Regulation of Interleukin-6 Expression in Human Decidual Cells and Its Potential Role in Chorioamnionitis

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Chorioamnionitis frequently precedes both genital tract and placental inflammation and is both a primary cause of maternal morbidity and a major antecedent of preterm premature rupture of the membranes (PPROM) as well as preterm delivery (PTD). In most cases of chorioamnionitis, neutrophils dominate the decidua. In a subset of these cases, a predominance of monocytes is uniquely associated with both neonatal intraventricular hemorrhage and death. The multifunctional cytokine, interleukin-6, promotes local monocyte dominance via several mechanisms. In this study, immunostaining of placental sections revealed significantly higher interleukin-6 HSCOREs in decidual cells (DCs) but not in interstitial trophoblasts, in chorioamnionitis versus gestational age-matched control placentas ($P < 0.05$). In confluent leukocyte-free term DCs, secreted interleukin-6 levels in incubations with estradiol-17 β were increased 2500-fold by IL-1 β ($P < 0.05$). This up-regulation was inhibited by more than 50% in parallel incubations that included medroxyprogesterone acetate ($n = 12$, $P < 0.05$). Western blotting data confirmed these enzyme-linked immunosorbent assay results; quantitative RT-PCR findings demonstrated corresponding changes in interleukin-6 mRNA levels. Specific inhibitors of signaling for both nuclear factor- κ B activation and p38-mitogen-activated protein kinase, but not for protein kinase C, significantly decreased IL-1 β -enhanced interleukin-6 expression levels in cultured DCs. In conclusion, *in situ* and *in vitro* results indicate that significantly enhanced interleukin-6 expres-

sion levels in DCs during chorioamnionitis could be pivotal in skewing decidual monocyte differentiation to macrophages. (Am J Pathol 2010, 177:1755–1764; DOI: 10.2353/ajpath.2010.090781)

During human pregnancy, chorioamnionitis (CAM) frequently precedes genital tract and placental inflammation. It is a primary cause of maternal morbidity and a major antecedent of preterm premature rupture of the membranes (PPROM) and preterm delivery (PTD).¹ The latter complicates about 13% of live births in the United States and is the leading cause of perinatal morbidity and mortality.^{2,3} During CAM, microbial species usually first ascend from the vagina and cervix to the uterus where they induce deciduitis. Despite the presence of intact membranes, microorganisms can ultimately invade the chorion, amnion, amniotic fluid, and fetus. Although positive microbial cultures are found in the amniotic fluid in 23% of women with PPROM,^{4,5} 19% displayed no signs of overt amniotic fluid infection.⁵ However, these samples contained elevated levels of neutrophil collagenase and elastase^{5,6} suggesting that pathogens remained localized to the decidua or the inciting microorganisms escaped detection.⁵

Recently, the large prospective Alabama Preterm Birth Study confirmed the strong association between CAM and the detection of bacterial infections. This study also observed a positive correlation between neutrophil infiltration of the fetal membranes, chorionic plate, and umbilical cord, which serve as markers of CAM, with positive intrauterine bacterial cultures and the occurrence of both

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the neonatal inflammatory response syndrome and necrotizing enterocolitis.⁷ However, in a significant subset of cases, a mononuclear infiltrate in the fetal membranes and decidua basalis proved to be a harbinger of neonatal intraventricular hemorrhage. Specifically, mononuclear cell infiltration was present in 10% of the placental free membranes and decidua basalis, and within this group there was a 24% increase in neonatal intraventricular hemorrhage. This study also found a positive correlation between PTD and significantly elevated umbilical cord blood levels of interleukin (IL)-6.⁷ The latter is a multifunctional cytokine that regulates hematopoiesis, the acute phase reaction, and both pro- and anti-inflammatory events.⁸ Our laboratory recently demonstrated that decidual cells (DCs), the predominant endometrial cell type throughout pregnancy⁹ are a major source of elevated maternal plasma IL-6 levels¹⁰ implicated in inducing systemic endothelial cell activation and vascular damage in preeclampsia.¹¹

In the current study we hypothesized that DCs could also contribute to the well documented CAM-related increase in IL-6 levels in maternal plasma¹² as well as cervical and amniotic fluid IL-6 levels¹³ that reliably predict PPRM and PTD¹⁴ while acting as an autocrine/paracrine modulator of the local immune cell population. To shed light on these questions, immunohistochemistry was used to localize IL-6 at the maternal-placental interface in DCs of placental sections from patients with CAM and gestational age-matched control placentas. The effects of IL-1 β , a classic proinflammatory cytokine present at elevated levels in the amniotic fluid of women with CAM,¹⁵ were evaluated on IL-6 mRNA and protein expression in cultured human term DCs. In these experiments, IL-1 β was added with either estradiol (E₂) as the control incubation or with E₂ plus medroxyprogesterone acetate (MPA) to mimic the steroid milieu of pregnancy. To eliminate the confounding effects of resident immune cells, experimental incubations were performed with DCs that were passaged until fluorescence-activated cell sorter analysis indicated that they were essentially leukocyte-free. To elucidate a potential mechanism regulating IL-1 β -enhanced IL-6 expression in human decidual cells, experimental incubations included specific inhibitors of the intracellular signal transduction pathways for p38 mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and nuclear factor (NF)- κ B activation, which are all known to mediate IL-1 β -enhanced IL-6 expression.^{16,17} A functional *in vitro* assay determined whether elevated IL-6 levels in the conditioned medium of IL-1 β -treated term DCs could promote differentiation of monocytes away from dendritic cells and toward macrophages.

