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IL-6 Trans-Signaling System in Intra-Amniotic Inflammation, Preterm Birth, and Preterm Premature Rupture of the Membranes

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Classic IL-6 signaling is conditioned by the transmembrane receptor (IL-6R) and homodimerization of gp130. During trans-signaling, IL-6 binds to soluble IL-6R (sIL-6R), enabling activation of cells expressing solely gp130. Soluble gp130 (sgp130) selectively inhibits IL-6 trans-signaling. To characterize amniotic fluid (AF) IL-6 trans-signaling molecules (IL-6, sIL-6R, sgp130) in normal gestations and pregnancies complicated by intra-amniotic inflammation (IAI), we studied 301 women during second trimester (n = 39), third trimester (n = 40), and preterm labor with intact (n = 131, 85 negative IAI and 46 positive IAI) or preterm premature rupture of membranes (PPROM; n = 91, 61 negative IAI and 30 positive IAI). ELISA, Western blotting, and real-time RT-PCR were used to investigate AF, placenta, and amniochorion for protein and mRNA expression of sIL-6R, sgp130, IL-6R, and gp130. Tissues were immunostained for IL-6R, gp130, CD15+ (polymorphonuclear), and CD3+ (T cell) inflammatory cells. Real-time RT-PCR was used to investigate AF, placenta, and amniochorion for protein and mRNA expression of sIL-6R, sgp130, IL-6R, and gp130.

The ability of sIL-6R and sgp130 to modulate basal and LPS-stimulated release of amniochorion matrix metalloprotease-9 was tested ex vivo. We showed that in physiologic gestations, AF sgp130 decreases toward term. AF IL-6 and sIL-6R were increased in IAI, whereas sgp130 was decreased in PPROM. Our results suggested that fetal membranes are the probable source of AF sIL-6R and sgp130. Immunohistochemistry and RT-PCR revealed increased IL-6R and decreased gp130 expression in amniochorion of women with IAI. Ex vivo, sIL-6R and LPS augmented amniochorion matrix metalloprotease-9 release, whereas sgp130 opposed this effect. We conclude that IL-6 trans-signaling molecules are physiologic constituents of the AF regulated by gestational age and inflammation.

PPROM likely involves functional loss of sgp130.
The height of the inflammatory response elicited by microbial invasion of the amniotic fluid (AF) cavity and amnionchorion is critical for pregnancy outcome (1). For instance, cytokine activation triggers release of matrix metalloproteases (MMPs), which, in turn, leads to degradation of extracellular matrix and preterm premature rupture of membranes (PPROM) (13). That the levels of AF IL-6 in pregnancies complicated by intra-amniotic inflammation (IAI) are significantly increased remains undisputed (1, 14). Still, our understanding of IL-6’s biological function in AF remains incomplete unless placed in the context of its two signaling pathways. We hypothesized that aside from IL-6, human AF contains IL-6 trans-signaling molecules with independent ability to modulate downstream end points with pathogenic significance for preterm birth (PTB).

In this study, we investigated the presence and activation of IL-6 trans-signaling in AF and reproductive tissues of pregnancies complicated by IAI. Furthermore, we provide functional evidence that sgp130 is a modulator of fetal membrane MMP-9 release, thereby highlighting the involvement of IL-6 trans-signaling in PPROM and PTB.

Materials and Methods
Patient population and AF samples
A flowchart of the women enrolled in the study outlining the subgroups of analyzed samples is presented in Supplemental Fig. 1. AF was retrieved from 301 consecutive women who had an indicated amniocentesis at Yale-New Haven Hospital from March 2004 to December 2008. All women provided informed consent. The study was approved by the Human Investigation Committee of Yale University.

We studied the following clinical groups: 1) second-trimester women undergoing genetic amniocentesis who delivered a healthy baby at term (gestational age [GA] median [interquartile range]: 19 [17–20] wk; n = 39); 2) third-trimester women undergoing fetal lung maturity testing before delivery (GA: 36 [36–37] wk; n = 40); and 3) women presenting with preterm labor or PPROM who had an amniocentesis to rule out infection (GA: 28 [25–31] wk; n = 222). This last group was further divided into women with negative IAI and intact membrane (GA: 28 [25–32] wk; n = 85), women with negative IAI and PPROM (GA: 30 [28–32] wk; n = 61), women with positive IAI and intact membrane (GA: 26 [24–29] wk; n = 46), and women with positive IAI and PPROM (GA: 28 [26–31] wk; n = 30). Of the 85 cases with negative IAI and intact membranes, 31 women delivered a healthy term baby. These cases were analyzed together with the genetic and lung maturity samples for GA correlation of AF IL-6, sIL-6R, and sgp130.

Second- and third-trimester control groups were designed to identify possible GA-mediated changes in AF levels of IL-6, sIL-6R, and sgp130. A cutoff of 27 completed weeks GA was chosen to delineate the second (≥28 wk GA) from third trimester (≥28 wk GA). The indications for the second-trimester amniocentesis included advanced maternal age, personal preference for testing, abnormal serum screening, or presence of minor ultrasonic markers suggestive of aneuploidy (choroid plexus cyst, intracardiac echogenic foci). Amniocentesis results for all women included in this group showed normal fetal karyotypes. The third-trimester group consisted of healthy women where confirmation of fetal lung maturity was clinically indicated before induction of labor or surgical delivery (cesarean section). PTB was defined as delivery of the fetus <37 wk GA. Preterm labor was defined as the presence of regular uterine contractions and documented cervical effacement and/or dilation in patients <37 wk GA. PPROM was confirmed by visualization of vaginal “pooling” at sterile speculum examination and positive “nitrazine” or “ferning” tests. When necessary, an intra-amniotic “dye test” (infusion of indigo carmine) was used (n = 25). Exclusion criteria for the study population included chromosomal aneuploidy, fetal structural abnormalities, multiple gestation, uterine contractions for the second- and third-trimester controls, known maternal medical conditions, anhydramnios, viral infection (human immunodeficiency or hepatitis), and fetal heart rate abnormalities at enrollment (i.e., bradycardia, prolonged/ repeated variable decelerations) requiring emergent delivery.

Protocol implemented for the clinical care of the preterm labor patients
Management of the patients was left to the clinical team. Based on the American Congress of Obstetrics and Gynecology recommendations, in the absence of clinical laboratory results suggestive of infection, or signs/symptoms of clinical chorioamnionitis (fever > 100.4°F, abdominal tenderness, fetal tachycardia), and/or abnormalities of fetal heart rate (variable or late decelerations), and/or abruption, PPROM was managed expectantly (15). If PPROM, digital examinations were not allowed. Patients received corticosteroids for lung maturity if ≤32 wk GA and antibiotic therapy as clinically indicated. Most preterm labor/PPROM women (80%) had their amniocentesis procedure performed before antibiotic or steroid therapy. Intrapartum women were monitored by cardiotocography at least twice daily for the presence of fetal heart abnormalities, uterine contractions, or both.

The decision for amniocentesis, expectant management, tocolysis, or indicated delivery was made by the primary physician independent of our research protocol. The clinical management team was not aware of our research test results. After amniocentesis, each woman was followed prospectively to delivery. Indicated delivery through induction of labor or cesarean section was performed for such clinical indications as AF laboratory results traditionally considered to indicate IAI/infection, fetal lung maturity, prolapsed umbilical cord, and/or GA ≥ 34 wk.

