

# Soluble TLR2 Is Present in Human Amniotic Fluid and Modulates the Intraamniotic Inflammatory Response to Infection<sup>1</sup>

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TLRs are pattern recognition transmembrane receptors that play key roles in innate immunity. A recently discovered soluble truncated form of TLR2 (sTLR2) acts as a decoy receptor, down-regulating the host inflammatory response to bacteria. To identify the presence and functional role of sTLR2 in modulating the intraamniotic inflammatory response to infection, we studied 109 amniotic fluid samples of women with normal pregnancy outcomes ( $n = 28$ ) and women with ( $n = 39$ ) and without ( $n = 42$ ) intraamniotic infection. We sought to demonstrate a functional role of the amniotic fluid sTLR2 in modulating the TLR2 inflammatory signaling in vitro by using a villous explant system. Two sTLR2 forms were identified, and specificity was confirmed with neutralizing peptides. We showed that sTLR2 is present constitutively in amniotic fluid, its levels are gestational age dependent, and we determined that the sTLR2 quantity and functional engagement modulates the intensity of the intraamniotic inflammation elicited by Gram-positive bacteria. In vitro, we demonstrated that challenging placental villous explants with a specific TLR2 agonist (Pam3Cys) induced a significant cytokine response. Notably, preincubation of the preterm, but not near-term, amniotic fluid with Pam3Cys significantly inhibited the ability of this TLR2 agonist to elicit a cytokine reaction. Moreover, depletion of sTLR2 from preterm amniotic fluid removed its neutralizing property. Monensin significantly diminished sTLR2 immunoreactivity, indicating that sTLR2 is the result of intracellular posttranslational processing of TLR2. We conclude that sTLR2 is part of the amniotic fluid innate immune system and participates in regulating the inflammatory response to microbial pathogens. *The Journal of Immunology*, 2009, 182: 7244–7253.

The innate immune system is an archaic defense mechanism, phylogenetically preserved to be at the forefront of resistance to microbial infections (1). The human TLRs are essential for triggering an inflammatory innate immune response (2). To date, 13 mammalian TLRs have been identified and 10 of these are present in humans (3). At the maternal-fetal interface, TLRs are expressed not only in immune cells, but also in the trophoblast and decidual cells (4, 5). Moreover, their expression pattern varies according to the stage of pregnancy (5). Such findings provide evidence that during pregnancy, placental TLRs may play a key role in modulating the inflammatory response triggered by infection.

TLRs are transmembrane receptors that mediate host defense through the engagement of pathogen-associated molecular patterns (PAMPs)<sup>3</sup>, which are ubiquitous constituents of the bacterial wall (6). TLR2 was the first of 10 human TLRs, proven to be precisely involved in recognition of PAMPs (lipoproteins, peptidoglycan, glycolipids, nucleic acids), representing broad groups of microbial species such as Gram-positive bacteria, *Mycobacteria*, spirochetes, and Mycoplasmataceae (7, 8, 9). Traditionally, it has been thought that the extracellular receptor domain is capable of discriminating among pathogens by recognizing a specific PAMP (10). This indicates the crucial importance of the extracellular TLR2 domain in ligand recognition. Following engagement, the molecular basis of TLR2 downstream signaling depends on the intracellular receptor domain, which is highly conserved among TLRs (11). When activated, TLR2 has been specifically linked with secretion of antimicrobial proteins and peptides, as well as with immune-modulating cytokines and chemokines, which recruit immune cells to the site of infection (12).

The events that lead to TLR2 engagement and activation during human gestation are incompletely understood. Recently, it has been suggested that the biological activity of the TLRs is not exclusively dependent on PAMPs, but is also regulated by co-receptor molecules (CD14), intracellular signaling adaptors (MyD88, Mal, TRIF, TRAM, and SARM), and soluble receptor antagonists (13). Specifically, it was shown that saliva, human plasma, and breast milk's TLR2-mediated innate immune activity can be altered through a natural soluble TLR2 (sTLR2) polypeptide (14, 15). Although still unknown, it is thought that the sTLR2 peptide

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Received for publication October 20, 2008. Accepted for publication March 23, 2009.

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<sup>1</sup> This work was funded by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Grant RO1 HD 047321-01 (to I.A.B.) and departmental funds. The funding sources had no involvement in study design, interpretation of data, writing of the report or decision to submit the paper for publication.

A.T.D. and I.A.B. designed the study, performed the experiments, collected, analyzed, and interpreted the clinical and experimental data, and drafted the manuscript. C.S.B. participated in the study design, supervised the clinical enrollment of the patients, collected, analyzed, and interpreted the clinical and experimental data, and participated with A.T.D. and I.A.B. in writing the manuscript. G.Z. conducted the ELISA assays, performed part of the experiments, and participated in writing of the report. S.J. assisted with experiments and data interpretation and participated in writing of the report. E.O. and A.B. participated with aspects of the study design, performance of the experiments, and participated in writing of the report. All listed authors have reviewed and approved the submitted version of the paper.

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<sup>3</sup> Abbreviations used in this paper: PAMP, pathogen-associated molecular patterns; GA, gestational age; LDH, lactate dehydrogenase; Pam3Cys, Pam<sub>3</sub>Cys-Ser-(Lys)<sub>4</sub> hydrochloride; sTLR2, soluble TLR2.

may be the result of posttranslational processing of the extracellular domain or of the intact TLR2 molecule (15).

Human amniotic fluid has a broad range of antiinflammatory roles (16). Multiple factors of innate and adaptive immunity (immunoglobulins, cytokines, defensins, lysozyme, lactoferrin, LPS-binding protein) can be identified in the amniotic fluid to protect the mother and her fetus (17, 18, 19, 20, 21, 22, 23). Our hypothesis was that sTLR2 is present in human amniotic fluid and has a functional role in modulating the intraamniotic innate immune response to a microbial attack. To address this, we studied samples of amniotic fluid of women with normal pregnancy outcomes ( $n = 28$ ) and women with ( $n = 39$ ) and without ( $n = 42$ ) intraamniotic infection. Two sTLR2 forms, 42 kDa (dominant) and 30 kDa, were identified, and specificity was confirmed with neutralizing peptides. We observed that sTLR2 is constitutively present in amniotic fluid, but its expression is gestational age (GA) regulated. We further determined that the sTLR2 quantity and functional engagement modulates the intensity of the intraamniotic inflammation elicited by Gram-positive bacteria. The functional significance of sTLR2 was demonstrated in vitro through the ability of preterm amniotic fluid to inhibit the release of IL-8 in response to Pam3Cys (Pam<sub>3</sub>Cys-Ser-(Lys)<sub>4</sub> hydrochloride), a bacterial lipopeptide analog and specific TLR2 agonist. Additionally, we investigated the mechanisms responsible for presence of sTLR2 in human amniotic fluid and provide evidence that sTLR2 is most likely the product of trophoblast and amnion secretion.