Materials and Methods

Patients and Tissue

Immunohistochemistry

After written informed consent was received, biopsy samples of decidua basalis were obtained from the cen-

tral portion of the maternal side of vaginally delivered placentas from patients with CAM-related PTDs ($n = 10$) and idiopathic gestational age-matched preterm control subjects ($n = 9$). Mean $\pm$ SD gestational ages for CAM-complicated and control pregnancies were 35.4 ± 6.0 and 35.5 ± 2.8 weeks, respectively ($P = 0.957$). None of these sections were marginal, and none were taken from any women with placenta previa. Control patients had no clinical evidence of CAM including fever, leukocytosis, or tender uterus, and their placental specimens were histologically examined to rule out conditions of acute or chronic inflammation such as CAM and chronic villitis and decidual hemorrhage (ie, abruption). CAM specimens were histologically defined as having more than 10 neutrophils per high-power field in the subchorionic space, chorion, or placental plate, based on the criteria of Naeye.¹⁸ CAM was also clinically diagnosed as maternal fever ($>37.8^{\circ}\text{C}$), uterine tenderness, foul-smelling amniotic fluid or visualization of pus at the time of the speculum examination, maternal tachycardia (≥ 100 beats/min), and fetal tachycardia (≥ 160 beats/min).¹⁹ All specimens were collected at Yale-New Haven Hospital, and collection was approved by the Yale Human Investigation Committee.

Decidua Isolation

After written informed consent was received, placentas and attached fetal membranes were obtained from patients with uncomplicated pregnancies undergoing repeat cesarean deliveries at term at Yale-New Haven Hospital under Human Investigation Committee approval. None of the patients from whom specimens were obtained was in labor. The decidua was taken from the central portion of the maternal side of the placenta, separated from the amniochorionic tissues, and a small portion of the former was formalin-fixed and paraffin-embedded and then examined histologically for signs of underlying acute and chronic inflammation. The remainder was used for the isolation and culturing of DCs.

Immunohistochemistry

Five-micrometer sections of formalin-fixed, paraffin-embedded tissues were deparaffinized in xylene and rehydrated in a descending ethanol series. Antigen retrieval was performed by boiling the slides in citrate buffer (10 mmol/L; pH 6.0) for 15 minutes, and endogenous peroxidase was blocked by immersing the slides in 3% hydrogen peroxide (in 50% methanol/50% distilled water) for 15 minutes. The slides were then incubated with 5% blocking horse serum in Tris-buffered saline (TBS) in a humidified chamber for 30 minutes at room temperature. After excess serum was blotted off, serial sections were incubated with mouse monoclonal anti-human IL-6 antibody at 10 $\mu\text{g/ml}$ in 1% blocking horse serum in TBS (R&D Systems, Minneapolis, MN) or mouse monoclonal anti-vimentin antibody (1:100 dilution in TBS) (DakoCytomation, Carpinteria, CA) overnight at 4°C in a humidified chamber. Slides were also stained with nonspecific

mouse IgG isotype-matched antibodies at the same concentrations to serve as negative controls. After being washed three times for 5 minutes in TBS, the slides were incubated with biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA), diluted 1:400 in TBS, for 30 minutes at room temperature. The antigen-antibody complex was visualized with an avidin-biotin-peroxidase kit (Vector Laboratories). Diaminobenzidine (3,3-diaminobenzidine tetrahydrochloride dehydrate) (Vector Laboratories) was used as the chromogen. The slides were then counterstained with hematoxylin and mounted.

IL-6 immunostaining intensity was semiquantitatively evaluated by HSCORE, using the following categories: 0 (no staining), 1+ (weak, but detectable staining), 2+ (moderate staining), and 3+ (intense staining). An HSCORE value was determined for each specimen by calculating the sum of the percentages of cells that stained at each category and multiplying that by the value of the intensity category, using the formula $HSCORE = \sum_i i * P_i$, where i represents the intensity category and P_i is the corresponding percentage of cells. For each slide, three different fields and 100 cells per field were evaluated microscopically at a $\times 20$ objective magnification. The percentage of cells staining at each intensity category within each field was evaluated at different times by two blinded investigators, and the average score of both were used.