Biochemical and microbiological studies of AF
A diagnosis of intra-amniotic infection and/or IAI was established based on biochemical and microbiological test panels. AF was analyzed for glucose concentration, lactate dehydrogenase (LDH) activity, WBC count, and Gram stain. Standard culturing methods for aerobic and anaerobic bacteria, including Enterococcus and Mycoplasma species, were used. Positive Gram stain and/or culture results were considered suggestive of intra-amniotic infection. Once the clinical requirements were satisfied, the remaining AF was centrifuged at 3000 × g, 4°C for 10 min, aliquoted, and stored at −80°C for research purposes. The results for these studies were used for clinical case management of the cases.

Diagnosis of IAI by mass spectrometry and Mass Restricted score
In previous studies, our group demonstrated that proteomic profiling of AF using surface-enhanced laser desorption ionization time-of-flight mass spectrometry was the most accurate research method for diagnosing IAI (14, 16). In brief, the AF Mass Restricted score is composed of four proteomic biomarkers: defense-2, defense-1, S100A12 (calgranulin C), and S100A8 (calgranulin A). The Mass Restricted score ranges from 0 to 4, depending on the presence or absence of each of the four protein biomarkers. A value of 1 was assigned if a biomarker peak was present and 0 if absent. A Mass Restricted score of 3 or 4 indicates the presence of IAI. All mass spectrometry tracings were scored by one investigator (I.A.B.) who was unaware of the results of the biochemical or microbiological tests used to diagnose IAI/intra-amniotic infection.

Immunoasays for IL-6, sIL-6R, sgp130, and MPP-9
ELISA assays for IL-6 (eBioscience, San Diego, CA), sIL-6R (eBioscience), and sgp130 (R&D Systems, Minneapolis, MN) were performed to measure their respective levels in AF and explant media. The molar ratio between AF sIL-6R and sgp130 was calculated for each patient as previously described (17). Explant media was also immunoassayed for MPP-9. The assays were run in duplicate according to the manufacturer’s protocols. For all assays, samples were diluted from 1:10 to 1:100 to fall within the range of the standard curves. The interassay and intra-assay coefficients of variation was <10% for all the analytes.

Evaluation of histological chorioamnionitis
Paraffin-embedded tissues were available from 119/146 (82%) of the negative IAI and 76/76 (100%) of the positive IAI patients who provided AF samples. Placental and fetal membranes tissues biopsies were collected immediately after delivery. Tissues were formalin fixed and embedded in paraffin. For clinical purposes, a diagnosis of histological inflammation of the placenta and fetal membranes was based on well-established criteria (18).

Placental and fetal membrane IL-6R, gp130, CD3, and CD15 immunohistochemistry
Immunohistochemistry for IL-6R, gp130, CD3, and CD15 was performed on formalin-fixed paraffin-embedded placental sections. Paraffin sections were blocked with normal goat serum for CD3, CD15, and CD16 antibodies and with normal rabbit serum for IL-6R antibody. A biotin-avidin-peroxidase system was used to detect antibodies. Antibody staining was evaluated by experts who were unaware of the patient’s clinical status or study group assignment.
presentation (i.e., breech) or prior cesarean delivery. All their infants were appropriately grown for GA and had reassuring fetal heart rate patterns before surgery. Clinical characteristics of these cases are provided in Supplemental Table I.

Five-micrometer paraffin tissue sections were deparaffinized in xylene and rehydrated with graded ethanol to potassium-PBS solution, pH 7.2. After Ag retrieval with citrate buffer (pH 6), the sections were pretreated with 1% hydrogen peroxide for 15 min, followed by 1 h blocking with 5% goat serum. The following primary Abs were used: rabbit anti-human IL-6R (sc-661; 1:200, overnight incubation at 4°C; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human gp130 (sc-9999; 1:50 dilution, overnight incubation at room temperature; Santa Cruz Biotechnology), rabbit anti-human CD3 (T cell marker; A0452; 1:200, overnight incubation at 4°C; Dako, Denmark), mouse monoclonal CD15 (polymorphonuclear neutrophil marker; ab53907; 1:50, overnight incubation at 4°C). After 1 h incubation with appropriate secondary Abs (biotinylated goat anti-rabbit or anti-mouse IgG 1:600 dilution; Jackson Immunochemicals, West Grove, PA), the sections were developed using the avidin-biotin-peroxidase system (Vectorstain Elite ABC; Vector Laboratories, Burlingame, CA) with Vector NovaRed (Vector Laboratories) as chromogen and hematoxylin as counterstain. The tissue sections were dehydrated in graded ethanol, cleared, and mounted. Specificity of staining was confirmed by replacing the primary Abs with equivalent concentrations of mouse or rabbit non-immune IgG (Novus Biologicals, Littleton, CO). Immunohistochemical staining of the intensity of the chromogen deposited in the amnion epithelium, choriodecidual and placental villous trophoblast, and stromal and endothelial cells was evaluated semiquantitatively in a blind fashion by examining three fields per slide and subjectively scoring on a scale from 0 (no staining) to 3 (intense red-brown staining) the intensity of the chromogen deposited in the amnion epithelium, choriodecidual, and placental villous trophoblast, as previously described (21). Vimentin immunostaining was performed to identify decidual cells, whereas cytokeratin staining identified trophoblast cells (data not shown).

Quantitative real-time RT-PCR and Western blotting for IL-6R and gp130

For the RT-PCR experiments, we used tissues (placenta and amniochorion membranes) retrieved from the same cases where the IL-6R level of expression was evaluated by immunohistochemistry. Clinical characteristics of these cases are provided in Supplemental Table I. Immediately after delivery, tissues were frozen in liquid nitrogen and kept at −80°C. RNA was extracted and reverse transcribed into cDNA with random hexamer primers using standard procedures. Quantitative RT-PCR was performed using TaqMan (Applied Biosystems) chemistry in 20 μl reactions composed of 10 μl master mix (TaqMan Fast Universal PCR 2X Master Mix), 8 μl water, 1 μl cDNA template normalized, and 1 μl PCR probe set (TaqMan Gene Expression Assays on Demand). For the detection of IL-6R and gp130 mRNAs, TaqMan probes from Applied Biosystems were used: hs01069842_m1 (IL-6R) (22) and hs00174360_m1 (gp130) (23). For controls, we used TaqMan probes hs99999907_m1 (β2 microglobulin, B2M) and hs00265497_m1 (ribosomal protein L30, RPL30). The combination of the two endogenous control mRNAs (B2M and RPL30) was validated in preliminary experiments using pools of cDNA amplified in the TaqMan Human Endogenous Control Plate (Applied Biosystems). Selection of the two reference genes was based on low cycle threshold (Ct) values that were not different among the six cDNA pools.

For each target, amplification was performed in duplicate reactions in a 2-step cycle (denaturation, 95°C for 15 s, annealing/extension at 62°C for 60 s) for 40 cycles. Postprocessing calculations were performed using the StepOne Software (v.2.1). The values obtained were normalized to the geometric mean of endogenous control mRNAs using calculations of ∆Ct (Ct of the target – Ct mean of endogenous controls). A ∆Ct of 0 indicates a ratio of 1 between the target and housekeeping genes. This ratio may be used as an indication of relative abundance between different targets among different tissues. Calculations of ∆∆Ct (ΔCt of individual sample – ΔCt of same target in reference sample) adds an additional normalization step. Therefore, ∆∆Ct improves estimation of relative mRNA abundance among different biological groups (24, 25). For ∆∆Ct calculations, we used our reference sample RNA pools of the same tissue type.