## Materials and Methods

### *Patient population and amniotic fluid samples*

This study was approved by the Human Investigation Committee of Yale University, and all patients provided written informed consent. A flowchart of the women enrolled in the study and subgroups of samples analyzed are presented in the supplemental Fig.<sup>4</sup> Amniotic fluid was retrieved from 109 women who had a clinically indicated amniocentesis in the following different populations: second trimester normal genetic karyotyping (GA median (range), 19 (15–23) wk,  $n = 14$ ), third trimester fetal lung maturity testing before cesarean delivery (GA, 37 (35–39) wk,  $n = 14$ ), and women admitted with symptoms of preterm labor who had an amniocentesis to rule out infection (GA, 28 (17–36) wk,  $n = 81$ ). To avoid selection bias, women in this last group were selected from a prospective cohort of 463 consecutive patients enrolled at Yale New Haven Hospital from March 2004 to June 2008. To study the relationship between intraamniotic infection and amniotic fluid sTLR2, the preterm birth group was divided into two subgroups: women who delivered preterm in the setting of a positive amniotic fluid culture ( $n = 39$ ), and women with negative amniotic fluid culture results who ultimately delivered at term ( $n = 42$ ). In turn, the positive amniotic fluid culture group was subdivided by cultured bacterial categories into exclusively Gram-positive ( $n = 21$ ) or Gram-negative bacterial infections ( $n = 18$ ).

The clinical characteristics of the participating subjects are presented in supplemental Table I. Gestational age was established based on either the last menstrual period or a first or second trimester ultrasound evaluation. Eligible women had a singleton fetus without evidence of structural abnormalities, at the time of assessment or birth. Women with maternal medical complications (hypertension, preeclampsia, diabetes, thyroid disease, cholestasis, lupus), viral infections (HIV, hepatitis B or C), anhydramnios, and fetal intrauterine growth restriction (estimated fetal weight <10th percentile for GA) were excluded. Delivery of the fetus at  $\geq 37$  wk of gestation was considered at term. Preterm labor was defined as presence of regular uterine contractions, advanced cervical dilatation ( $\geq 3$  cm), or effacement at <37 wk of gestation. Rupture of the membranes was confirmed either by “pooling” on speculum examination, positive “nitrazine” and “ferning” tests, or by a positive amnio-dye test. Clinical chorioamnionitis was established in the presence of maternal fever ( $>37.8^{\circ}\text{C}$ ), maternal leukocytosis ( $\geq 15,000$  cells/mm<sup>3</sup>), uterine tenderness, foul smelling amniotic fluid or visualization of pus at the time of the speculum exam, and maternal or fetal tachycardia.

The clinical characteristics and the results of the amniotic fluid analysis of the women admitted with symptoms of preterm labor are presented in

supplemental Table II. For all women who underwent amniocenteses to rule out infection, the clinical laboratory performed the amniotic fluid glucose and lactate dehydrogenase (LDH) measurements as well as the white blood cell count. An amniotic fluid glucose cut-off of  $\leq 15$  mg/dl, an LDH level  $\geq 419$  U/L, and/or a white blood cell count  $\geq 100$  cells/ml were considered suggestive of intraamniotic infection/inflammation (24, 25, 20). Concurrently, the amniotic fluid was examined in the microbiology laboratory for the presence of microorganisms using the traditional Gram staining and culturing method. The fluid was cultured for aerobic and anaerobic bacteria, *Ureaplasma* and *Mycoplasma* species. The microbiological data for the women who had a positive microbial culture results are presented in supplemental Tables III and IV. The results of the clinical and microbiological laboratories were available for clinical management. The remaining amniotic fluid was centrifuged at  $3000 \times g$  and  $4^{\circ}\text{C}$  for 20 min, aliquoted, and stored at  $-80^{\circ}\text{C}$  for research purposes.

### *Placental villous explant culture*

Nine placentas were obtained from healthy women without any significant past medical history undergoing scheduled, elective cesarean delivery in the absence of labor (GA, 38–40 wk). Indications for abdominal delivery included elective repeat or primary cesarean delivery for fetal malpresentation (i.e., frank breech). No patient had abnormal placentation (placenta previa, abruption). All infants were appropriately grown for GA and had reassuring fetal heart rate patterns before surgery.

Placental cotyledons from the central part of the placenta were removed under sterile conditions and chorionic villi were dissected within 30 min of delivery. The villous tissue was cut into pieces of similar weight, washed thoroughly with ice-cold saline, and four pieces ( $\sim 100$  mg wet weight) were cultured as freely suspended villi in 24-well plates in 1.5 ml of RPMI 1640 medium (Invitrogen) containing 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (Invitrogen). Cultures were maintained at  $37^{\circ}\text{C}$  in a humidified gas mixture of 5%  $\text{CO}_2$ -95% air. After varying incubation times (1, 4, 18, or 24 h) the supernatants were collected, centrifuged to remove cellular debris, and stored at  $-80^{\circ}\text{C}$ . The incubated tissue was immediately homogenized in 1-ml cell extraction buffer (20 mmol/L Tris-HCl, 150-mmol/L NaCl, 1% Triton X-100, 1 mmol/L PMSF, and Complete protease inhibitor cocktail (Roche)). Specimens were spun at  $1000 \times g$  at  $4^{\circ}\text{C}$  for 15 min, and protein quantification in incubated tissue was performed using bicinchoninic acid (BCA) protein assay (Pierce) according to the manufacturer's instructions. The analytes' explant medium concentration was normalized to total protein in tissue extract to correct for variations in tissue incubated per each well. 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 fold change from the untreated level. Monensin and cycloheximide were dissolved first in DMSO at 20 mmol/L and 100 mg/ml, respectively, and then further diluted in culture medium. Parallel wells were also treated with the equivalent dose of DMSO alone. All drugs and chemicals were from Sigma-Aldrich unless specified otherwise.

