth weight <5th percentile) (25–27) (n = 8). These events occurred most often by 28–31 weeks' gestation. Preeclampsia was defined as new onset of elevated systolic blood pressure (≥140 mm Hg) and/or elevated diastolic blood pressure (≥90 mm Hg) after 20 weeks' gestation on 2 occasions at least 4 hours apart and proteinuria of 300 mg or greater in a 24-hour urine specimen or ≥1+ on dipstick on 2 occasions at least 4 hours apart in the absence of pyelonephritis or hematuria (26,28).

Each of 28 cases (SLE patients with a poor outcome) was matched by age and ethnicity/race to an SLE patient with an uncomplicated pregnancy (SLE patients without a poor outcome) and to a pregnant healthy control. Pregnant healthy control subjects had to have had ≥1 normal pregnancy and could not have a history of fetal death at >10 weeks' gestation or >1 miscarriage at <10 weeks' gestation. The racial composition of each group was 50% white, 25% African American, 14% Asian, and 11% mixed. Ethnicity was 20% Hispanic and 80% non-Hispanic. This study was approved by the Hospital for Special Surgery Institutional Review Board.

We analyzed a second group of patients without autoimmune disease who were followed up through pregnancy at Beth Israel Deaconess Medical Center (Boston, MA), including 9 patients who developed preeclampsia and 6 patients with uncomplicated pregnancies. Stored frozen samples from both the PROMISSE and Beth Israel Deaconess cohorts were used in IFN and angiogenic factor assays.

Assessment of IFNα activity by WISH reporter cell assay. Serum samples from the 3 matched pregnancies were assayed simultaneously for IFNα activity using a reporter cell assay (29). A total of 400 samples prospectively collected during pregnancy were tested. All analyses for relative expression of *MX1* messenger RNA (mRNA) were done on samples collected at 6–27 weeks' gestation, a time before poor outcomes were clinically apparent. Results for each culture condition are reported as relative expression compared with WISH cells cultured with medium alone. Details of the real-time quantitative polymerase chain reaction (qPCR) method and data analysis have been described previously (29). In preliminary studies, we confirmed that neutralizing antibodies to IFNα decreased the elevated IFN levels seen in SLE patients to levels seen in healthy donors. *MX1* was the gene transcript that drove the IFN score in this group of patients and was chosen as a single transcript to quantify serum IFNα activity.

Maternal angiogenic factor assays. Soluble Flt-1 and PlGF were measured in duplicate using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) as previously described (30). The minimal detectable levels of soluble Flt-1 and PlGF were 5 pg/ml for both assays, and interassay coefficients of variation were 7% and 11%, respectively. Soluble Flt-1 and PlGF levels are reported as pg/ml and as the soluble Flt-1:PlGF ratio.

Endothelial cell culture and reagents. Human umbilical vein endothelial cells (HUVECs) were cultured as previously described (31) and used between passages 1 and 8. For gene expression analysis, HUVECs (50,000 cells/well) were cultured for 3 days, then incubated for 6 hours with or without recombinant IFNα (100 units/ml; PBL InterferonSource), recombinant soluble Flt-1 (5 ng/ml; R&D Systems), or both, and RNA was then purified as described below.

The human endometrial endothelial cell (HEEC) line (32) was maintained as previously described (33). For analysis of soluble Flt-1 secretion, HEECs were plated into 60-mm tissue culture dishes overnight, and then treated in serum-free Opti-MEM (Invitrogen) with or without recombinant IFNα (1,000 units/ml). After 24 hours, soluble Flt-1 in supernatants was measured by ELISA.

Endothelial cell gene expression analysis by real-time qPCR. RNA was isolated using an RNeasy kit (Qiagen), and 0.5 μg of RNA per condition was used for complementary DNA synthesis using iScript Reverse Transcription Supermix (Bio-Rad) and an ABI Veriti Thermal Cycler (Life Technologies). SsoAdvanced Universal SYBR Green Supermix and a CFX96 Real-Time System from Bio-Rad were used following the manufacturer's instructions for volumes and reaction settings. Primer sequences for *sFLT1*, *MX1*, and *GAPDH* genes have been described previously (29,34,35), and were purchased from Integrated DNA Technologies.

Endothelial cell–trophoblast coculture assay. Endothelial cell–trophoblast interactions were evaluated using an established 3-dimensional culture system (36). Briefly, HEECs (32) stained with red PKH26 (Sigma) were seeded onto 24-well tissue culture plates coated with Matrigel. After 24 hours, tube-like structures were formed (36). Medium was removed, and the human first-trimester extravillous trophoblast cell line HTR8 (37), stained with green PKH67, was added in serum-free Opti-MEM in the presence or absence of either IFNα (100 units/ml), soluble Flt-1 (5 ng/ml), or both for 24 hours. Six fields per well were recorded by fluorescence microscopy, and the number of tubes per field were counted.