Western blotting for IL-6R and gp130 isoforms

We used fetal membrane, placental tissue lysates, and corresponding AF samples of a select group of preterm women with (n = 6) and without (n = 6) IAI and/or histological chorioamnionitis. Western blotting was performed on AF (5 μl/lane), fetal membrane, and placenta tissue lysates (20 μg total protein/lane) prepared by homogenization in a buffer containing 50 mM Tris buffer (pH 7.4), 0.1 mM EDTA, 0.14 μM l-2 ME, and complete protease inhibitor mixture (Roche Applied Sciences, Indianapolis, IN). Samples were mixed 1:2 with reducing sample buffer (Bio-Rad, La Jolla, CA), boiled, and applied to 4–20% SDS-PAGE gels. After electrophoretic transfer, nitrocellulose membranes were blocked with 5% milk and then incubated overnight at 4°C with either polyclonal rabbit anti–IL-6R Ab (sc-661, 1:200; Santa Cruz Biotechnology) or mouse monoclonal anti-gp130 Ab (clone B-P4; ab34325, 1:300; Abcam, Cambridge, MA). Detection was performed using appropriate HRP-linked secondary Abs and chemiluminescence (ECL Plus; Amersham Biosystems). OD of the bands of interest (sIL-6R: ~44–55 kDa [26]; sgp130: ~100 kDa [27]) was analyzed with ImageJ software (National Institutes of Health: http://rsb.info.nih.gov).

Fetal membranes explant culture system

Fetal membranes were obtained from healthy women (n = 7) without any maternal medical history undergoing scheduled, elective cesarean delivery in the absence of labor (GA: 39 [38–40] wk). Indications for abdominal delivery included elective repeat or prior cesarean delivery for fetal malpresentation (i.e., breech) or prior uterine scar. No patient had abnormal placenta (i.e., placenta previa, abruption). All their infants were appropriately grown for GA and had reassuring fetal heart rate patterns before surgery.

The setup for the fetal membrane culture system was established based on previously published procedures (28, 29). Membranes, distal from the point of iatrogenic rupture, were harvested under sterile conditions and dissected free from the placenta within 10 min of delivery. Ten-millimeter-diameter pieces were washed thoroughly to eliminate blood clots using cold HBSS with antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B). Two pieces of fetal membrane tissue were placed into each well of a 24-well tissue culture dish in 1 ml culture medium containing a 1:1 mixture of DMEM with low glucose, 10% FBS, and antibiotic-antimycotic solution. Prior studies demonstrated that relative cellular quiescence is achieved in untreated membrane cultures after a minimum period of 48 h (30). Therefore, in this study, fetal membranes were initially maintained in the medium for 48 h (37°C, 5% CO2/95% air) and performed in healing, fibroblast-like explant medium and stimulated for 24 h with LPS (50 ng/ml; Sigma, St. Louis, MO), sIL-6R alone (10 ng/ml; R&D Systems), or sgp30 alone (500 ng/ml; R&D Systems). In addition, the effect of sgp30 in the presence of LPS or sIL-6R was also investigated. The LPS dose was chosen based on both a dose–response experiment (5, 50, 500 ng/ml LPS) where we determined that the peak of MMP-9 and IL-6 release in the explant medium was seen after stimulation with 50 ng/ml LPS (Supplemental Fig. 2). Optimal concentrations of sIL-6R and sgp30 were chosen in our attempt to mimic as close as possible our in vivo data.

After 24 h of incubation, the supernatants were collected, centrifuged to remove cellular debris, and stored at −80°C. The incubated tissue was immediately homogenized in 1 ml cell extraction buffer (20 mM Tris-HCl, 150 mmol/l NaCl, 1% Triton X-100, 1 mM PMSF, and complete technology, Rockford, IL), according to the manufacturer’s instructions. To correct for variations in tissue incubated per each well, we normalized the explant medium concentration of the sought analytes (MMP-9, IL-6, sIL-6R, sgp130) to total protein in tissue extract. For each experimental condition, values were derived by averaging normalized values from duplicate wells either without (untreated) or with the various treatments. Values were further interpreted as percent change from basal release.

To assess tissue viability during in vitro incubations, we determined the release of the intracellular enzyme LDH into the incubation medium as previously described using the LDH LiquidUV Assay (Stainbo, Boerne, TX) (31). The interassay and intra-assay coefficients of variation were <5%.

We found no significant change in LDH release during the incubation period and after treatments in support of sustained tissue viability.

Statistical analysis

Normality testing was performed using the Kolmogorov–Smirnov test. Data were compared with one- or two-way ANOVA followed by Student–Newman–Keuls tests (parametric) or Kruskal–Wallis on ranks followed by Dunn’s tests (nonparametric), to adjust for multiple comparisons as appropriate. Statistical analysis of data derived subsequently was performed after logarithmic transformation. Spearman or Pearson correlations were used to measure colinearity between the selected independent variables, as well as other relevant relationships between dependent and
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Table I. Demographic clinical and outcome characteristics of the women who provided AF samples for sgp130, sIL-6R, and IL-6 levels (n = 301)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Second-Trimester Genetic Amniocenteses (n = 39)</th>
<th>Third-Trimester Lung Maturity Amniocenteses (n = 40)</th>
<th>Rule-Out Infection Amniocenteses (n = 222)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, y&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33 (28–38)</td>
<td>30 (26–36)</td>
<td>28 (22–33)</td>
<td>0.002</td>
</tr>
<tr>
<td>Parity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0–1)</td>
<td>1 (0–2)</td>
<td>1 (0–1)</td>
<td>0.017</td>
</tr>
<tr>
<td>Gravidity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (1–3)</td>
<td>3 (2–4)</td>
<td>2 (1–3)</td>
<td>0.162</td>
</tr>
<tr>
<td>GA, wk&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19 (17–20)</td>
<td>36 (36–37)</td>
<td>29 (25–31)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Uterine contractions&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>109 (49)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cervical dilatation &gt; 3 cm&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>72 (32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PPROM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>91 (41)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clinical chorioamnionitis&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>18 (8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Outcome characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Term delivery (≥37 wk)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39 (100)</td>
<td>23 (58)</td>
<td>31 (14)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PTB at &lt; 34 wk&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>175 (79)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GA at delivery&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39 (39–40)</td>
<td>37 (36–37)</td>
<td>31 (27–33)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Amniocentesis-delivery time, d&lt;sup&gt;a&lt;/sup&gt;</td>
<td>143 (134–154)</td>
<td>1 (1–4)</td>
<td>3 (0–17)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data presented as median (interquartile range) and analyzed by Kruskal–Wallis ANOVA on ranks.

<sup>b</sup>Data are presented as n (%) and analyzed by χ² tests.