Isolation and Culture of DCs

The decidua was scraped from the maternal surface of the chorion, minced, and digested in Ham's F-10 + 10% charcoal-stripped calf serum (Flow Laboratories, Rockville, MD) containing 25 mg/ml of collagenase (200 U/mg) (Worthington Biochemical Corp., Freehold, NJ) in a shaking water bath at 37°C for 30 minutes. After addition of 6.25 units of DNase (Sigma-Aldrich, St. Louis, MO)/ml of digestate, the incubation was continued for another 45 minutes. Cell clusters in the final digestate were dissociated via aspiration with a 23-gauge needle. The isolated cells were centrifuged at 1500 rpm for 5 minutes at 4°C and then washed three times in Ham's F-10, and the resulting cell pellet was resuspended (1 g of tissue/ml) in 20% Percoll (Sigma-Aldrich), layered onto a (60%:50%:40%) discontinuous Percoll gradient, and centrifuged at 22,000 rpm for 20 minutes at 4°C. The top cell layer was collected, washed, resuspended in Ham's F-10, and centrifuged at 1500 rpm for 5 minutes at 4°C. After this procedure was repeated, the cell pellet was resuspended in 40% Percoll (1 g of tissue/ml), layered on a discontinuous (55%:50%:40%) Percoll gradient, and centrifuged at 22,000 rpm for 20 minutes at 4°C. The top cell layer was washed twice in serum-free Ham's F-10 and centrifuged at 500 rpm for 5 minutes at 4°C. The cell pellet was resuspended in Ham's F-10 + 10% charcoal-stripped calf serum, and decidual cells were counted in a hemocytometer. After the isolation procedure, trypan blue exclusion indicated that more than 95% of isolated cells were viable.

Isolated decidual cells (5×10^5 cells/ml) were suspended in basal medium, a phenol red-free 1:1 v/v mix of Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) and Ham's F-12 (Flow Laboratories) with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml Fungizone supplemented with 10% charcoal-stripped calf serum. The cells were seeded onto polystyrene tissue culture dishes coated with 2% type B gelatin (Sigma-Aldrich). The cultures were grown to confluence in a standard 95% air-5% CO₂ incubator at 37°C and passaged three times. Fluorescent antibody cell sorting for the presence of CD45⁺ demonstrated that unpassaged cultures contained 12 to 15% CD45⁺ cells, whereas passaged cultures were >99% negative for this common leukocyte marker. The latter were used for experimental cell incubations. The cultured cells were vimentin-positive and cytokeratin-negative. Cultured cells also showed decidualization-related morphological changes and expressed biochemical end points of decidualization including enhanced tissue factor and plasminogen-activator inhibitor-1 and reduced interstitial collagenase and stromelysin-1 under the influence of progesterin (data not shown).

Experimental Cell Incubations

Confluent decidual cells were primed for 7 days in basal medium supplemented with 10% charcoal-stripped calf serum containing either vehicle control (0.1% ethanol), 10^{-8} mol/L E₂, 10^{-7} mol/L MPA (Sigma-Aldrich) or E₂ plus MPA with one change of medium. Because circulating levels of both E₂ and progesterone are high during the third trimester, E₂ was used with MPA to mimic the gestational steroidal milieu. MPA was used in place of progesterone because the latter is rapidly metabolized *in vitro*.²⁰ The cultures were washed twice with PBS and switched to a serum-free defined medium (DM) consisting of basal medium plus ITS+ premix (BD Biosciences, Bedford, MA), 5 μ mol/L FeSO₄, 0.5 μ mol/L ZnSO₄, 1 nmol/L CuSO₄, 20 nmol/L Na₂SeO₃, 50 μ g/ml ascorbic acid (Sigma-Aldrich), and 50 ng/ml epidermal growth factor (BD Biosciences, San Jose, CA). The corresponding vehicle or steroid(s) with and without 0.01 to 10 ng/ml of IL-1 β (R&D Systems) was added to the DM.

In a parallel set of experiments, the cultured DCs were also incubated with or without the following specific inhibitors within ranges recommended by the manufacturer (EMD Chemicals Inc., Gibbstown, NJ): either a p38 MAPK inhibitor (p38KI) at 10^{-5} mol/L (SB203580), or its corresponding negative control (p38KI neg cont) at 10^{-5} mol/L (SB202474), or a protein kinase C inhibitor at 10^{-7} mol/L (calphostin C), or an NF- κ B activation inhibitor at 10^{-5} mol/L (NF- κ B activation inhibitor III). All of the inhibitors and negative control were added as pretreatments for 30 to 60 minutes before the addition of 1 ng/ml IL-1 β .

After the test period, cells were harvested by scraping in ice-cold lysis buffer solution of Tris-buffered saline with 1% Triton X-100, 1 mmol/L phenylmethanesulfonyl fluoride (Sigma-Aldrich), and Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) and then briefly

sonicated. Conditioned medium supernatants and cell lysates were stored at -70°C . Total RNA was extracted from a different set of parallel incubations (6 hours) with Tri Reagent (Sigma-Aldrich).

Enzyme-Linked Immunosorbent Assay

Total cell protein levels were measured by a modified Lowry assay (Bio-Rad Laboratories, Hercules, CA). An enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems was used to measure IL-6 levels in the cell-conditioned DM according to the manufacturer's instructions. The sensitivity of the ELISA is 0.7 pg/ml with intra-assay and interassay coefficients of variation of 3.1 and 2.7%, respectively.