Statistical analysis. Relative *MX1* levels were determined at multiple time points between 6 and 27 weeks' gestation in each patient. Because levels did not vary systematically between visits, the median value of each subject's available measurements over the time period of interest was computed and used as the unit of analysis in subsequent statistical tests. All samples for each patient were included. The repeated-measures data were also analyzed using linear mixed-effects models based on the log-1 transformed data, but results are not shown because they were nearly identical to those of the first approach. Relative *MX1* expression and other continuous variables were compared between disease groups using Wilcoxon's rank sum test. Spearman's rank correlation coefficients were estimated to assess relationships between relative *MX1* expression and SLEPDAI, anti-Ro, and antibody titers. Unadjusted and adjusted odds ratios for preeclampsia were estimated by fitting logistic regression models. *P* values less than 0.05 (2-sided) were considered significant. Endothelial gene expression data were analyzed using the $\Delta\Delta C_t$ method and GraphPad Prism software version 6.0b.

RESULTS

Elevated functional IFNα activity in early pregnancy in SLE patients who develop preeclampsia. Clinical features at enrollment in the PROMISSE study were similar for SLE patients with poor pregnancy outcomes and those with uncomplicated pregnancies (Table 1). Patients with a poor outcome were more likely to have anti-DNA antibodies at screening. All patients had low disease activity as assessed by SLEPDAI scores at screening. Serum samples were obtained monthly, and we assayed samples obtained at 6–27 weeks' gestation (average of 3.6 samples/patient), a period that generally antedated clinically apparent pregnancy complications in our patients. IFNα activity was assessed using the

Table 1. Clinical and laboratory features of SLE patients at screening*

	Patients with a poor pregnancy outcome	Patients with an uncomplicated pregnancy
Clinical features†		
Cutaneous	18/28 (64)	15/27 (56)
Arthritis	20/28 (71)	19/27 (70)
Serositis	5/28 (18)	7/27 (26)
Hematologic	14/28 (50)	14/27 (52)
Renal	15/28 (54)	10/27 (37)
Laboratory features		
Anti-DNA	16/28 (57)	9/26 (35)
Anti-Ro	9/25 (36)	16/25 (64)‡
Anti-La	2/25 (8)	6/25 (24)
Low C3	16/28 (57)	7/27 (26)§
Medications		
Hydroxychloroquine use	17/28 (61)	16/27 (59)
Steroid use	16/28 (57)	11/27 (41)
Age, mean ± SD years	31.6 ± 3.9	31.4 ± 4.5
SLEPDAI, mean ± SD	4.6 ± 3.5	2.2 ± 2.7¶

* Except where indicated otherwise, values are the number of patients affected/number of patients assessed (%). SLEPDAI = Systemic Lupus Erythematosus Pregnancy Disease Activity Index.
† Clinical features were defined according to the American College of Rheumatology criteria for systemic lupus erythematosus (SLE).
‡ $P = 0.05$ versus patients with a poor pregnancy outcome.
§ $P = 0.02$ versus patients with a poor pregnancy outcome.
¶ $P < 0.01$ versus patients with a poor pregnancy outcome.

WISH reporter cell assay, and the serial measures for each subject were summarized using the median relative expression of *MX1* (an IFNα-responsive gene). As expected, levels of IFNα activity, defined as relative *MX1* expression, were higher in serum from the SLE group than the control group (median 1.50 for all SLE patients versus 0.71 for healthy controls; $P < 0.007$). IFNα activity was higher in the SLE patients with a poor outcome compared to the healthy controls ($P < 0.004$), but was not significantly different from that in the SLE patients without a poor outcome (Figure 1A). When patients with poor outcomes were further stratified according to whether or not their pregnancy was complicated by preeclampsia, SLE patients with preeclampsia showed higher median levels of IFNα activity than SLE patients with a poor outcome other than preeclampsia (4.33 versus 1.25; $P < 0.006$) and SLE patients without a poor outcome (4.33 versus 1.33; $P = 0.04$).

Because relative *MX1* levels were similar in SLE patients without poor outcomes and those with outcomes other than preeclampsia ($P = 0.83$), we combined these groups and compared them to SLE patients with preeclampsia to increase the sample size to further evaluate the association between relative *MX1* expres-