Results

Gestational regulation of AF trans-signaling molecules IL-6, sIL-6R, and sgp130

Demographic clinical and outcome characteristics of the women who provided AF samples are presented in Table I. Women in the second-trimester genetic amniocentesis group were significantly older compared with those who had an amniocentesis to rule out infection. Third-trimester control women were of higher parity compared with the second-trimester genetic amniocentesis group and, as expected, were evaluated at a more advanced GA compared with the other two groups. Given the clinical circumstances at the time of recruitment, the rule-out infection group had a greater frequency of uterine contractions, advanced degree of cervical dilatation, PPROM, clinical symptoms of chorioamnionitis, and a greater frequency of spontaneous or induced PTB. Seventy-five percent of the third-trimester patients had reassuring AF lung maturity test results (lecithin/sphingomyelin ratio > 2.5). Thus, the third-trimester group experienced a greater frequency of medically indicated PTB and the shortest amniocentesis-to-delivery interval.

Our analysis of GA regulation of IL-6 trans-signaling molecules was limited to genetic (n = 39), lung maturity (n = 40), and rule-out infection amniocenteses from women who ultimately had a term delivery (n = 31). All three trans-signaling analytes (Fig. 1) were constitutively present in human AF across gestation. The AF IL-6 and sIL-6R concentrations did not vary significantly among groups or with GA (Fig. 2A, 2B; absolute levels are provided in Supplemental Table II). Conversely, AF sgp130 was inversely correlated with GA, with lower levels approaching term (Fig. 2C; p < 0.001). Because the balance between the stimulatory sIL-6R and the inhibitory sgp130 may have considerable functional relevance, we were interested in how their relative ratio varied with GA (17). There was a significant GA regulation of sIL-6R/sgp130 molar ratio, which increased toward term (Fig. 2D; p < 0.001).

Changes in AF IL-6 trans-signaling molecules in human pregnancies complicated by IAI, PPROM, or both

The demographic, clinical, and laboratory characteristic of the preterm women are presented in Table II. PPROM patients were significantly older irrespective of IAI status (two-way ANOVA PPROM: p < 0.001; IAI: p = 0.171). We also determined that both IAI and PPROM impacted on latency interval (time from PPROM to delivery) with significant interaction between the two variables (IAI: p < 0.001; ROM: p = 0.010; interaction: p = 0.002). Moreover, women in the IAI groups delivered at earlier GA, and their newborns had lower birth weights. Both GA and birth weight were synergistically impacted by IAI and membrane status (IAI: p < 0.001; PPROM: p < 0.001; interaction: p = 0.001).

The results of the biochemical and microbiological studies of the AF showed lower glucose and higher LDH activity in the IAI groups, which were significantly influenced by membrane status (IAI: p < 0.001; PPROM: p < 0.001; interaction: p = 0.001).

![FIGURE 1. Diagram illustrating the downstream pathways of IL-6 signaling.](http://www.jimmunol.org/Downloadedfrom)
Greater AF WBC counts were seen in the IAI groups with significant interaction with PPROM (IAI: \( p < 0.001 \); PPROM: \( p < 0.001 \); interaction: \( p = 0.005 \)). Lastly, women in IAI groups experienced a higher frequency of positive Gram stain and positive microbial culture results, again significantly influenced by the membrane status (IAI: \( p < 0.001 \); PPROM: \( p < 0.001 \); interaction: \( p = 0.004 \)). The pathological examination of the placenta revealed higher stages and grades of histological chorioamnionitis and funisitis in the IAI groups, independent of membrane status.

### Table II. Demographic, clinical, laboratory, and outcome characteristics of women with symptoms of preterm labor or PPROM who provided AF samples for sgp130, sIL-6R, and IL-6 levels

<table>
<thead>
<tr>
<th>Variable</th>
<th>No IAI and intact ( (n = 85) )</th>
<th>No IAI and PPROM ( (n = 61) )</th>
<th>Yes IAI and intact ( (n = 46) )</th>
<th>Yes IAI and PPROM ( (n = 30) )</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical and outcome characteristics at amniocentesis and at delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age, y(^a)</td>
<td>25 (20–32)</td>
<td>30 (26–33)</td>
<td>24 (21–32)</td>
<td>33 (25–38)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Parity(^b)</td>
<td>1 (0–1)</td>
<td>1 (0–2)</td>
<td>0 (0–1)</td>
<td>1 (0–3)</td>
<td>0.030</td>
</tr>
<tr>
<td>Gravidity(^b)</td>
<td>2 (1–3)</td>
<td>3 (1–4)</td>
<td>2 (1–3)</td>
<td>2 (2–5)</td>
<td>0.365</td>
</tr>
<tr>
<td>History of PTB(^b)</td>
<td>21 (25)</td>
<td>16 (26)</td>
<td>11 (24)</td>
<td>7 (23)</td>
<td>0.989</td>
</tr>
<tr>
<td>GA at amniocentesis, wk(^b)</td>
<td>28 (25–32)</td>
<td>30 (28–32)</td>
<td>26 (24–29)</td>
<td>28 (26–31)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Expectant management(^b)</td>
<td>72 (87)</td>
<td>42 (69)</td>
<td>16 (36)</td>
<td>10 (33)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>GA at delivery, wk(^b)</td>
<td>33 (28–38)</td>
<td>32 (30–33)</td>
<td>26 (24–29)</td>
<td>29 (27–32)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Amniocentesis-delivery interval, h(^c)</td>
<td>428 (52–1379)</td>
<td>107 (31–223)</td>
<td>6 (4–11)</td>
<td>18 (7–69)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Amniocentesis-delivery &lt; 7 d</td>
<td>33 (39)</td>
<td>37 (61)</td>
<td>43 (93)</td>
<td>26 (87)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Birth weight, g(^d)</td>
<td>2210 (1285–3060)</td>
<td>1820 (1430–2126)</td>
<td>955 (731–1246)</td>
<td>1350 (1000–1710)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>AF laboratory test results</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mg/dl(^e)</td>
<td>30 (22–39)</td>
<td>28 (21–40)</td>
<td>3 (2–13)</td>
<td>8 (2–18)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>LDH activity, U/l(^f)</td>
<td>168 (126–236)</td>
<td>150 (105–257)</td>
<td>924 (541–2083)</td>
<td>639 (424–1118)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>WBC count, cells/mm(^3)</td>
<td>3 (1–7)</td>
<td>8 (2–22)</td>
<td>715 (194–1445)</td>
<td>687 (52–2380)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Positive Gram stain(^g)</td>
<td>0 (0)</td>
<td>4 (7)</td>
<td>8 (57)</td>
<td>11 (37)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Positive cultures(^h)</td>
<td>1 (1)</td>
<td>14 (23)</td>
<td>30 (65)</td>
<td>22 (73)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Placental histology results</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 59</td>
<td>n = 60</td>
<td>n = 46</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chorionic plate inflammation, stage(^i)</td>
<td>1 (0–2)</td>
<td>1 (0–3)</td>
<td>3 (3–4)</td>
<td>3 (3–3)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Amnionitis, grade(^j)</td>
<td>0 (0–0)</td>
<td>0 (0–2)</td>
<td>3 (2–4)</td>
<td>3 (2–3)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Chorioamnionitis, grade(^k)</td>
<td>1 (0–3)</td>
<td>2 (0–3)</td>
<td>3 (3–4)</td>
<td>3 (2–4)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Funisitis, grade(^l)</td>
<td>0 (0–0)</td>
<td>0 (0–2)</td>
<td>3 (0–4)</td>
<td>2 (0–4)</td>
<td>(&lt;0.001)</td>
</tr>
</tbody>
</table>

\(^a\)Data are presented as median (interquartile range) and analyzed by Kruskal–Wallis ANOVA on ranks.