Western Blotting

Western blot analysis was performed on conditioned DM supernatants that were concentrated up to ninefold using centrifugal filter devices with a 3000 molecular weight cutoff (Microcon, Millipore Corporation, Bedford, MA). For some of the blots a positive control was also used (10 ng/lane of recombinant human IL-6, R&D Systems). The concentrated supernatants and positive control were diluted 1:1 in Laemmli nonreducing sample buffer and then boiled for 3 minutes. The centrifuged media and positive control were subjected to electrophoresis on either a 10 to 20% SDS-polyacrylamide linear gradient Tris-HCl gel (Bio-Rad Laboratories) or 12% SDS-polyacrylamide TGX gel (Bio-Rad Laboratories) with subsequent electroblotting transfer onto a 0.2- μm nitrocellulose membrane (Bio-Rad Laboratories). After transfer, the membrane was blocked overnight in TBS with 4% nonfat dry milk and then incubated for 2 hours with 0.2 $\mu\text{g/ml}$ mouse anti-human IL-6 monoclonal antibody (R&D Systems). Membranes were rinsed in PBS and 0.2% Tween 20 before and after incubation with horseradish peroxidase-conjugated anti-mouse IgG (ICN Biomedicals, Aurora, OH). Chemiluminescence was detected with ECL reagents (PerkinElmer Life and Analytical Sciences, Boston, MA) and autoradiography film (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions.

Real-Time Quantitative RT-PCR

To verify that the IL-6 and β -actin probes yielded the correct bands, extracted RNA from experimental cell incubations were subjected to semiquantitative RT-PCR using a kit from Invitrogen, performing 35 cycles with the Mastercycler (Eppendorf, Westbury, NY). To perform quantitative real-time RT-PCR, reverse transcription was initially performed with avian myeloblastosis virus reverse transcriptase (Invitrogen). A quantitative standard curve was created using cDNA with a Roche LightCycler (Roche, Indianapolis, IN) by monitoring increasing fluorescence of PCR products during amplification. On establishing the standard curve, quantification of the unknowns was determined with the Roche LightCycler and adjusted to the quantitative expres-

sion of β -actin from the corresponding unknowns. Melting curve analysis determined the specificity of the amplified products and the absence of primer-dimer formation. All products obtained yielded correct melting temperatures. Products were then run on a 1.2% agarose gel along with a 100-bp DNA ladder and then stained with ethidium bromide for visualization. The following primers were synthesized and gel-purified at the Yale DNA Synthesis Laboratory, Critical Technologies: β -actin: forward 5'-CGTACCACTGGCATCGTGAT-3' and reverse 5'-GTGTTGGCGTACAGGTCTTTG-3', 452 bp and IL-6: forward 5'-ATGCAATAACCACCCCT-3' and reverse 5'-AGTGTCTTAACGCTCATAC-3', 277 bp.

Functional Assay for in Vitro Monocyte Differentiation

Monocyte Culture

Monocytes were isolated from peripheral blood of healthy donors by Ficoll-Paque (GE Healthcare, Piscataway, NJ) density gradient centrifugation and then purified using anti-CD14-paramagnetic beads according to the manufacturer's instructions (Milenyi Biotec, Auburn, CA). Purified monocytes were seeded at 1×10^6 cells/well in six-well plates containing 1 ml of AIM-V serum-free medium (Invitrogen).

Term DC Culture

Confluent term DC monolayers were incubated for 24 hours with 10^{-8} mol/L E_2 or $\text{E}_2 + \text{IL-1}\beta$ (1 ng/ml) and collected using sterile technique (see "Isolation and Culture of DCs" and "Experimental Cell Incubations" described above). A neutralizing antibody against human IL-1 β (R&D Systems) was added at 1 $\mu\text{g/ml}$ (approximately a twofold excess) to this conditioned DM for 2 hours followed by its removal using protein G Sepharose (Zymed Laboratories, South San Francisco, CA).

Monocyte and Conditioned DM from DCs Culture

Monocytes in the six-well plates were incubated for 5 days with the conditioned DM from the term DC cultures plus an antibody against human IL-6 ($\alpha\text{-IL6}$, R&D Systems) at 15 $\mu\text{g/ml}$ (approximately a threefold excess). Using a modified protocol²¹ that differentiates monocytes to macrophages and dendritic cells, 30 ng/ml of recombinant human granulocyte macrophage-colony (GM)-stimulating factor (CSF), 10 ng/ml of recombinant human IL-4, and 20 ng/ml of recombinant human macrophage-CSF (M-CSF) (R&D Systems) were added to the culture medium. Fresh medium (30% volume) containing equal amounts of M-CSF, GM-CSF, and IL-4 was added to the conditioned DM on day 3. The expression of cell surface markers was determined by flow cytometric analysis using anti-human CD11C-FITC (for dendritic cells), CD68-PE, or CD14-FITC monoclonal antibody (for macrophages) (R&D Systems).

Statistical Analysis

Comparisons of control and treatment groups were performed with the Kruskal-Wallis analysis of variance on ranks test followed by either the Student-Newman-Keuls post hoc test (when the groups were of equal sample size) or Dunn's post hoc test (when the groups were of unequal sample size). $P < 0.05$ represented statistical significance. Gestational ages were normally distributed and compared by Student's *t*-test. HSCOREs from the immunohistochemical analysis of control and CAM samples were normally distributed (Kolmogorov-Smirnov test) and analyzed by one-way analysis of variance, followed by post hoc Holm-Sidak testing.