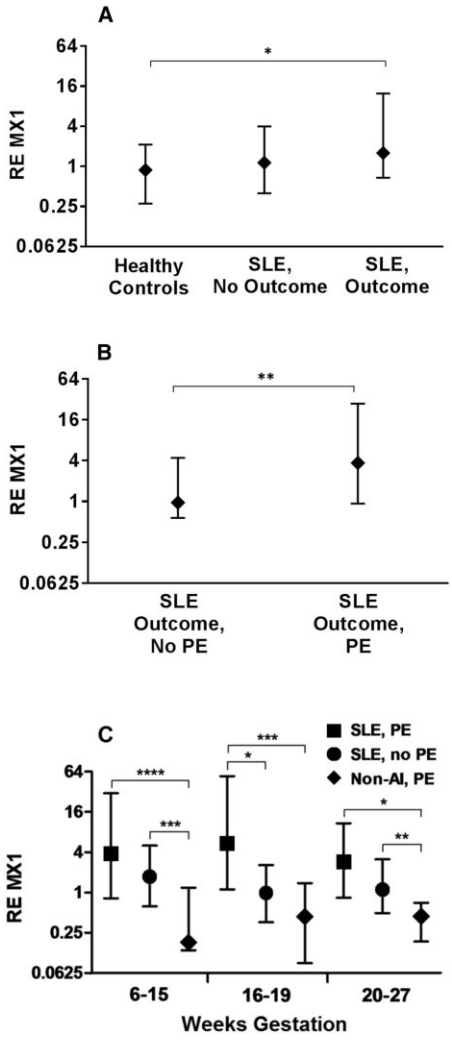

Figure 1. Interferon-α (IFNα) activity in patients in the Predictors of Pregnancy Outcome: Biomarkers In Antiphospholipid Antibody Syndrome and Systemic Lupus Erythematosus (PROMISSE) study. Samples obtained at 6–27 weeks' gestation (average 3.6 samples/patient), a period that antedated clinically apparent pregnancy complications, were assayed for IFNα activity using the WISH reporter cell assay. **A**, Relative expression (RE) of *MX1* in healthy controls (n = 28), patients with systemic lupus erythematosus (SLE) without poor outcomes (SLE, no outcome; n = 27), and patients with SLE with poor outcomes (SLE, outcome; n = 28). * = $P < 0.004$. $P = 0.07$ for healthy controls versus SLE patients without poor outcomes; $P = 0.23$ for SLE patients with poor outcomes versus SLE patients without poor outcomes. **B**, Relative expression of *MX1* in SLE patients with poor outcomes other than preeclampsia (PE) (n = 14) and in SLE patients with preeclampsia (n = 14). ** = $P = 0.006$. **C**, Relative expression of *MX1* in healthy subjects without autoimmune disease who developed preeclampsia (Non-AI PE; n = 9), SLE patients without preeclampsia (no outcomes and outcomes other than preeclampsia; n = 41), and SLE patients with preeclampsia (n = 14) at the indicated weeks of gestation. * = $P = 0.02$; ** = $P = 0.04$; *** = $P = 0.008$; **** = $P < 0.005$. Values are the median and 25th to 75th percentiles.

sion and preeclampsia. Relative *MX1* levels were higher in the SLE patients with preeclampsia than in the combined group of SLE patients without preeclampsia ($P = 0.006$) (Figure 1B). For every 10-unit increase in relative *MX1* expression, the odds ratio for preeclampsia in a logistic regression analysis was 1.37 (95% confidence interval [95% CI] 1.09–1.72). Relative *MX1* expression levels were consistently higher in the preeclampsia group throughout pregnancy, with the largest difference occurring between 16 and 19 weeks ($P = 0.02$) (Figure 1C). We performed similar analyses using an IFN score calculated by measuring the relative expression of 3 IFNα-responsive genes (*IFT1, IFI44*, and *MX1*), as previously described (29). Results showed trends similar to those of *MX1*, but were less robust (data not shown).

The elevation in IFNα activity in patients who developed preeclampsia was not attributable to disease activity or autoantibody profile. In nonpregnant SLE patients, higher disease activity and specific autoantibody profiles are associated with IFN signature (15). At baseline, mean ± SD SLEPDAI values were low and did not differ between SLE patients who developed preeclampsia and those who had other poor outcomes (4.6 ± 3.8 versus 4.6 ± 3.5; $P = 0.98$), though SLEPDAI scores for SLE patients with any complication were higher than those for patients without any complication (4.6 ± 3.5 versus 2.2 ± 2.7; $P = 0.007$). We found no correlation between SLEPDAI and relative *MX1* expression. Similarly, the association between relative *MX1* expression and anti-Ro antibodies at 6–11 weeks' gestation was not significant. Paradoxically, anti-Ro antibodies were less frequent in patients who had poor pregnancy outcomes (Table 1).

Antimalarials, potent inhibitors of endosomal Toll-like receptors, have been associated with relatively low IFNα levels in SLE (15). The use of hydroxychloroquine did not differ between patient groups (Table 1). In addition, the difference in relative *MX1* expression in patients taking hydroxychloroquine compared to those not taking the medication at screening was not significant (median 1.1 versus 2.4; $P = 0.94$). Taken together, these results indicate that there is increased IFNα activity before the appearance of clinical symptoms in SLE patients who develop preeclampsia, and this increase in IFNα levels is not related to disease activity, autoantibody profiles, or exposure to hydroxychloroquine. After adjusting for these factors in a multivariable logistic regression model, the odds ratio for preeclampsia associated with a 10-unit increase in relative *MX1* expression was 1.85 (95% CI 1.27–2.68) ($P = 0.001$).