### *Amniotic fluid incubations ex vivo*

Stored amniotic fluid was filtered through a 0.22- $\mu\text{m}$  syringe filter (Millipore) and incubated with or without the TLR2-specific ligand Pam<sub>3</sub>Cys-Ser-(Lys)<sub>4</sub> hydrochloride (Pam3Cys, 1  $\mu\text{g}/\text{ml}$ ; Calbiochem) for 1 h at  $37^{\circ}\text{C}$  with shaking. Pseudoamniotic fluid (118.5 mM NaCl, 4.8 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.15 mM  $\text{KH}_2\text{PO}_4$ , 1.15 mM  $\text{MgSO}_4$ , 25.0 mM  $\text{NaHCO}_3$ , 2.0 mM glucose, 6.0 mM urea, and 0.2% BSA (pH 7.0)) was prepared as previously described and used for control incubations (26). Placental explants were plated as previously described except that incubation medium was replaced with either the preincubated amniotic or pseudoamniotic fluid.

### *Tissue viability*

To assess tissue viability during in vitro incubations, the release of the intracellular enzyme LDH into the incubation medium was determined in explant medium and tissue extract and as described previously using the LDH Liqui-UV assay (Stanbio Laboratory) (27). The interassay and intraassay coefficients of variation were  $<5\%$ . LDH release was expressed as a percentage of total tissue control, which was calculated as LDH activity in the medium divided by total tissue LDH activity multiplied by 100. The LDH activity measured in the amniotic fluids before addition of the tissue served as baseline for ex vivo amniotic fluid incubation experiments.

### *Western blot*

Gel electrophoresis was conducted on 10% SDS-PAGE gels using a Bio-Rad Miniprotein II gel apparatus. Ten microliters of amniotic fluid were diluted 1/2 (v/v) with electrophoresis sample buffer (Bio-Rad) and reduced

<sup>4</sup> The online version of this article contains supplemental material.

by boiling for 5 min. After electrophoretic transfer to a polyvinylidene difluoride membrane (Bio-Rad) at 100 V for 60 min and blocking with 5% milk, the blots were incubated overnight at 4°C with either goat anti-TLR2-C19 or goat anti-TLR2-N17 Ab (Santa Cruz Biotechnology) diluted 1/200. Blots were subsequently subjected to ECL using a Western blotting detection system (Amersham) with enzyme conjugate anti-rabbit IgG-HRP as secondary Ab. Autoradiography film was applied to the blot until satisfactory exposure was achieved. OD of each band was quantified using ImageJ v.1.33 software (28). Inter-gel comparison was achieved by expressing the OD of each band relative to that of an amniotic fluid sample pool loaded on each gel. Ab specificity was confirmed by omitting the primary Ab and by preadsorbing the primary Ab with neutralizing peptide (N17) from the same manufacturer.

#### Immunodepletion of amniotic fluid sTLR2

Protein G-Sepharose beads were washed according to the manufacturer's instructions (Sigma-Aldrich) and then incubated at 4°C overnight with filtered amniotic fluid to preclear the specimens of endogenous IgG. Human anti-TLR2 (anti-N17) was then added to the precleared samples and incubated with shaking at 4°C. After 2 h, newly washed beads were added to the mixture and incubation was continued overnight. Supernatant was then collected and stored at -80°C until ready for use. The level of immunodepletion was assessed by Western blot as described above by comparing sTLR2 immunoreactivity with that of the native (nondepleted) fluid.

#### Immunoassays for human IL-6 and IL-8

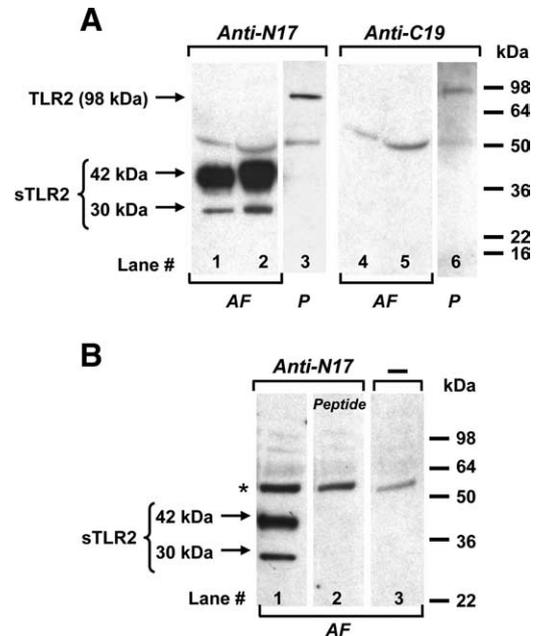
An IL-6 ELISA system (Pierce/Endogen) was used to measure levels in amniotic fluid with a minimal detectable concentration of 1 pg/ml. IL-8 was measured in explant culture media and amniotic fluid using ELISA according to the manufacturer's instructions (R&D Systems). The minimal detectable concentration was 1.5–7.5 pg/ml. All measurements were performed in duplicate, and the inter- and intraassay coefficients of variation were <10% for both IL-6 and IL-8 assays.