Results

IL-6 Immunoreactivity Is Elevated in DCs in CAM-Complicated Specimens

Serial sections of CAM-complicated (Figure 1, A and B) and control (Figure 1, C and D) decidua were immuno-

Figure 1. Immunohistochemical analysis of IL-6 expression in CAM-complicated decidua. CAM-complicated (A and B) and control (C and D) decidual specimens were immunostained to detect expressed IL-6 and vimentin in serial sections. Vimentin staining identified DCs (arrows) of CAM-complicated specimens (A) than control specimens (C). Interstitial trophoblasts (arrowheads) displayed similar levels of IL-6 immunoreactivity in both specimen groups, but the localization of the staining was more perimembranous in CAM-complicated specimens, particularly when immediately adjacent to DCs (A, inset). The inset in A is a magnification of the outlined area. E: Staining with an isotype-matched, negative, control antibody. F: IL-6 immunoreactivity HSCOREs (mean $\pm$ SEM) in control ($n = 9$) and CAM-complicated ($n = 10$) specimens. * $P < 0.05$ in both CAM-complicated versus control DCs and DCs versus interstitial trophoblast in CAM-complicated specimens and Scale bars: 50 μ m (A–F); 25 μ m (inset).

stained for either IL-6 or vimentin. DCs were identified by positive vimentin staining and morphologically by their larger cell size and larger euchromatic nuclei compared with vimentin-positive decidual leukocytes (Figure 1, B and D). Immunoreactivity for IL-6 was greater in DCs from CAM-complicated (Figure 1A) versus control specimens (Figure 1C). In CAM-complicated but not control specimens IL-6 immunoreactivity was significantly greater in DCs than in interstitial trophoblasts. In contrast with cytoplasmic IL-6 immunostaining in DCs, IL-6 immunostaining in interstitial trophoblasts was perimembranous and particularly prominent in trophoblasts adjacent to DCs; this effect was more prominent in CAM-complicated specimens (Figure 1A). Immunostaining with an isotype-matched negative control antibody revealed no staining (Figure 1E). HSCORE analysis (Figure 1F) confirmed that IL-6 staining in DCs of CAM-complicated specimens was significantly higher than in those of control specimens (HSCORE mean $\pm$ SEM: 201 $\pm$ 20 versus 152 $\pm$ 23, respectively; $P < 0.05$) and that DCs displayed higher IL-6 staining than trophoblasts in CAM-complicated specimens (HSCORE mean $\pm$ SEM: 201 $\pm$ 20 versus 133 $\pm$ 9, respectively; $P < 0.05$). In contrast, IL-6 staining intensity was similar in trophoblasts of CAM-complicated and control specimens (HSCORE mean $\pm$ SEM: 133 $\pm$ 9 and 130 $\pm$ 8, respectively; not significant) and in DCs and trophoblasts of control specimens (HSCORE mean $\pm$ SEM: 152 $\pm$ 23 and 130 $\pm$ 8, respectively; not significant).

Regulation of IL-6 Protein Expression in DCs

Elevated circulating E_2 and progesterone levels during gestation prompted use of E_2 as the control during parallel incubations of leukocyte-depleted term DCs with E_2 plus MPA. Figure 2, A and B, displays the results of 12 separate experiments. It indicates that IL-6 levels were similar in conditioned DM of DCs incubated in parallel with E_2 (0.34 $\pm$ 0.15 pg/ml/ μ g of protein) or with E_2 + MPA (0.24 $\pm$ 0.15 pg/ml/ μ g of protein); (mean $\pm$ SEM, $n = 12$). Incubation of E_2 -treated and E_2 + MPA-treated DCs with 1.0 ng/ml of IL-1 β markedly enhanced IL-6 output to respective 577 $\pm$ 218 pg/ml/ μ g of protein ($P < 0.05$) and 157 $\pm$ 35 pg/ml/ μ g of protein ($P < 0.05$). The IL-1 β -enhanced response in incubations with E_2 of 2474 $\pm$ 586-fold was reduced to 792 $\pm$ 146-fold in incubations with E_2 + MPA. This MPA-mediated blunting of IL-1 β -augmented IL-6 output proved to be statistically significant ($P < 0.05$).

The Western blot depicted in Figure 3 indicates that the conditioned DM from term DCs incubated with 1 ng/ml of IL-1 β contains a doublet that corresponds to a previously described major band at 21 to 24 kDa and a minor band at 25 to 29 kDa that arises from differences in O-glycosylation of IL-6.²² Inspection of Figure 3 confirms the ELISA results of Figure 2, ie, when compared with E_2 alone, coincubation with E_2 and MPA reduced the magnitude of the marked IL-1 β -mediated increase of the doublet representing IL-6.

Figure 4 displays the effects of increasing concentrations of IL-1 β on secreted levels of IL-6 as determined by

Figure 2. Effects of MPA and IL-1 β on IL-6 output by DC monolayers. Confluent, leukocyte-free term DCs were incubated for 7 days in 10^{-8} mol/L E₂ or E₂ + 10^{-7} mol/L MPA then switched to DM with corresponding steroid(s) $\pm$ 1 ng/ml IL-1 β for 24 hours. IL-6 levels were measured by ELISA in conditioned DM and normalized to cell protein amount. Decidual specimens were obtained from separate patients ($n = 12$; mean $\pm$ SEM). The ordinate represents IL-6 (pg/ml/ μ g of cell protein) as linear plot (A) and log scale (B). *Versus E₂ or E₂ + MPA; **versus E₂ + IL-1 β ($P < 0.05$).