No increase in functional IFNα levels in patients without autoimmune disease who develop preeclampsia. To determine whether the increase in functional IFNα present before the onset of preeclampsia in patients with SLE is also a harbinger of preeclampsia in patients without autoimmune disease, we assessed relative *MX1* expression induced in WISH cells by serum obtained at 12–28 weeks' gestation from 9 healthy patients (average of 2.7 samples/patient) who subsequently developed preeclampsia and compared them to healthy controls who did not develop preeclampsia (6 patients from the same cohort and 28 patients from the PROMISSE study). IFNα activity was not increased by serum from these patients without autoimmune disease. SLE patients who developed preeclampsia showed higher relative *MX1* expression than the patients without autoimmune disease as early as 6–15 weeks' gestation (median 3.31 versus 0.18; $P < 0.005$), and this difference persisted through 20–27 weeks' gestation (Figure 1C). These data indicate that preeclampsia, in and of itself, is not associated with elevated IFNα activity.

Association of minimal angiogenic factor dysregulation with preeclampsia in patients with elevated IFNα levels, but not in those with low IFNα levels. The major clinical phenotypes of preeclampsia, hypertension and proteinuria, are due, at least in part, to an excess of the antiangiogenic factor soluble Flt-1 and reduced PIGF in the maternal circulation (12). IFNα represses transcription of angiogenic factors in endothelial cells and consequently could increase the vulnerability of vascular beds that require VEGF, such as those in glomeruli (21). If this were true, pregnant SLE patients with high IFNα levels could manifest endothelial dysfunction in the absence of marked alterations in soluble Flt-1/PlGF, whereas those with preeclampsia without marked elevations in IFNα activity should demonstrate angiogenic imbalance. To test this possibility, we divided SLE patients with preeclampsia into 2 groups, those with high IFNα levels and those with low IFNα levels, defined as above or below the median relative *MX1* expression in SLE patients with uncomplicated pregnancies (1.33), and compared soluble Flt-1 and PlGF levels at 18–21 weeks' gestation, a time before clinical manifestations of preeclampsia were present.

As expected, SLE patients with lower IFNα levels who developed preeclampsia had higher soluble Flt-1 levels than those who did not (median 5,425 pg/ml versus 1,793 pg/ml, respectively; $P < 0.08$) (Figure 2A). In contrast, in SLE patients with higher IFNα levels, there was no difference in soluble Flt-1 levels between those

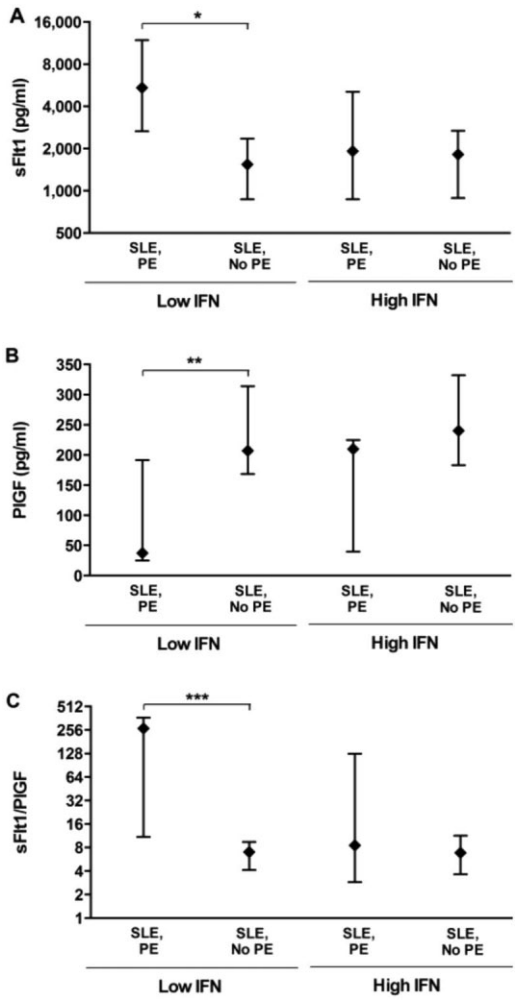

Figure 2. Levels of angiogenic factors in SLE patients who developed preeclampsia. SLE patients were classified as having low IFNα levels (n = 22) or high IFNα levels (n = 17). Plasma obtained at 18–21 weeks' gestation was assayed for soluble Flt-1 (sFlt-1) and placenta growth factor (PlGF) by enzyme-linked immunosorbent assay. **A** and **B,** Levels of soluble Flt-1 (**A**) and PlGF (**B**) in SLE patients with low IFNα levels with preeclampsia (n = 5) compared to those without preeclampsia (no outcome and outcomes other than preeclampsia) (n = 17) and in SLE patients with high IFNα levels with preeclampsia (n = 7) compared to those without preeclampsia (n = 10). $* = P < 0.08$ by Wilcoxon's rank sum test; $** = P < 0.03$. **C,** Ratio of soluble Flt-1 to PlGF in the indicated patient groups. $*** = P < 0.07$. For reference, in healthy controls enrolled in the PROMISSE study, the median levels of angiogenic factors at 18–21 weeks' gestation were 1,026 pg/ml for sFlt-1, 249 pg/ml for PlGF, and 6.21 for the sFlt-1:PlGF ratio. Values are the median and 25th to 75th percentiles. See Figure 1 for other definitions.