#### Immunohistochemistry

Five-micrometer paraffin sections were deparaffinized in xylene and rehydrated with graded ethanol to potassium-PBS solution (pH 7.2). Following Ag retrieval with citrate buffer, the sections were pretreated with 1% hydrogen peroxide for 15 min, followed by overnight incubation at 4°C with a rabbit polyclonal anti-human TLR2 (amino-terminal end) Ab (1/250 dilution; ab47840; Abcam). Detection was performed with biotinylated donkey anti-rabbit IgG (1/600; Jackson Immunochemicals) followed by avidin-biotin staining (Vectastain Elite ABC; Vector Laboratories) and with 3,3'-diaminobenzidine/nickel sulfate as chromogen solution. Specificity of staining was confirmed by omitting the primary Ab. Immunohistochemical staining of the intensity of the chromogen deposited in the amnion epithelium, choriondecidua, placental villous trophoblast, and stromal and endothelial cells was graded using the HSCORE (histoscore) system, according to the method described by McCarty et al., which considers the intensity and percentage of cells staining at each intensity (29, 30). Slides stained immunohistochemically were purposely not counterstained so that morphological changes were hidden to the examiner. Three to five randomly selected areas were imaged ( $\times 400$  magnification) under a light microscope (Olympus IX71) and images acquired using a Coolpix camera (Nikon) under the same light intensity settings. Cells in each field were scored for staining intensity in the following categories: 0, no staining; 1+, weak; 2+, moderate; 3+, intense staining. HSCORE values (cellular or nuclear component) were calculated for each area using the formula  $\sum Pi(i + 1)$ , where  $i$  represents one of the four degrees of intensity staining and  $Pi$  is its corresponding percentage of cells, which fluctuates from 0% to 100%. The HSCORE is a numerical figure from 100 to 400. A lack of immunoreactivity results in an HSCORE value of 100, while an HSCORE of 400 is the highest possible (when 100% of cells are stained at a 3+ level) (29). Tissue HSCOREs were derived by averaging values from the individual areas. The coefficient of variation for HSCORE was <7% for all tissues. For illustration purposes, the substrate utilized was Vector NovaRed (Vector Laboratories), and sections were counterstained with hematoxylin. Adjacent sections were either stained histologically with Masson's trichrome or immunostained with mAbs against cytokeratin-7 (epithelial cell marker) or vimentin (mesenchymal cell marker) (1/100 dilution; Zymed Laboratories/Invitrogen) to distinguish among cellular types expressing TLR2.

#### Statistical analysis

Data were tested for normality using the Kolmogorov-Smirnov test and reported as median and range. Comparisons between two groups were performed using Student's  $t$  tests or Mann-Whitney rank-sum tests



**FIGURE 1.** Human amniotic fluid (AF) contains two sTLR2 isoforms (42 and 30 kDa). The figure is a composite of Western blot data (10% SDS-PAGE reducing gel), which shows that a TLR2-specific polyclonal Ab raised against the extracellular (anti-N17, sc8689) but not intracellular (anti-C19, sc8690) domain detects two sTLR2 isoforms in the AF samples from a patient with second trimester genetic amniocentesis (A, lanes 1 and 4) and a patient with symptoms of preterm labor that delivered at term (A, lanes 2 and 5). Placental tissue homogenate (P) from a healthy woman who delivered at term in the absence of labor was used to demonstrate reactivity of both Abs to the full-length transmembrane TLR2 (98-kDa band) (A, lanes 3 and 6). The specificity of the detection with the anti-N17 Ab was confirmed by performing peptide competition by immunoblotting (B). Shown is a result of a representative Western blot with the anti-N17 Ab (B, lane 1) in AF from a patient in the third trimester. Preadsorption of the anti-N17 Ab with neutralizing peptide (N17, sc8689P) eliminated detection of the bands at 42 and 30 kDa (B, lane 2). The band at 55 kDa (\*) was deemed nonspecific since it remained after omission of the primary anti-N17 Ab (B, lane 3).

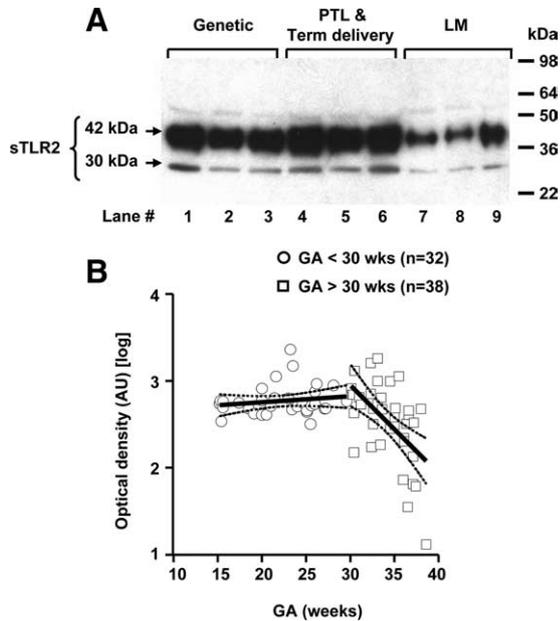
as appropriate. Multiple comparison procedures were performed using one-way or Kruskal-Wallis ANOVA followed by Student-Newman-Keuls or Dunn's post hoc analysis, respectively. Time course data were analyzed by two-way repeated measures ANOVA and Student-Newman-Keuls post hoc comparisons. Proportions were compared with  $\chi^2$  of Fischer exact tests. Relationships between variables (correlations) were explored using Pearson's product moment rank-order correlations. Comparison between correlations was achieved based on  $z$  statistic (31). Med-Calc and SigmaStat statistical softwares (RockWare) were used for analysis.

## Results

### Two sTLR2 isoforms are present in the human amniotic fluid

We first searched for the presence of sTLR2 polypeptides in samples of human amniotic fluid retrieved from healthy women during the second trimester (genetic testing: GA range, 15–23 wk,  $n = 14$ ), third trimester (fetal lung maturity testing: GA, 35–39 wk,  $n = 14$ ), and women in preterm labor with negative amniotic fluid cultures who ultimately delivered at term (GA, 17–36 wk,  $n = 42$ ); groups are defined in *Materials and Methods*.

Western blot analysis of amniotic fluid using TLR2-specific polyclonal Abs raised against peptides mapping at either the C terminus (intracellular domain: anti-C19) or N terminus (extracellular domain: anti-N17) showed the presence of specific bands only when the anti-N17 Ab was used as primary Ab (Fig. 1A). The