ELISA in leukocyte-free term DCs incubated with E₂ + MPA. Exogenous IL-1 β elicited a statistically significant concentration-dependent increase in IL-6 output between 0.01 and 10 ng/ml of exogenous IL-1 β .

Figure 5, A and B, indicates that changes in steady-state IL-6 mRNA levels as determined by quantitative real time RT-PCR in term DC monolayers correspond to those of IL-6 protein. Specifically, IL-1 β added at 1.0 ng/ml enhanced IL-6 mRNA levels by 1883 ± 749 -fold when incubated with E₂ alone and by 577 ± 122 -fold in parallel incubations with E₂ + MPA ($n = 8$; mean $\pm$ SEM; $P < 0.05$). As with the effects of MPA on secreted IL-6 protein levels displayed in Figure 2, the blunting exerted by MPA on IL-1 β -enhanced IL-6 mRNA levels proved to be statistically significant ($P < 0.05$).

Figure 3. Western blot of MPA and IL-1 β effects on IL-6 output by DC monolayers. Confluent, leukocyte-free term DCs were incubated for 7 days in 10^{-8} mol/L E₂ or E₂ + 10^{-7} mol/L MPA, then switched to a DM with corresponding steroid(s) $\pm$ 1 ng/ml IL-1 β for 24 hours. Conditioned DM was subjected to 10 to 20% SDS-polyacrylamide gel electrophoresis and Western blotting (see *Materials and Methods*). A representative blot at a 10-second exposure time is shown. Only the lanes relevant to this study are shown; the others have been omitted from the image.

Figure 4. Concentration-dependent effects of IL-1 β on IL-6 output by DC monolayers maintained in E₂ + MPA. Confluent, leukocyte-free term trimester DCs were incubated for 7 days in 10^{-8} mol/L E₂ + 10^{-7} mol/L MPA and then were switched to a DM with steroid(s) $\pm$ the indicated amount of IL-1 β (0.01 to 10.0 ng/ml). IL-6 levels were measured by ELISA in conditioned DM and normalized to cell protein amount. Decidual specimens were obtained from separate patients ($n = 5$, mean $\pm$ SEM). *Versus E₂ + MPA; **versus 0.01 ng/ml IL-1 β ; ***versus 0.1 ng/ml IL-1 β ($P < 0.05$).

Figure 6A indicates that the specific inhibitors NF- κ B activation inhibitor III and the p38 MAPK inhibitor SB203580 each blunted IL-1 β -enhanced secreted IL-6 levels in term DC monolayers incubated with E₂ + MPA by at least half during incubation with E₂ + MPA. In contrast, the specific protein kinase C inhibitor calphostin C did not affect IL-1 β -enhanced IL-6 output nor did the negative control for the p38 MAPK inhibitor. In addition, Figure 6B indicates that none of the inhibitors significantly affected basal IL-6 output. Western blotting confirmed these ELISA results (Figure 7).

Figure 5. Effects of MPA and IL-1 β on IL-6 mRNA levels in DC monolayers. Confluent leukocyte-free term DCs were incubated for seven days in 10^{-8} mol/L E₂ or 10^{-8} mol/L E₂ + 10^{-7} mol/L MPA and then were switched to a DM with corresponding steroid(s) $\pm$ 1 ng/ml IL-1 β for six hours. Aliquots of extracted total RNA were used to measure IL-6 mRNA levels by quantitative real-time RT-PCR. Decidual specimens were from separate patients ($n = 8$; mean $\pm$ SEM). The ordinate represents IL-6 mRNA/ β -actin as a linear plot (A) and log scale (B). *Versus E₂ or E₂ + MPA; **versus E₂ + IL-1 β ($P < 0.05$).

Figure 6. Effects of signaling pathway inhibitors on IL-6 secretion by term DC monolayers. Confluent, leukocyte-free term DCs were incubated for seven days in 10^{-8} mol/L E_2 + 10^{-7} mol/L MPA. Cultures were then switched for 24 hours to a DM with E_2 + MPA $\pm$ signaling pathway inhibitors $\pm$ 1 ng/ml IL-1 β . The following specific inhibitors were used: p38 MAPK inhibitor (P38KI; SB203580), protein kinase C inhibitor (PKCI; calphostin C), and NF- κ B activation inhibitor (NFkBBI). All of the inhibitors were added as pretreatments for 30 to 60 minutes before the addition of IL-1 β . Levels of IL-6 were measured by ELISA in conditioned DM and normalized to cell protein amount. Decidual specimens were obtained from separate patients ($n = 6$ each for E_2 + MPA and p38KI groups; $n = 3$ each for PKCI and NFkBBI groups; mean $\pm$ SEM). The ordinate represents IL-6 (pg/ml/ μ g of cell protein). **A:** Effect of inhibitors on IL-1 β -induced IL-6. **B:** Effect of inhibitors on IL-6 under basal conditions (E_2 + MPA). *Versus E_2 + MPA; **versus E_2 + MPA + IL-1 β ($P < 0.05$). neg cont, negative control.