who did and those who did not develop preeclampsia (1,916 pg/ml versus 1,873 pg/ml, respectively; $P = 0.83$), and these patients showed minimal elevations in soluble Flt-1 compared to healthy pregnant patients. For reference, in healthy controls enrolled in the PROMISSE study, the median level of soluble Flt-1 at 18–21 weeks was 1,026 pg/ml, and for nonpregnant SLE patients, the median level of soluble Flt-1 was 81 pg/ml, over 10-fold lower.

Likewise, PlGF levels were significantly decreased (median 37 pg/ml versus 240 pg/ml) and soluble Flt-1:PlGF ratios were increased (268 versus 8.0) in patients with low IFNα levels who developed preeclampsia compared to those who did not develop preeclampsia ($P < 0.03$ and $P < 0.07$, respectively). Again, in SLE patients with high levels of IFNα, levels of angiogenic factors were indistinguishable between those who did and those who did not develop preeclampsia (240 pg/ml versus 210 pg/ml and 10.1 versus 8.5, respectively) (Figures 2B and 2C). In pregnant healthy controls, the median level of PlGF was 249 pg/ml, and the soluble Flt-1:PlGF ratio was 6.21. Taken together, these data indicate that marked angiogenic imbalance precedes maternal manifestations of preeclampsia in SLE patients with low levels of IFNα, whereas in patients with high levels of IFNα, preeclampsia occurs without evidence of systemic levels of antiangiogenic factors in the range associated with preeclampsia.

Exposure to IFNα increases sensitivity of HUVECs to soluble Flt-1. There is a slow and steady increase in soluble Flt-1 levels during normal pregnancy, but the rate of change and levels are markedly greater in those who develop preeclampsia (12). Given that maternal endothelial dysfunction, manifested as preeclampsia, occurs in the absence of elevated antiangiogenic factors in SLE patients with high IFNα levels, we considered the possibility that IFNα increases the vulnerability of endothelial cells to the relative VEGF deficiency induced by increasing levels of soluble Flt-1. We discovered that incubation of HUVECs with soluble Flt-1 (5 ng/ml, levels comparable to those in the second trimester of pregnancy) induced the expression of *sFLT1* mRNA. The effects of soluble Flt-1 on *MX1* (an IFNα-responsive gene) (Figure 3) and *VEGF* mRNA expression were minimal (data not shown). When modest doses of IFNα (100 IU/ml) were present (16), a greater than 10-fold increase in the capacity of soluble Flt-1 to amplify its own production was seen (Figure 3A). Our results identify a novel mechanism by which IFNα can potentiate the antiangiogenic effects of soluble Flt-1. IFNα can lower the threshold for endothelial dysfunction by antagonizing the autocrine function of VEGF and amplify the feed-forward loop of soluble Flt-1 production, and as