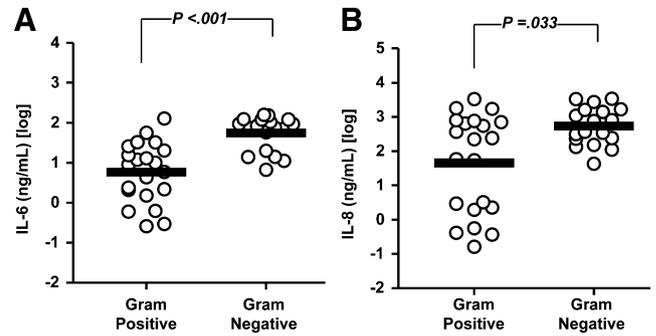


**FIGURE 2.** Levels of amniotic fluid (AF) sTLR2 isoforms (42 and 30 kDa) are GA regulated. *A*, Representative sTLR2 Western blot data (10% SDS-PAGE reducing gel) of AF retrieved from women in each of the following subgroups: second trimester genetic testing (lanes 1–3), preterm labor (PTL)-negative cultures and term delivery (lanes 4–6), and third trimester fetal lung maturity (LM) testing (lanes 7–9). Each lane represents a sample from a different woman. *B*, Summary of the quantification of sTLR2 levels in AF of 70 women in the three subgroups described above (genetic,  $n = 14$ ; PTL and term delivery,  $n = 42$ ; LM,  $n = 14$ ). All women had a normal pregnancy outcome. Densitometric image analysis of the sum of the 42- and 30-kDa polypeptides demonstrated that AF sTLR2 levels are lower toward the end of pregnancy and decrease logarithmically after 30 wk of gestation.

major polypeptide band was  $\sim 42$  kDa and the minor band was  $\sim 30$  kDa. This occurred in contrast to placental villous tissue that is known to express the full-length TLR2 receptor (98 kDa), which was detected by using both anti-C19 and anti-N17 Abs. Competition studies with blocking peptides (N17 and C19) were conducted to confirm specificity of the detection. As seen in Fig. 1*B*, preincubation of the anti-N17 Ab with the N17 blocking peptide eliminated the detection of the amniotic fluid bands at 42 and 30 kDa. These data indicate that the human amniotic fluid contains two sTLR2 polypeptides that are likely to be derived from conversion of the extracellular domain of TLR2.

#### Levels of the sTLR2 in the amniotic fluid are gestational age regulated

Next, we tested for GA regulation in expression of amniotic fluid sTLR2. This analysis was limited to amniotic fluid retrieved from healthy women during the second trimester (genetic testing: GA, 15–23 wk), third trimester (fetal lung maturity testing: GA, 35–39 wk) and women in preterm labor with negative amniotic fluid cultures who ultimately delivered at term (GA, 17–36 wk). Western blotting with the anti-N17 Ab demonstrated that the presence of sTLR2 in amniotic fluid is GA regulated. Specifically, we showed by densitometric image analysis that the intensity of both the 42 and 30 kDa sTLR2 bands were decreased in the third trimester compared with earlier in gestation (Fig. 2*A*). When amniotic fluid sTLR2 immunoreactivity (both bands) was plotted against GA, we found that sTLR2 remained elevated until 30 wk of gestation. This was followed by a logarithmic decrease in sTLR2 immunoreactivity



**FIGURE 3.** Gram-positive bacteria elicit in vivo an intraamniotic inflammatory response of lower intensity compared with Gram-negative bacteria. Amniotic IL-6 (*A*) and IL-8 levels (*B*) were compared between intraamniotic infections with exclusively Gram-negative ( $n = 18$ ) or Gram-positive ( $n = 21$ ) infections as indicated by amniotic fluid cultures. The thick line illustrates the median analyte level. Statistical analysis was conducted using Mann-Whitney tests.

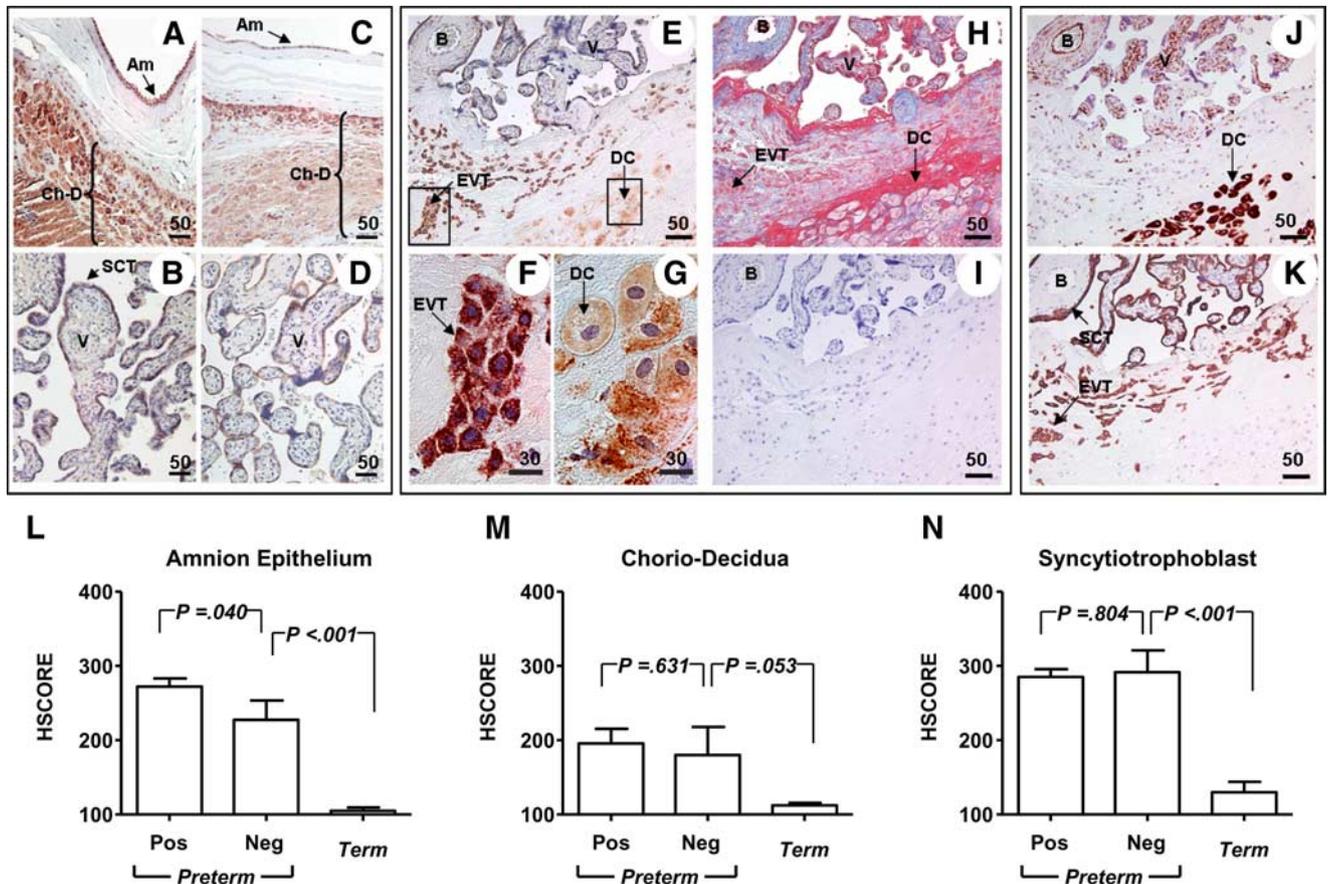
thereafter (Pearson  $r = -0.536$ ,  $p < 0.001$ ), which differed significantly from the steady-state observed earlier in pregnancy ( $z$  statistic vs  $<30$  wk, 3.07;  $p = 0.002$ ) (Fig. 2*B*). These results indicate that sTLR2 levels are GA regulated and decreased at term.