\(^b\)Data are presented as n (%) and analyzed by \( \chi^2 \) test.
The absolute values for IL-6, sIL-6R, and sgp130 in AF of preterm women evaluated for IAI are presented in Supplemental Table III. IL-6 was significantly increased in women with IAI, although its levels were lower in IAI with PPROM compared with IAI with intact membranes (two-way ANOVA; Fig. 3A; IAI: \( p < 0.001 \); membrane status: \( p < 0.001 \); interaction: \( p < 0.001 \)). The levels of AF sIL-6R were increased in women with IAI independent of PPROM (Fig. 3B; IAI: \( p < 0.001 \); membrane status: \( p = 0.866 \); interaction: \( p = 0.973 \)). Conversely, PPROM women had decreased sgp130 levels regardless of whether they had IAI (Fig. 3C; IAI: \( p = 0.247 \); membrane status: \( p < 0.001 \); interaction: \( p = 0.129 \)). The sIL-6R/sgp130 molar ratio was significantly increased by PPROM alone and further increased in the setting of PPROM with IAI (Fig. 3D; IAI: \( p < 0.001 \); membrane status: \( p < 0.001 \); interaction: \( p < 0.001 \)). When we restricted our analysis to PPROM cases with negative Gram stain and microbial culture results \( (n = 40) \) managed expectantly postamniocentesis, we determined that women with amniocentesis-to-delivery intervals longer than 7 d \( (n = 16) \) had greater AF sgp130 concentrations compared with cases with shorter latencies \( (n = 24) \); latency \( \geq 7 \) d: 50.1 [38.4–61.4] versus latency \( < 7 \) d: 39.2 [31.1–51.2] ng/ml; \( p = 0.034 \). All results maintained following GA correction.

Transcriptional changes in IL-6R and gp130 in human fetal membranes and placenta

Fig. 4 displays mRNA levels of the full-length IL-6R and gp130 in reproductive tissues of term and preterm women with and without IAI. In the absence of IAI, transcripts of IL-6R and gp130 were present in both placenta and fetal membranes (Fig. 4A). Compared with IL-6R, gp130 mRNA was more abundant \( (p < 0.001) \). IL-6R mRNA levels were similar in fetal membranes and placental villous tissues with absent GA regulation \( (p < 0.001) \). The gp130 mRNA was significantly decreased in term amniochorion compared with preterm tissues \( (p = 0.002) \). This change was not observed in placental villous tissue. We noted a significant increase in IL-6R mRNA \( (p = 0.029; \text{Fig. 4B}) \) concurrent with significantly decreased gp130 expression in amniochorion of IAI cases \( (p = 0.018; \text{Fig. 4C}) \). The differences remained significant after correcting for GA. Placental IL-6R and gp130 mRNA levels remained unaffected by inflammation (Supplemental Fig. 3).

Immunoreactive forms of sIL-6R and sgp130 in human AF, placenta, and fetal membranes

The Western blots in Fig. 4D depict several specific bands corresponding to IL-6R \( (80–110 \text{ kDa}) \) and sIL-6R \( (38–55 \text{ kDa}) \). The multiple bands likely resulted from posttranslational modifications of both targets as previously described \( (33) \). In the setting of IAI, we observed marked differences in banding patterns in both AF \( (\text{Fig. 4D, lanes 1–4}) \) and fetal membranes \( (\text{Fig. 4D, lanes 5–8}) \), but not in placental lysates \( (\text{Fig. 4D, lanes 9, 10}) \). A significant increase in 80- to 110-kDa bands was identified in both AF and fetal membranes of patients with IAI compared with those without IAI \( (p < 0.001) \). In the absence of IAI, immunoreactive bands corresponding to sgp130 \( (∼100 \text{ kDa}) \) were present in the AF \( (\text{Fig. 4E, lanes 1–4}) \) but absent in the fetal membranes and placenta, which appear to express only the full-length gp130 isoform \( (∼130 \text{ kDa}) \). AF bands characteristic to sgp130 were significantly decreased in the context of IAI \( (\text{Fig. 4E, lanes 1–4}; p = 0.026) \). This phenomenon appeared to be mirrored by a decrease in the expression of gp130 protein in fetal membranes but not placenta \( (\text{Fig. 4E, lanes 5–10}) \).

Immunostaining of IL-6R and gp130 in human placenta and fetal membranes

Fig. 5A–D shows IL-6R immunostaining in fetal membranes and placental tissue. In preterm fetal membranes without IAI, IL-6R was localized predominantly in extravillous trophoblasts \( (\text{EVts}) \) and decidual cells, whereas the amnion epithelium exhibited little positive staining \( (\text{Fig. 5A}) \). In IAI, a marked increase in IL-6R immunoreactivity was observed in preterm amnion epithelium and in infiltrating inflammatory cells \( (\text{Fig. 5B}) \). In term fetal membranes, IL-6R remained localized only at the periphery of vacuolated EVTs, whereas decidual cells maintained their staining.
intensity (Fig. 5C). In the placenta, IL-6R was identified in EVTs and villous syncytiotrophoblast (Fig. 5D) without notable changes with either IAI or GA (data not shown). Fig. 5E–H illustrates gp130 immunostaining in fetal membranes and placental tissues in the same tissues as shown for IL-6R. In preterm fetal membranes absent IAI, amnion and chorionic decidual cells displayed conspicuous gp130 staining (Fig. 5E), which decreased significantly with IAI (Fig. 5F) and at term (Fig. 5G). In the placentas, the strongest gp130 signal was noted in villous cytotrophoblasts and in EVTs of the basal and chorionic plate without remarkable impact from histological chorioamnionitis or GA. Given the role of the IL-6 trans-signaling in directing transition from innate to acquired immunity, we characterized the nature of the inflammatory infiltrates in tissues with confirmed histological chorioamnionitis. Fig. 5I and 5J demonstrate concurrent choriodedecial homing for both CD15+ (Fig. 5I) and CD3+ inflammatory cells (Fig. 5J). Both CD15+ and CD3+ cells populated perivascular areas in the decidua as shown in Fig. 5L and 5M. Results of histological scoring of the amnion and choriodedecia for IL-6R and gp130 are shown in Fig. 5O and 5P.

Effect of sIL-6R and sgp130 on MMP-9 release by fetal membrane explants

We explored the functional role of sIL-6R and sgp130 in modulating the release of MMP-9 and IL-6 by using an amniochorion explant system. Recombinant sIL-6R alone, but not sgp130, significantly upregulated MMP-9 (IL-6R versus basal: p = 0.035; Fig. 6A), an effect similar to that of LPS alone (LPS versus basal: p = 0.020; LPS versus sIL6-R: p = 0.330). Addition of recombinant sgp130 over sIL-6R or LPS had an antagonistic effect by reversing the MMP-9 release (p < 0.001 for both) to the basal level (sIL-6R + sgp130 versus basal: p = 0.910; LPS + sgp130 versus basal: p = 0.141; Fig. 6A). sgp130, sIL-6R, and their combination had no effect on IL-6 (Fig. 6B). In contrast, LPS alone significantly upregulated IL-6 levels (LPS versus basal: p < 0.001), an effect unaltered by addition of sgp130 (LPS + sgp130 versus LPS: p = 0.706).