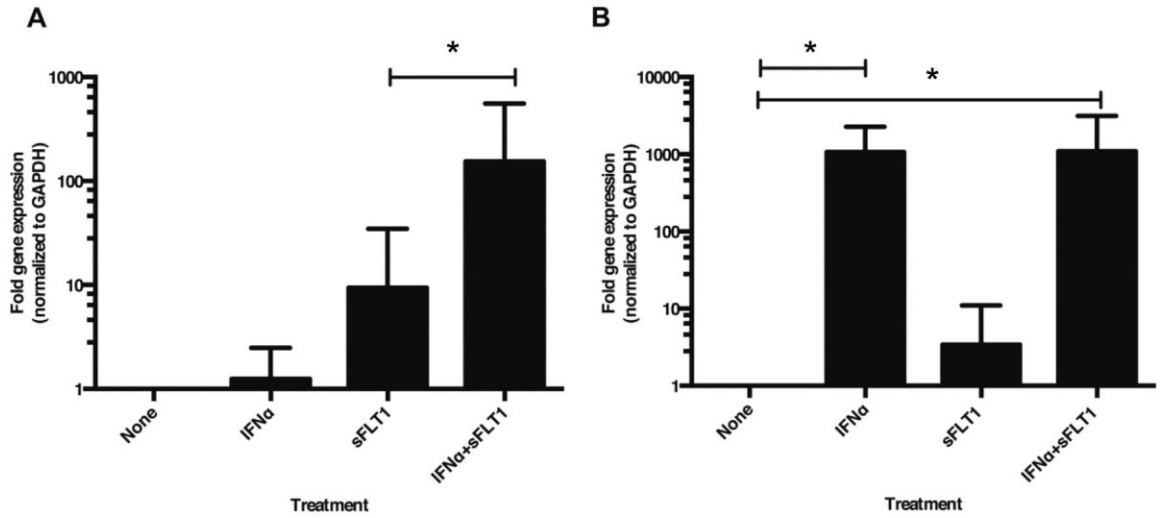

Figure 3. Regulation of gene expression in human umbilical vein endothelial cells (HUVECs) by interferon-α (IFNα) and soluble Flt-1 (sFlt-1). The transcriptional regulation of **A**, *sFLT1* and **B**, *MX1* in HUVECs after treatment with recombinant IFNα and sFlt-1 was analyzed by quantitative real-time polymerase chain reaction. HUVECs were incubated for 6 hours with no treatment (none), IFNα (100 units/ml), sFlt-1 (5 ng/ml), or both proteins. Fold gene expression was calculated using $\Delta\Delta C_t$ normalized to the housekeeping gene GAPDH and to the baseline condition without treatment. Bars show the mean ± SEM of at least 5 different experiments. ∗ = $P < 0.05$ by Wilcoxon's 2-tailed matched pairs signed rank test.

shown by others, decrease the production of VEGF (16,17,19).

Disruption of endothelial cell–trophoblast interactions by the combination of IFNα and soluble Flt-1. During normal placentation, the trophoblast invades the maternal uterine vasculature and remodels it by replacing endothelial cells (38,39). Insufficient spiral artery transformation is a hallmark of preeclampsia (40). Using an in vitro model of spiral artery transformation (36,41), we explored the implications of the antiangiogenic effects of IFNα and soluble Flt-1 on HEEC interactions with extravillous trophoblast cells (HTR8 cells). In this in vitro system, HTR8 cells stabilize HEEC tube structures over time (Figure 4). As shown in Figure 5A, compared to untreated control (not treated [NT]), IFNα or soluble Flt-1 alone had no effect on the ability of HTR8 cells to invade the tube-like structures, replacing the HEECs. However, the combination of IFNα and soluble Flt-1 dramatically reduced the ability of the HTR8 cells to remodel HEEC tubes, resulting in their destabilization (Figure 5A), reflected in a significant reduction in the number of tubes (Figure 5B). Moreover, fewer HTR8 cells (green) were observed within the HEEC tube structures (red) under these conditions. These changes were not due to altered viability of HEEC or HTR8 cells in the presence of IFNα, soluble Flt-1, or both. After 24 hours, cell viability measured by colorimetric CellTiter assay (Promega) was >95%.

To expand upon our observations showing that IFNα amplifies *sFLT1* mRNA in HUVECs, we exposed HEECs to recombinant IFNα and found that IFNα increased soluble Flt protein secretion by 27.3 ± 8.9% ($P = 0.03$). These data show that in two different endothelial cells lines, IFNα modulates soluble Flt-1 expression.

DISCUSSION

Preeclampsia complicates 13–35% of pregnancies in SLE patients, compared to 4–6% of all first pregnancies and less than 1% of subsequent pregnancies in otherwise healthy women (42). We have shown in a case–control study that SLE patients who develop preeclampsia are more likely to have increased circulating IFNα activity before the onset of clinical symptoms compared to patients who do not develop preeclampsia; that this increase cannot be explained by higher levels of disease activity, autoantibodies, or exposure to hydroxychloroquine; and, in a group of patients without autoimmune disease, that preeclampsia, in and of itself, is not associated with elevated IFNα levels.

In patients without autoimmune disease, preeclampsia has been associated with excessive placenta-derived circulating antiangiogenic proteins, such as soluble Flt-1, which causes a shift toward a more "antiangiogenic" state in the mother (30). We found that