#### sTLR2 human amniotic fluid levels are independent of intraamniotic infection or bacterial category

We asked whether intraamniotic infection modulates the amount of amniotic fluid sTLR2. To answer this question we compared the expression of the 42- and 30-kDa sTLR2 bands in the amniotic fluid of women with a positive ( $n = 39$ ) and a negative microbial culture ( $n = 42$ ) result. Infection did not affect amniotic fluid sTLR2 band intensity (42-kDa band:  $p = 0.795$ ; 30-kDa band:  $p = 0.102$ ). Furthermore, the microbial category (Gram-positive,  $n = 21$  vs Gram-negative,  $n = 18$ ) did not appear to affect sTLR2 immunoreactivity in the amniotic fluid (42-kDa band:  $p = 0.337$ ; 30-kDa band:  $p = 0.241$ ). These findings led us to the conclusion that sTLR2 is present constitutively in preterm amniotic fluid and its levels are independent of intraamniotic infection or microbial category.

#### Gram-positive bacteria elicit a lower intraamniotic inflammatory response, and amniotic fluid sTLR2 may be responsible for this effect

In our original hypothesis we postulated that sTLR2 acts as a decoy for the TLR2 receptor, thereby down-regulating the intraamniotic inflammatory response to infection. To test this premise, we made use of amniotic fluid culture results as an indicator of presence of TLR2 ligands, and amniotic fluid chemokine (IL-8) and cytokine (IL-6) levels as indicators of intraamniotic inflammation, in vivo. We found that compared with Gram-negative microbes (TLR4-engaging,  $n = 18$ ), Gram-positive bacteria (TLR2-engaging,  $n = 21$ ) induced a significantly lower intraamniotic (median (range)): Gram-positive, 223.2 (0.2–3329.1) vs Gram-negative, 564.5 (43.6–3408.8) IL-8 ng/ml,  $p = 0.033$  and IL-6 (Gram-positive, 9.1 (0.2–129) vs Gram-negative, 80.9 (7–158) ng/ml,  $p < 0.001$ ) inflammatory response (Fig. 3). These data suggest that in vivo the amniotic fluid sTLR2 may prevent engagement of TLR2 ligands of Gram-positive bacteria to the TLR2 receptor, thereby lowering the downstream cytokine and chemokine response.



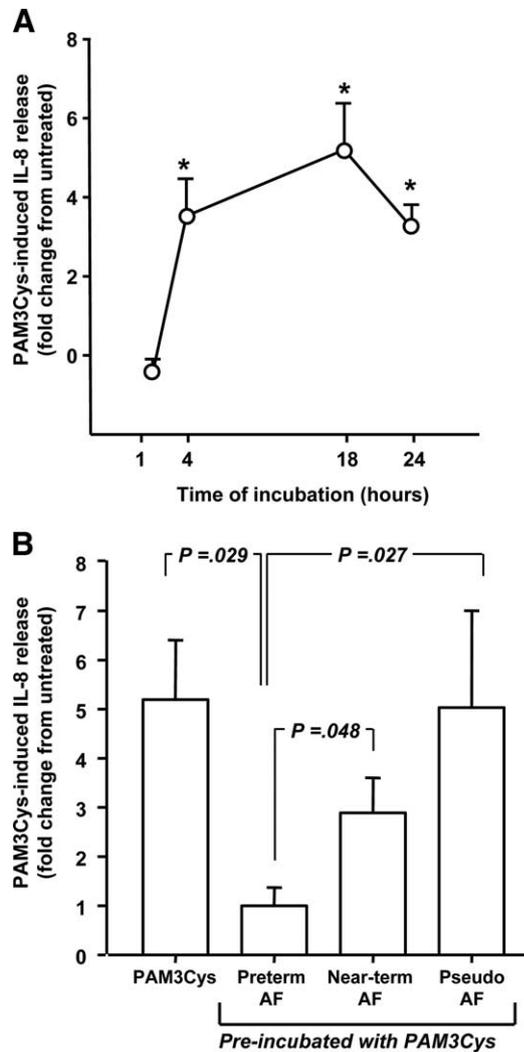
**FIGURE 4.** Presence and localization of TLR2 immunoreactivity in placenta and amniochorion. *A–D*, TLR2 immunostaining in a representative patient with preterm birth (*A*, fetal membranes; *B*, placental villous tissue) and one representative patient at term (*C*, fetal membranes; *D*, placental villous tissue). Both cases had negative amniotic fluid cultures, no evidence of clinical or histological chorioamnionitis, and both deliveries were by cesarean section. Of note is the decreased TLR2 staining intensity in the term amnion epithelium (Am) and choriodecidua (Ch-D) and syncytiotrophoblast (SCT) cells surrounding placental villi (V). *E* and *H–K*, Serial sections of placental basal plate in a preterm birth patient. *E*, TLR2 immunostaining is localized predominantly in villous (V) syncytiotrophoblast (SCT), extravillous trophoblasts (EVT) (*E*; marked area shown at higher magnification in *F*), and decidual cells (DC) (*E*; marked area shown at higher magnification in *G*). *H*, Masson's trichrome (nuclei are red, collagen is light blue, cytoplasm is dark red in EVTs and light pink in DCs). *I*, Negative control (omitted primary Ab). *J*, Vimentin immunostaining identified cells of mesenchymal origin such as decidual cells (DC), endothelial cells lining the blood vessels (B), and stromal cells within the placental villi (V). *K*, Cytokeratin immunostaining identified cells of epithelial origin such as syncytiotrophoblasts (SCT) and extravillous trophoblasts (EVT). The number above the bar marks the magnification (in  $\mu\text{m}$ ) for each panel. *L–N*, Results of HSCORE analysis (mean  $\pm$  SEM) showing decreased TLR2 immunostaining in amnion epithelium ( $p < 0.001$ ) and syncytiotrophoblast ( $p < 0.001$ ) at term. Although visually cells in term choriodecidua specimens also showed a decreased staining intensity, this did not reach significance (*M*) after correcting for the number of cells counted in each field, as required in HSCORE analysis. Data are presented as mean  $\pm$  SEM and analyzed by one-way ANOVA followed by Student-Newman-Keuls tests.