Figure 8 indicates that in monocyte incubations with conditioned media from term DCs with E_2 , treatment with an IL-6-neutralizing antibody did not alter the percentage of either CD14-positive macrophages (left panel) or

Figure 7. Western blot of effects of signaling pathway inhibitors on IL-6 secretion by term DC monolayers. Confluent, leukocyte-free term DCs were incubated for seven days in 10^{-8} mol/L E_2 + 10^{-7} mol/L MPA. Cultures were then switched for 24 hours to a DM with E_2 + MPA $\pm$ signaling pathway inhibitors $\pm$ 1 ng/ml IL-1 β . The following specific inhibitors were used: p38 MAPK inhibitor (P38KI), protein kinase C inhibitor (PKCI), and NF- κ B activation inhibitor (NFkBBI). All of the inhibitors were added as pretreatments for 30 to 60 minutes before the addition of IL-1 β . Conditioned DM was subjected to 12% SDS-polyacrylamide gel electrophoresis and Western blotting (see Materials and Methods). A representative blot at a 2-minute exposure time is shown. Positive control (Pos Cont): 10 ng of recombinant human IL-6.

CD11c-positive dendritic cells (right panel). However, in parallel monocyte incubations with conditioned media from term DCs with E_2 plus IL-1 β , which elevated secreted levels of IL-6 greater than 2000-fold in the term DCs (seen in Figure 2), a statistically significant increase in CD14-positive cells was observed (left panel). Furthermore, this increase was blocked by coincubation with an IL-6-neutralizing antibody (left panel). In comparison, the right panel indicates that these monocyte incubations with conditioned media from term DCs with E_2 + IL-1 β led to a small nonsignificant increase in numbers of CD11c-bearing cells. Paradoxically, coincubation with the antibody against IL-6 resulted in a statistically significant increase in CD11c-bearing cells.

Discussion

Elevated IL-6 levels in amniotic fluid, maternal serum, and cervicovaginal secretions each reliably predict preterm birth.^{12-14,23} Augmented IL-6 levels in amniotic fluid in combination with evaluation of cervical characteristics by vaginal sonography and elevated levels of fetal fibronectin in cervicovaginal secretions have proven to be the best method of predicting PTD.²⁴ Previous studies suggested that IL-6 derived from human decidual and chorion laeve cells^{25,26} contribute to elevated amniotic fluid IL-6 levels involved in the pathophysiology of infection-related PTD. Levels of IL-6 in the amniotic fluid of women with infection were found to enhance the production of uterine contraction-mediating prostaglandin E_2 when incubated with amnion or DCs, thereby suggesting a mechanism to account for CAM-associated PTD.²⁷

As in our evaluation of preeclamptic versus control decidual specimens,¹⁰ the current study observed IL-6 immunostaining localized primarily in the cytoplasm of DCs that increased significantly in CAM versus control placental sections (HSCOREs, $P < 0.05$). In contrast, IL-6 HSCOREs were significantly lower in adjacent interstitial trophoblasts where the staining is primarily perimembranous and similar between patients and control subjects. Consistent with DCs as a source of CAM-related new IL-6 protein synthesis, the current study found that IL-1 β markedly up-regulated IL-6 mRNA and protein expression in leukocyte-free term DC monolayers. These *in vivo* and *in vitro* results suggest that DCs, the predominant cell-type in human decidua, contribute to elevated IL-6 levels in maternal serum as well as amniotic fluid during CAM.^{12,23} Incubation with a specific p38 MAPK inhibitor and a specific NF- κ B activation inhibitor significantly blunted IL-1 β -enhanced IL-6 output by term DCs. As for other inflammatory conditions, these results suggest that CAM-associated augmented IL-6 expression in DCs is probably mediated at least in part by both the p38 MAPK and the NF- κ B activation signaling pathways.

As is typical of an initial host-mediated protective response against microbial invasion, during CAM neutrophil trafficking into the decidua promotes acute inflammation.²⁸ The previously cited Alabama Preterm Birth Study noted a strong association between positive intrauterine microbial cultures and neutrophil infiltration of the

Figure 8. Functional *in vitro* assay. IL-6 skews monocyte differentiation to macrophages. Monocytes from human peripheral blood were cultured for five days in a combination of conditioned medium obtained from term decidua cells that had been treated for 24 hours with E₂ or E₂ + IL-1β in the presence or absence of anti-IL-6 antibody (α-IL6) and 20 ng/ml M-CSF, 30 ng/ml GM-CSF, and 10 ng/ml IL-4. Differentiation was determined at day 5 by the expression of the macrophage markers CD14 (left) and CD68 (data not shown for CD68) and the dendritic cell marker CD11c (right). Data are shown as the mean ± SEM (*n* = 4). **P* < 0.05; ***P* < 0.005.

free membranes, chorionic plate, and umbilical cord.⁷ Consistent with this acute inflammatory response, our laboratory²⁹ and that of Dudley³⁰ found that in cultured term DCs IL-1β enhances expression of well established neutrophil chemoattractants and activators such as IL-8, epithelial neutrophil-activating peptide (ENA-78), growth-related oncogene-α (Gro-α), and granulocyte-chemotactic protein-2 (MCP-2).