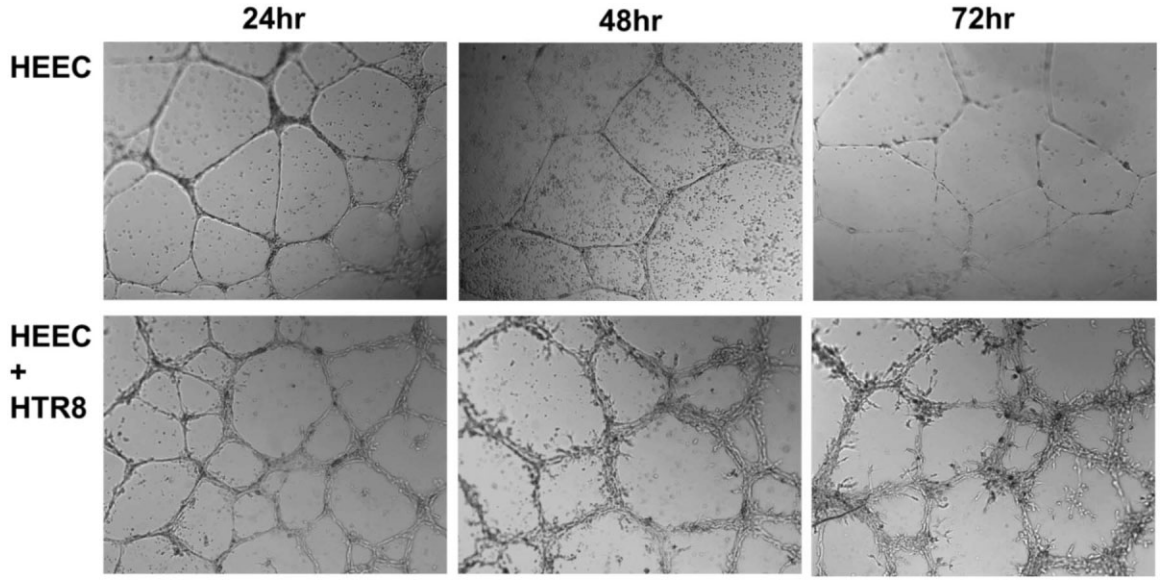

Figure 4. Stabilization of human endometrial endothelial cell (HEEC) tubes by HTR8 cells over time. HEECs were seeded in Matrigel overnight to allow tube formation. Medium was then removed from the wells and replaced, with or without the first trimester extravillous trophoblast cell line HTR8. After 24 hours, in the absence of HTR8 cells, HEEC tubes are established. However, by 48 hours, the HEEC tubes begin to destabilize, and by 72 hours, they are mostly disintegrated. In contrast, in the presence of HTR8 cells, the tube structures are maintained over 72 hours, and increase in size as the trophoblast cells remodel the endothelium.

in SLE patients with lower levels of IFNα, there was also a marked increase in soluble Flt-1 and soluble Flt-1: PlGF ratios before the appearance of clinical signs of preeclampsia. In contrast, SLE patients with higher IFNα levels who developed preeclampsia showed no evidence, in serum, of angiogenic factor dysregulation. Unexpectedly, their soluble Flt-1 levels were similar to levels in patients who had uncomplicated pregnancies or

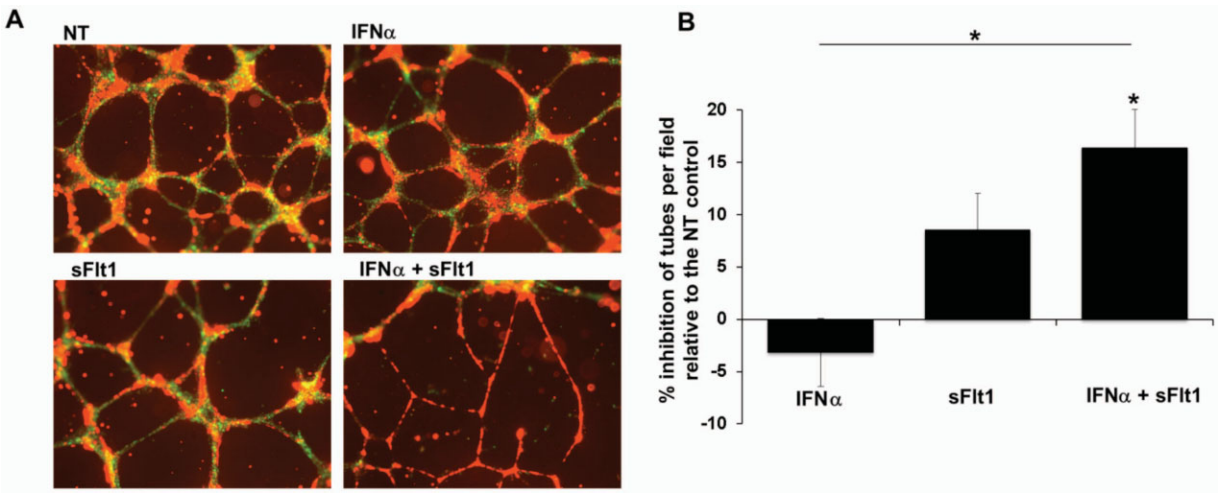

Figure 5. Effect of interferon-α (IFNα) and soluble Flt-1 (sFlt-1) on endothelial cell–trophoblast interactions. HTR8 cells were added to human endometrial endothelial cell (HEEC) tube cultures that were left untreated (no treatment [NT]) or were treated with IFNα (100 units/ml), sFlt-1 (5 ng/ml), or both and cultured for 24 hours. **A**, Interactions between HEECs (red) and HRT8 (green), as visualized by fluorescence microscopy. Images from a representative experiment are shown. Colocalization of the cells is shown in yellow. **B**, Percent inhibition of the number of tubes per field relative to the untreated control. Bars show the mean ± SEM of 4 independent experiments. For untreated controls, the mean ± SEM number of tubes was 18.1 ± 1.0. $* = P < 0.05$ by analysis of variance with Bonferroni correction.

complications other than preeclampsia. These findings suggested that elevated IFNα levels may contribute to the pathogenesis of preeclampsia in some women with lupus by sensitizing the maternal vascular endothelium to the antiangiogenic effects of even normal levels of soluble Flt-1, as well as by inhibiting transcription of proangiogenic VEGF. Indeed, our in vitro studies showed that incubation with physiologic doses of soluble Flt-1 induces expression of endogenous *sFLT1* mRNA in endothelial cells and that IFNα dramatically increases this effect, thereby potentiating the blockade of autocrine VEGF signaling.