Discussion

Considerable progress has been made in understanding the downstream molecular events of a large array of decidual, placental, fetal membranes, and AF cytokines including TNF-α, IL-1, IL-8, and in particular, IL-6 (1, 3, 8, 34, 35). The latter is a pleiotropic factor that belongs to the family of gp130 cytokines (36). Investigation of IL-6’s bioactivity has been focused primarily on its proinflammatory properties given that augmented AF IL-6 levels are associated with PTB (37). However, as our understanding continues to evolve, we have gained a better appreciation of the physiologic and pathologic roles of IL-6 in human gestation. Consistent with previous reports, our study demonstrated that IL-6 was present in AF of pregnancies with normal outcomes and absent infection (38, 39). That the human amnion, choriodedecidua, and fetus are potential sites of IL-6 synthesis and probable sources of AF IL-6 has been previously proposed (34, 35). Therefore, a relevant question relates to the physiologic function of AF IL-6 across normal human gestation. This study provides firsthand evidence that, if any, the biological role of AF IL-6 can be exercised through both classic and trans-signaling pathways. In support of the classic pathway, we demonstrated that both IL-6R and gp130 are expressed in resident cells of the fetal
**Figure 5.** Representative photomicrographs of IL-6R and gp130 immunoreactivity in fetal membranes and placental sections of preterm and term pregnancies. Preterm amnion (am) stained higher for IL-6R in the presence of IAI compared with no IAI (A, B) and term fetal membranes (C). Intense IL-6R immunostaining was identified in EVT9s (evt; arrows) and decidual cells (open arrowheads) of the chorio-decidua (cd), as identified by cytokeratin- or vimentin-positive staining (data not shown). In the placenta (D), IL-6R was identified in evt and villous syncytiotrophoblasts (st) without notable changes with either IAI or GA (data not shown). Localization of IL-6R staining in evt was both perimenstruous and intracytoplasmic, consistent with the ability of the Ab to recognize both membrane-bound IL-6-R and sIL-6R. The am stained less positive for gp130 than cd with no discernible differences with histological inflammation or GA (E–G). Both evt (arrows) and decidual cells (open arrowheads) stained intensely for gp130 in the absence of histological inflammation (E). In the placenta (H), the strongest gp130 signal was noted in villous cytotrophoblasts (ct) and evt (arrows) of chorionic plate. Outlined area in B is shown in I and J at higher magnification aimed to illustrate concurrent homing of neutrophils (CD15+)(I) and T lymphocytes (CD3+, closed arrowheads) (J) in the inflammatory infiltrate of the cd of women affected by IAI. CD15+ and CD3+ cells were also identified in the vicinity of cd blood vessels (bv) suggestive of transendothelial migration of inflammatory cells (L, M). Specificity of staining was confirmed by incubation of slides with rabbit IgG as control for IL-6R and CD3 staining (K). Mouse nonimmune IgG served as negative control for gp130 and CD15 mAbs (N). IL-6R and gp130 histological scores in the am and cd of preterm (no and yes IAI) and term patients (T) is shown in O and P, respectively. Data are presented as mean + SEM and analyzed by one-way ANOVA followed by post hoc Student–Newman–Keuls tests. Means sharing at least one common superscript letter are not statistically significant (p > 0.05). Scale bars, 30 μm.

membranes and placental villous tissues. It is critical to recognize that IL-6 exercises not only a proinflammatory but also an anti-inflammatory role (1). The anti-inflammatory function of IL-6 is facilitated through downregulation in expression of proinflammatory cytokines (i.e., TNF-α, IFN-γ) and induction of soluble IL-1 and TNF-αR antagonists (40). These mediators act then in an autocrine/paracrine fashion and target normal biological processes occurring within villous trophoblast, decidua, and amnion (41). As a result, regardless of its source, the AF and decidual IL-6 could be of significant relevance for fetal development.

Our novel finding that sIL-6R and sgpl30 are constituents of the normal human AF supports the notion that any biological role assigned to AF IL-6 could be initiated or repressed via the trans-signaling pathway. Similar to IL-6, the source of sIL-6R and sgpl30 in AF is debatable. Our real-time RT-PCR, Western blot, and immunohistochemistry results point to fetal membranes as primary and placenta as secondary sources. Cleavage of the amniochorion and villous trophoblast IL-6R or gp130 could be responsible for the presence of these two soluble factors in AF (9, 42, 43). That this mechanism occurs in fetal membranes and placental tissues needs to be confirmed. However, in the case of sIL-6R, the inducers of the shedding process (i.e., TNF-α converting enzyme) are expressed in amnion and chorio-decidua of healthy women (42). There is a high regulatory complexity of the splicing mechanisms responsible for sIL-6R and sgpl30 synthesis (9). Inducers of the IL-6R mRNA splicing process include oncostatin M (cytokine synthesized by activated T lymphocytes and monocytes) and IL-1 (44). The splicing process for the sgpl30 mRNA continues to be poorly understood, but oncostatin M, IL-1, and IL-6 may play a role (44). The well-documented expression of oncostatin M, IL-1, and IL-6 in the human decidua (34, 45) suggests that physiologic immune processes occurring at the maternal–fetal interface may be responsible for the synthesis of sIL-6R and sgpl30, and their subsequent release into the AF. The contribution of enzymatic cleavage and mRNA splicing mechanisms to the total pool of AF sIL-6R and sgpl30, in addition to the rates of metabolism and clearance for the two aforementioned factors, needs to be clarified in the future.

We showed that AF levels of sgpl30 progressively decrease toward term. This suggests that the inflammatory modulator effect of this molecule is GA dependent. This observation is remarkable because sgpl30 specifically inhibits sIL-6R–reliant responses and does not interfere with the cell-surface expressed IL-6R (6). Our data raise important questions regarding the identity of the inhibitory mechanisms leading to the decreased cellular release or synthesis of sgpl30 at term. Understanding the subtleties of this...
regulatory process is important because an increased level of sgp130 can render cells unresponsive to IL-6 and the family of gp130 cytokines. Data derived from the human endometrium demonstrated that sgp130 was downregulated in the proliferative but upregulated during the secretory phase of the menstrual cycle (46). This suggests that hormonal factors such as estrogen and progesterone may impact the synthesis and release of sgp130 (46, 47). Whether the functional progesterone withdrawal characteristic to human pregnancy is responsible or linked to the decreased AF levels of sgp130, at term, remains unknown.

Mitchell et al. (48, 49) indicated that IL-6 signaling through the classic pathway was responsible for the increased production of PGs by amnion and decidual cells both term and preterm. The observed increased sIL-6R/sgp130 molar ratio at term implies that the IL-6 trans-signaling pathway becomes more active toward the end of gestation. Withdrawal of the trans-signaling system inhibition could enhance the process of IL-6-induced PG production and subsequently facilitate the onset of term human parturition. The MMP-9 release was inhibited when amniochorion was incubated with sgp130 plus sIL-6R or LPS reversed the release of MMP-9. Incubation of the tissues with sgp130, sIL-6R, and their combination had no effect of IL-6 release in the conditional media of the amniochorion explant (B). The IL-6 levels were significantly upregulated after incubation with LPS. sgp130 did not impact the stimulatory effect of LPS. These results indicate that sIL-6R and sgp130 modulate MMP-9 activity through mechanisms independent of IL-6 levels in the system. Data are presented as mean ± SEM of seven independent experiments and analyzed by one-way ANOVA followed by post hoc Student–Newman–Keuls tests. Means with at least one common superscript are not statistically significant. p > 0.05.