*Expression of TLR2 receptor (probable source of sTLR2) is primarily localized in trophoblasts, decidual cells, and amnion epithelium*

Because sTLR2 is thought to originate from processing of the TLR2 molecule, we searched for TLR2 expression in human placenta and amniochorion using an immunohistochemistry-compatible Ab raised against the extracellular domain of TLR2. This Ab detects both the full-length TLR2 receptor and the sTLR2 isoforms in amniotic fluid by Western blotting (data not shown). We analyzed random histological sections from women that delivered preterm in the presence (Gram-negative,  $n = 6$ ; Gram-positive,  $n = 10$ ) or absence ( $n = 7$ ) of intraamniotic infection or histological chorioamnionitis. We also tested amniochorion and placental tissues of healthy women delivered by elective cesarean at term, with no amniotic fluid infection or histological chorioamnionitis ( $n = 10$ ). Our findings suggested that the TLR2 immunostaining was primarily localized in amnion epithelium, syncytiotrophoblasts, extravillous trophoblasts, and decidual cells (Fig. 4A–K).

Our HSCORE analysis further illustrated that TLR2 intensity in amnion epithelium ( $p < 0.001$ ) and syncytiotrophoblast ( $p <$

$0.001$ ) appear decreased at term (Fig. 4, *L* and *N*). Although visually choriodecidua staining displayed decreased intensity at term, HSCORE analysis did not reach significance ( $p = 0.053$ ). This could be attributed to the cellular heterogeneity in choriodecidua and to variations in the relative number of decidual and extravillous trophoblast cells in each prepared section. In preterm patients, the HSCORE did not vary with amniotic fluid infection presence or absence in choriodecidua ( $p = 0.631$ ) or syncytiotrophoblast ( $p = 0.804$ ) as shown in Fig. 4, *M* and *N*. In this same group of patients, Gram stain bacterial status, or intensity of inflammation as indicated by stages of histological chorioamnionitis (data not shown) in either placenta, choriodecidua, or amniochorion did not differ. However, we did observe an up-regulation of the TLR2 HSCORE in infected, preterm amnion epithelium, which reached statistical significance ( $p = 0.04$ , Fig. 4*L*) and remained significant even when the analysis was confined to specimens taken from women with Gram-positive intraamniotic infection ( $p = 0.021$ ). In summary, our results indicate that in the absence of intraamniotic infection, the expression of TLR2 in reproductive tissues is down-regulated at term, which may provide an explanation for our

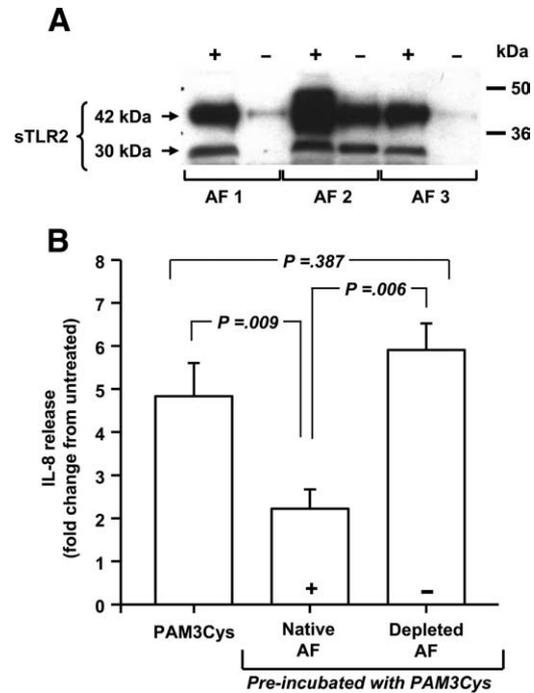


**FIGURE 5.** Production of IL-8 by placental villous explants in response to Pam3Cys (PAM). *A*, In time course experiments, exposure of placental villous tissue to 1  $\mu$ g/ml Pam3Cys induced a significant increase in IL-8 at 4 h of treatment. The maximal level of IL-8 was measured at 18 h of incubation. *B*, Amniotic fluid (AF) from preterm women (<30 wk,  $n = 3$ ) who delivered at term or near term (>36 wk,  $n = 3$ ) had a significantly inhibitory effect on IL-8 release compared with PAM alone or pseudoamniotic fluid (PAF) preincubated with PAM. \*,  $p < 0.01$  vs 1 h of incubation. Data are presented as mean  $\pm$  SEM and analyzed by one-way ANOVA followed by Student-Newman-Keuls tests.

finding that the levels of amniotic fluid sTLR2 is decreased near term. Although the TLR2 Ab would recognize both membrane-bound TLR2 and sTLR2, we further noted that villous trophoblasts stained primarily in a membrane-bound pattern suggestive of TLR2 expression. In contrast, amnion epithelial cells as well as decidual cells (vimentin-positive and cytokeratin-negative) and extravillous trophoblasts cells (vimentin-negative and cytokeratin-positive) within the basal plate of the placenta were positive for TLR2 immunostaining in a cytoplasmic pattern likely consistent with sTLR2.