As the first immune cells to arrive at inflammatory sites, neutrophils phagocytose and kill invading microorganisms and provide further protection by initiating the acute inflammatory host response via release of reactive oxygen species.³¹ Neutrophils are also a rich source of extracellular matrix (ECM)-degrading proteases such as neutrophil elastase and the matrix metalloproteinases (MMPs) including MMP-8, which preferentially degrades fibrillar collagens, and MMP-9, which degrades basement membrane-associated collagens IV and V.^{32,33} Our previous study³⁴ showed that incubation of leukocyte-free term DCs with IL-1β markedly enhanced the expression of MMP-1, which preferentially degrades fibrillar collagens, and MMP-3, which degrades several ECM substrates and can mediate an ECM-degrading cascade by activating secreted pro-MMP-1 and MMP-9.^{35,36} The interactive effects of a fibrillar collagen-rich ECM scaffolding in the amnion and chorion/decidua normally withstands disruptive physical forces stemming from the fetus, amniotic fluid, and myometrial contractions by providing greater than additive tensile strength.³⁷ Degradation of these ECMs by proteases derived from neutrophils or DCs impairs this structural integrity to promote PPRM.³⁸

Chronic deciduitis in the absence of vilitis as defined by mononuclear cell infiltration of the decidua basalis occurs in 5 to 10% of placentas and leads to PTD.²⁸ In a significant subset of patients, the decidua basalis contains a mononuclear infiltrate composed primarily of plasmacytes (B-lymphocytes) and secondarily of histiocytes (macrophages) that is uniquely associated with severe pathological changes, ie, neonatal intraventricular hemorrhage and death.⁷ Decidual cell-derived IL-6 is likely to play an integral role in promoting this increase in the local monocyte population. Specifically, in several pathological conditions, IL-6 mediates the transition from acute to chronic inflammation by inhibiting neutrophil infiltration.^{21,39}

During inflammation IL-6 also is involved in promoting monocyte differentiation away from dendritic cells toward

macrophages.^{21,39} The existence of this mechanism in human DCs near or at term is indicated by the results of a functional assay presented in Figure 8. Specifically, incubation of monocytes isolated from the circulation with conditioned medium (DM) from IL-1β-treated leukocyte-free term DC monolayers induced the differentiation of monocytes to macrophages and had a slight but not significant increase on the dendritic cell population. However, preincubation of this same conditioned medium with a specific IL-6-neutralizing antibody blocked this differentiation to macrophages while inducing a reciprocal increase in dendritic cells. This finding suggests that among several agents present in IL-1β-treated DC-conditioned medium, IL-6 plays a decisive role in promoting differentiation to macrophages and this conversion is normally kept in check by the presence of excess IL-6.

In addition to neutrophil chemoattractants, human DCs at term express a complex array of other immune cell modulators. These include GM-CSF, which is both a potent neutrophil and macrophage chemoattractant and activator,⁴⁰ as well as the monocyte/macrophage chemoattractant and activators, M-CSF⁴¹ and monocyte chemoattractant protein-1 (MCP-1).⁴² The decidua of patients with CAM-complicated pregnancies also contains several factors that can alter the immune response. These include i) the initial decidual influx of neutrophils, which can protect against the transition to chronic inflammation and its potential pathological consequences⁴³ by expressing Th2 mediators, such as the cytokine inhibitors tumor necrosis factor soluble receptor type 2 and the IL-1 receptor antagonist⁴⁴ and ii) a constitutively expressed excess of macrophage migration inhibitory factor, a 12.5-kDa cytokine that in some contexts inhibits migration and chemotaxis of macrophages.⁴¹ Moreover, inhibition of IL-6 expression by MPA in cultured term DCs described in the current study suggests a mechanism by which maintenance of high circulating progesterone levels blocks conversion of acute to chronic inflammation in the decidua. The specificity of this progestin-mediated inhibition of IL-6 expression is indicated by our observation that MPA did not affect expression of either GM-CSF,⁴⁰ M-CSF,⁴¹ MCP-1,⁴² or IL-8²⁹ by the DCs.

The severe pathological complications associated with monocyte-associated chronic inflammation in the decidua described in the Alabama Preterm Birth Study were not experienced by the majority of patients with CAM in

whom infiltration of neutrophils remained dominant. This suggests that persistence of acute local inflammation leading to PTD in most women with CAM may have developed as an evolutionary protective alternative. Uncovering the mechanisms that normally prevent DC-derived IL-6 from promoting a local chronic inflammatory response presents an important challenge. In this regard, human term DCs also express IL-11,⁴⁵ another member of the IL-6 cytokine family, that generally differs from IL-6 in exerting anti-inflammatory actions.^{46,47} The possibility that progesterin-induced prolactin expression in DCs protects against IL-6-mediated chronic inflammation deserves investigation. Both rodent and human DCs continue to express prolactin throughout term.^{48–50} The rodent decidua does not normally express IL-6, whereas active IL-6 expression by the decidua of transgenic prolactin or prolactin receptor knockout mice⁵¹ promotes aberrant local inflammation associated with premature abortion and fetal death⁵¹ reminiscent of the fetal inflammatory response syndrome in humans, which is characterized by systemic inflammation and elevated fetal plasma IL-6 levels.⁵² Further studies are needed to elucidate the mechanisms underlying the overexpression of decidual cell IL-6 in chorioamnionitis.

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