There is evidence to sustain the view that the sharp increase in the AF IL-6 levels occurs secondary to microbial invasion of the amnion and choriodendua (34, 35). This is further supported by our in vitro experiments that demonstrate the amplified release of IL-6 after incubation of fetal membranes with LPS. What mediates the transmembrane passage of IL-6 into AF remains largely unknown (35), but angiopoietins may play a significant role (50). We determined that the fetal membrane IL-6-R mRNA levels were upregulated, whereas the expression of gp130 was reduced in IAI. First, these results imply that in IAI, the amniochorion is a functional target for IL-6 classic signaling. Second, incubation of the amniochorion with sIL-6R, sgp130, and their combination did not enhance the release of IL-6. From this perspective, components of the IL-6 trans-signaling pathway play a limited role in the process of amniochorion IL-6 protein synthesis or release, or both.

In our study, it was evident that AF sIL-6R levels were upregulated in relation with IAI. The steady-state amniochorion, IL-6-R mRNA, and sIL-6R protein levels also were significantly enhanced by IAI. The mechanisms responsible for the proteolytic release of the ectodomain of IL-6-R and IL-6-R alternative splicing rearrangements are likely to play an integral role in promoting an increase of the AF sIL-6R levels during microbial (i.e., *Streptococcus, Escherichia coli*) invasion of fetal membranes. The proof of concept that exacerbation of the TNF-α converting enzymatic activity and shedding of the IL-6-R could be evoked by bacterial MMPs and toxins (i.e., streptolysin-O, *E. coli*-hemolysin) was previously provided (51). In addition, bioavailability of the AF sIL-6R could occur through functional amplification of the alternative splicing apparatus in the amnion and choriodendua. Experiments to substantiate this hypothesis are needed.

We found that women with PPROM and IAI have lower IL-6 levels than women with IAI but intact membranes. No prospective study has targeted a direct comparison of the two clinical categories. Yet, studies in PPROM women report lower optimal diagnostic cutoffs for IL-6 than studies limited to intact subjects, which would be in agreement with our data (14). To our knowledge, our finding that PPROM and especially PPROM and IAI women have lower sgp130 in AF is novel. One possible explanation may be that similar to other soluble antagonists (i.e., sRAGE) (21), sgp130 becomes consumed while binding the IL-6–IL-6R complex in the process of trans-signaling inhibition (52). Alternatively, gp130 itself may be downregulated as processes terminating IL-6 signaling become activated (53). Lastly, transcriptional and/or shedding processes responsible for synthesis and release of sgp130 may be altered in a subgroup of women, and these events may facilitate rupture of the membranes.

Real-time RT-PCR and Western blotting results revealed downregulation in expression of fetal membrane gp130 in the setting of IAI. In addition, by Western blotting, we demonstrated a significant downregulation in the synthesis and release of sgp130 protein in relation to IAI. This could be an alternative explanation for the decreased sgp130 in women with PPROM and IAI. Binding of IL-6 to sIL6-R is known to prolong IL6’s half-life (54). Although relevant for complex systems harboring all IL-6 trans-signaling components (i.e., amniotic cavity), the direct role played by sgp130 in modulating the clearance of IL-6 or IL-6–sIL6-R complexes has not been explored.

A relevant question is whether IL-6 plays a direct role in events leading to PTB. In an infection-induced mouse model of PTB, acute administration of rIL-6 did not result in significant shortening of gestation (55). This observation would argue that IL-6 has limited biological relevance for parturition. However, recent evidence suggests the contrary (56). By using IL-6–deficient animals, Robertson et al. (56) demonstrated that IL-6 deletion results in delayed normal timing of delivery (~24 h). Chronic infusion of IL-6 in IL-6−/− mice restored the normal timing of delivery. Maternal progesterone levels remained unaltered by IL-6 manipulation, despite remarkable changes of a wide array of genes with critical roles in parturition. Together, these data support the argument that IL-6 is instrumental in regulating the timing of delivery in normal gestation and in infection-induced PTB in a manner independent of luteolysis.

**FIGURE 6.** Ex vivo production of MMP-9 and IL-6 after incubation of the fetal membranes with sgp130, sIL-6R, and LPS. Recombinant sIL-6R and LPS stimulated the release of MMP-9 (A). Addition of recombinant sgp130 over sIL-6R or LPS reversed the release of MMP-9. Incubation of the tissues with sgp130, sIL-6R, and their combination had no effect of IL-6 release in the conditional media of the amniochorion explant (B). The IL-6 levels were significantly upregulated after incubation with LPS. sgp130 did not impact the stimulatory effect of LPS. These results indicate that sIL-6R and sgp130 modulate MMP-9 activity through mechanisms independent of IL-6 levels in the system. Data are presented as mean ± SEM of seven independent experiments and analyzed by one-way ANOVA followed by post hoc Student–Newman–Keuls tests. Means with at least one common superscript are not statistically significant. p > 0.05.
We found that women with PPROM and longer latency intervals had greater levels of sgp130. This argues that in these women, the inflammatory process involved in activation of uterine contractility could be suppressed for a longer period. The observation that PPROM women had decreased AF sgp130 levels and an increased sIL-6R/sgp130 molar ratio was provocative. Our in vitro experiments provide evidence for a mechanism through which AF sgp130 may prevent weakening of the fetal membranes in pregnancies complicated by infection. As shown, incubation of the fetal membranes with LPS induced a significant increase in the release of MMP-9. The recombinant sgp130 reversed the process.

The acute inflammatory response involves immediate access of the neutrophils at the site of a bacterial attack (1, 3, 18, 34). Our immunohistochemistry results established concurrent homing of CD15+ neutrophils and CD3+ T cells in the choriodicida of women with IAI. Given that resident cells of these tissues express IL-6, IL-6R, sIL-6R, gp130, and sgp130, we argue that in chorioamnionitis, the classic and trans-signaling mechanisms are both engaged in the process of leukocyte trafficking and activation (34). Generation of sIL-6R may therefore represent a rate-limiting event in the regulation of these processes distinguishing between physiological and pathological processes orchestrated by IL-6.

Decidual T cells are sparse in early pregnancy (57). At term, however, 45–50% of the leukocytes in the decidua basalis are CD3+ representing one of the most abundant leukocyte populations. The choriodicida T cell population is thought to participate in defense mechanisms against pathogens, particularly during parturition and in regulation of chronic inflammatory processes localized at the fetal–maternal interface (57). Studies have documented key roles for IL-6 trans-signaling in leukocyte trafficking and activation. In particular, IL-6 trans-signaling–driven STAT3 activity is important for T cell recruitment during successful resolution of any inflammatory response and transition from innate to acquired immunity (58). Therefore, we posit that downregulation of gp130 approaching term may facilitate IL-6 trans-signaling and physiological T cell recruitment in the choriodicida, in anticipation of parturition.