There is precedent for the concept that endothelial health and angiogenesis, which is promoted by VEGF, is antagonized by type I IFNs, and multiple mechanisms have been described. IFNα has been shown to induce an antiangiogenic signature in endothelial progenitor cells from lupus patients, as well those from healthy subjects; addition of VEGF restores their capacity to survive, differentiate, and proliferate (17). IFNα inhibits VEGF activation of FAK, ERK, Akt, and endothelial cell nitric oxide synthase, and induces transcription of hypoxia-inducible factor 1α in human endothelial cells, which limits their proliferative capacity (19,43). Recent studies have shown that inhibition of type I IFN receptor signaling, as a consequence of VEGF-induced degradation of receptors for type I IFNs, is required for efficient VEGF-stimulated angiogenesis (44). That IFNα induces an antiangiogenic state in vivo in nonpregnant SLE patients is suggested by findings of vascular rarefaction in renal blood vessels and repression of VEGF in kidney and serum (16,17).

Our in vitro studies identify a new mechanism by which IFNα induces an antiangiogenic milieu locally in the vasculature. We show that treatment with exogenous soluble Flt-1, which creates a VEGF-deficient state, induces expression of endogenous soluble Flt-1 mRNA in HUVECs, leading to a local positive feedback loop, and that IFNα dramatically amplifies the response to soluble Flt-1. Enhanced production of soluble Flt-1 promoted by IFNα could blunt uterine spiral artery remodeling early in pregnancy and alter placental perfusion. Indeed, using an in vitro model of the uterine vasculature, our studies also show that IFNα, in combination with soluble Flt-1, has a dramatic effect on the ability of trophoblast cells to interact with and transform the endothelium. Similarly, later in pregnancy, IFNα could sensitize the maternal vasculature to the effects of even modest elevations in soluble Flt-1 that occur in normal pregnancies and intensify local VEGF insufficiency. This is particularly important in glomerular endothelial beds which require podocyte production of VEGF for health; loss of free VEGF in experimental models promotes renal microvascular injury and thrombotic microangiopathy, clinically manifested as proteinuria and hypertension, characteristics of preeclampsia (6,21). Our findings support the possibility that glomerular endothelial cells primed by IFNα in SLE have exaggerated responses to decreased VEGF availability. Recent studies have shown that soluble Flt-1 also sensitizes endothelial cells to the proinflammatory and proadhesive effects of TNFα (45).

We propose that in a subset of SLE patients, the gradual increase in soluble Flt-1 levels that occurs in all pregnancies, superimposed upon the increase in soluble Flt-1 levels and previously described decrease in VEGF produced by endothelial cells in response to IFNα, is sufficient to disrupt podocyte-derived VEGF function. Indeed, the increased frequency of preeclampsia in patients who have active SLE early in pregnancy, particularly active nephritis (46–49), may be related in part to vasculopathic effects of elevated IFNα present in many such patients. Although the major source of soluble Flt-1 in pregnant patients is the placenta (4,5), that circulating levels of soluble Flt-1 are not correlated with IFNα suggests that modest local increases in soluble Flt-1 production by endothelial cells have adverse clinical consequences in vulnerable vascular beds.

There were some limitations to our study that should be considered. The study included a small number of patients. We matched cases and controls according to demographic characteristics and excluded those with other risk factors for preeclampsia. Nonetheless, SLE patients are highly heterogeneous. We focused on soluble Flt-1 and PlGF, but there are multiple other factors that may contribute to the risk of preeclampsia, including soluble endoglin, microparticles, and agonistic antiangiotensin receptor antibodies (30,50,51). Our small pilot study could not address more analytes. In addition, it is possible that concentrations of cytokines and angiogenic factors at the maternal–fetal interface or within glomeruli, which may not be reflected in the circulation, alter trophoblast function and trigger vasculopathic effects. Despite these limitations, our study provides a new model to explain the increased vulnerability of some SLE patients to antiangiogenic factors.

Our findings extend the role of type I IFNs to the risk of maternal vascular disease characteristic of preeclampsia. They support the concept that in the presence of elevated IFNα, endothelial cell–trophoblast interactions are impaired, a potential basis for inadequate spiral artery remodeling, and the resultant placen-

tal dysfunction and angiogenic dysregulation, even if modest, may lead to maternal endothelial responses characteristic of preeclampsia. In addition, they raise the possibility that elevated IFNα levels may be used to risk-stratify pregnancies in SLE patients.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Salmon had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Andrade, Koo, Kirou, Crow, Abrahams, Kaplan, Salmon.

Acquisition of data. Andrade, Blanco, Koo, Redecha, Kirou, Alvarez, Mulla, Kaplan, Salmon.

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