In summary, we found that IL-6 trans-signaling molecules are physiologic constituents of the AF with both GA and inflammatory regulation. The decreased AF levels of sgp130 in women with ruptured membranes and the demonstrated inhibitory effect of sgp130 on MMP-9 release suggest that IL-6 trans-signaling may play a critical role in PPROM. Similar to inflammatory arthritis, where intra-articular injection of recombinant sgp130 effectively prevented joint destruction (59), we suggest that targeting of IL-6 trans-signaling pathway may have potential for preventing PTB and PPROM (56).

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Disclosures
The authors have no financial conflicts of interest.

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**Supplemental Legends**

**Figure 1 Supplemental Data:** *Flowchart of women and biological samples used in this study.* Abbreviations: IAI: intra-amniotic inflammation; CRL: control; IHC: immunohistochemistry; HCA: histological chorioamnionitis; ROM: rupture of membranes.

**Figure 2 Supplemental Data:** *Fetal membranes explant culture system.* The LPS dose was chosen based on a dose response experiment (5, 50, 500 ng/mL LPS) where we determined that the peak of IL-6 and MMP-9 release in the explants medium. The peak of MMP-9 and IL-6 release was seen following stimulation with 50 ng/mL LPS. The figure shows dose-response curves of LPS on IL-6 and MMP-9 release from amniochorion explants (n=4) after 24 hours challenge. Means with different superscripts are different at P<0.05.

**Figure 3 Supplemental Data:** *Transcriptional regulation of IL-6R and gp130 in human placenta.* Relative mRNA expression of IL-6R (A) and gp130 (B) in placental villous tissue from preterm (PT) women and term (T, n=9) women. Preterm women were grouped by presence or absence of intra-amniotic inflammation (IAI: no IAI, n=5, yes IAI, n=15). Data presented as mean and standard error.
FIGURE 1 (Supplemental Data)

Flowchart of women and biological samples used in this study.

Subject enrollment groups

- 2nd trimester genetic amniocenteses n = 39
- 3rd trimester lung maturity amniocenteses n = 40
- Rule-out infection amniocenteses n = 222

Amniotic fluid groups studied with ELISA

- 2nd trimester CRL n = 39
- 3rd trimester CRL n = 40

Reproductive tissues studied with real-time PCR and IHC

- 3rd trimester elective Cesarean delivery n = 7
- Negative IAI & term delivery n = 10
- Negative IAI & preterm delivery n = 5
- Positive IAI & preterm delivery n = 17
FIGURE 2 (Supplemental Data)

Fetal membranes explant culture system.
TABLE 1 (Supplemental Data)

**Absolute levels of AF IL-6, IL-6R, sgp130 and IL-6R/sgp130 molar ratio for women with normal outcomes.**

<table>
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<th>Variable</th>
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<th>2nd trimester genetic amniocenteses n = 39</th>
<th>Rule-out infection amniocenteses &amp; term delivery n = 31</th>
<th>3rd trimester lung maturity amniocenteses n = 40</th>
<th>P value</th>
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<td>IL-6, ng/mL †</td>
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<td>0.18 [0.08 – 0.44]</td>
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<td>0.24 [0.10 – 0.40]</td>
<td>0.581</td>
</tr>
<tr>
<td>sIL-6R, ng/mL †</td>
<td></td>
<td>1.35 [1.20 – 1.68]</td>
<td>1.57 [1.27 – 1.83]</td>
<td>1.35 [0.97 – 1.64]</td>
<td>0.097</td>
</tr>
<tr>
<td>sgp130, ng/mL †</td>
<td></td>
<td>69.75 [59.10 – 90.58]</td>
<td>70.36 [59.60 – 98.81]</td>
<td>48.56 [35.87 – 61.53]</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

† Data presented as median [interquartile range] and analyzed by Kruskal-Wallis ANOVA on Ranks.
TABLE 2 (Supplemental Data)

Absolute levels of AF IL-6, IL-6R, sgp130 and IL-6R/sgp130 molar ratio for rule out infection amniocenteses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Rule out infection amniocenteses (n=222)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no IAI &amp; intact n = 85</td>
<td>no IAI &amp; PPROM n = 61</td>
</tr>
<tr>
<td>IL-6, ng/mL ‡</td>
<td>0.43 [0.16 – 1.89]</td>
<td>0.58 [0.23 – 1.58]</td>
</tr>
<tr>
<td>sIL-6R, ng/mL ‡</td>
<td>1.54 [1.18 – 1.87]</td>
<td>1.49 [1.08 – 2.09]</td>
</tr>
<tr>
<td>sgp130, ng/mL ‡</td>
<td>71.19 [55.81 – 102.13]</td>
<td>43.85 [35.90 – 59.80]</td>
</tr>
</tbody>
</table>

† Data presented as median [interquartile range] and analyzed by Kruskal-Wallis ANOVA on Ranks.
TABLE 3 (Supplemental Data)

Demographical characteristics of the women who provided tissues for RT-PCR, Western blot and immunohistochemistry.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Negative IAI, negative HCA &amp; preterm delivery n = 5</th>
<th>Positive IAI, positive HCA &amp; preterm delivery n = 15</th>
<th>Term elective Cesarean section n = 9</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age, years, †</td>
<td>23 [18 – 27]</td>
<td>32 [27 – 37]</td>
<td>25 [22 – 29]</td>
<td>0.082</td>
</tr>
<tr>
<td>Parity †</td>
<td>0 [0 – 1]</td>
<td>1 [0 – 3]</td>
<td>1 [1 – 3]</td>
<td>0.242</td>
</tr>
<tr>
<td>PPROM †</td>
<td>3 (60%)</td>
<td>8 (53%)</td>
<td>0 (0%)</td>
<td>0.018</td>
</tr>
<tr>
<td>Positive AF cultures †</td>
<td>0 (0%)</td>
<td>15 (100%)</td>
<td>NA</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clinical chorioamnionitis ‡</td>
<td>0 (0%)</td>
<td>3 (20%)</td>
<td>0 (0%)</td>
<td>0.210</td>
</tr>
<tr>
<td>Cesarean delivery ‡</td>
<td>1 (20%)</td>
<td>6 (40%)</td>
<td>9 (100%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Gestational age at delivery, weeks †</td>
<td>31 [29-33]</td>
<td>25 [24-29]</td>
<td>39 [38-40]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Birthweight, grams †</td>
<td>1,395 [1,175-2,099]</td>
<td>800 [596-1,249]</td>
<td>3,235 [3,070-4,479]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Placental pathology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chorionic plate inflammation, stage †</td>
<td>0 [0-0]</td>
<td>3 [3-3]</td>
<td>0 [0-0]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Amnionitis, grade †</td>
<td>0 [0-0]</td>
<td>3 [2-4]</td>
<td>0 [0-0]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chorio-deciduitis, grade †</td>
<td>0 [0-0]</td>
<td>3 [3-4]</td>
<td>0 [0-0]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Funisitis, grade †</td>
<td>0 [0-0]</td>
<td>3 [2-4]</td>
<td>0 [0-0]</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

† Data presented as median [interquartile range] and analyzed by Kruskal-Wallis ANOVA on Ranks.
‡ Data presented as n [%] and analyzed by Chi square test.
FIGURE 3 (Supplemental Data)

Transcriptional regulation of IL-6R and gp130 in human placenta.