#### Amniotic fluid has the ability to modulate the TLR2-mediated inflammatory response *in vitro*

To explore the functional role of the amniotic fluid sTLR2, we first sought to determine, using a placental villous explant system, the effect of a synthetic lipopeptide and specific TLR2 agonist (Pam3Cys) on IL-8 release. We were guided in the system choice by the preferential expression of TLR2 in villous syncytiotropho-

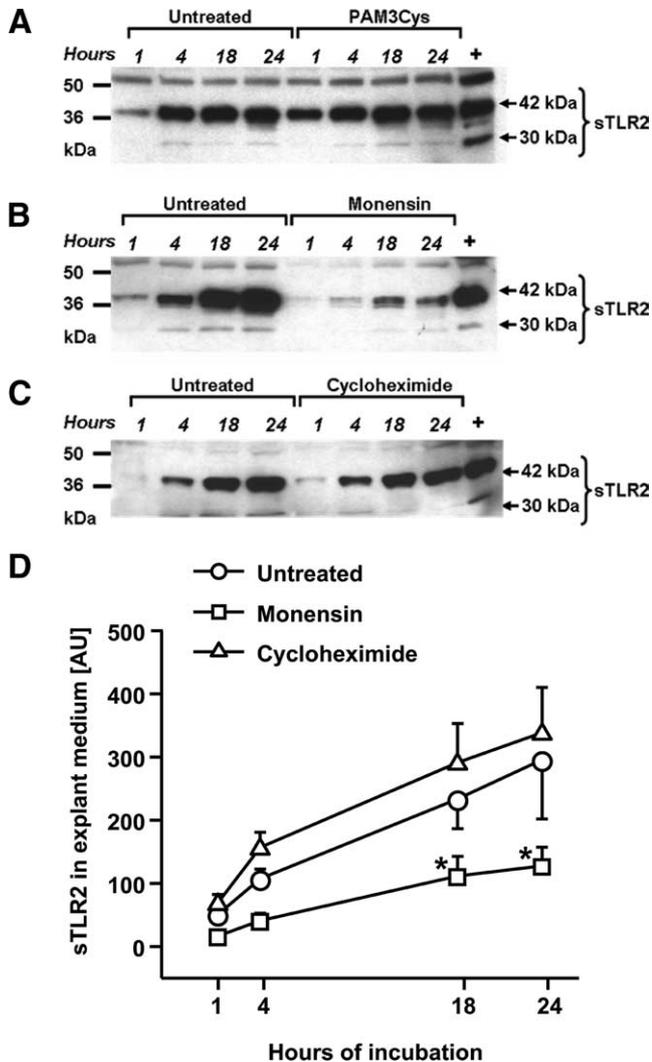


**FIGURE 6.** Effect of sTLR2 immunodepletion on IL-8 production by placental villous explants in response to Pam3Cys. *A*, Representative Western blot of sTLR2 detection in three different samples of preterm (<30 wk) amniotic fluid (AF) before (+) and after (-) immunodepletion with the anti-N17 Ab (sc8689). *B*, sTLR2-depleted fluid is unable to inhibit the stimulatory effect of Pam3Cys (PAM) on IL-8 production in the villous explant. In contrast, the native AF (nonimmunodepleted) retained the ability to down-regulate IL-8 levels. IL-8 levels were measured in the supernatant at 18 h, normalized to tissue total protein, and reported as fold change from the incubation in the absence of Pam3Cys. Data are presented as mean  $\pm$  SEM and analyzed by one-way ANOVA followed by Student-Newman-Keuls tests.

blast, and by the ability to measure the level of TLR2 activation by IL-8 (an NF- $\kappa$ B-inducible chemokine) levels in the supernatant.

In time course experiments ( $n = 9$ ), we demonstrated that exposure of placental villous tissue to Pam3Cys induced a significant increase in IL-8 at 4 h of treatment (Fig. 5*A*). A maximal level of IL-8 was measured at 18 h of incubation. As a result, this time point was chosen for subsequent experiments. We next postulated that should the amniotic fluid sTLR2 act as a receptor decoy, it would competitively bind Pam3Cys, prevent its engagement to TLR2, and thus down-regulate IL-8 levels. To test this hypothesis we took a stepwise approach. We first used amniotic fluid from women at either <30 wk (preterm,  $n = 3$ ) or >36 wk (near term,  $n = 3$ ) of gestation who delivered at term. By Western blotting we confirmed that these samples had different endogenous sTLR2 levels ( $p < 0.001$ ) consistent with the differences in GA. We incubated the amniotic fluid with the synthetic TLR2 agonist Pam3Cys. Following incubation, we determined that the preterm, but not the near-term fluid, had a significant inhibitory effect on IL-8 release (Fig. 5*B*). This was in contrast to the effect of Pam3Cys alone or when Pam3Cys was preincubated with pseudoamniotic fluid, which lacks sTLR2. Collectively, these results suggest that a soluble factor in preterm amniotic fluid is capable of counteracting the stimulatory effect of bacterial mimics engaged with TLR2 and that this effect may be GA dependent.

To provide evidence that this inhibitory amniotic fluid factor is indeed sTLR2, in separate experiments, we removed sTLR2 from four samples of preterm amniotic fluid by immunodepletion. By Western blot we determined that this procedure was able to lower



**FIGURE 7.** sTLR2 release by placental villous explants. *A*, Representative Western blot of sTLR2 released in incubation medium of a villous explant preparation of a term placenta in the absence (untreated) or presence of the TLR2 agonist Pam3Cys (1  $\mu$ g/ml). Explant medium was assayed at 1, 4, 18, and 24 h (10  $\mu$ l/lane) and shows that sTLR2 accumulation is Pam3Cys-independent. *B*, Representative Western blot of sTLR2 released in incubation medium in the absence (untreated) or presence of monensin (2  $\mu$ M). Explant medium was assayed at 1, 4, 18, and 24 h (10  $\mu$ l/lane) and shows a decrease in sTLR2 (both 42- and 30-kDa bands) following monensin treatment. *C*, Representative Western blot of sTLR2 released in incubation medium in the absence (untreated) or presence of cycloheximide (10  $\mu$ g/ml), demonstrating a lack of change following treatment. Explant medium was assayed at 1, 4, 18, and 24 h (10  $\mu$ l/lane) and shows a decrease in sTLR2 (both 42- and 30-kDa bands) following monensin treatment. *D*, Kinetic of sTLR2 accumulation in explant medium over 24 h of incubation in the absence (untreated) or presence of monensin (20  $\mu$ M) or cycloheximide (10  $\mu$ g/ml). Data from three separate placental experiments are presented as mean  $\pm$  SEM. \*,  $p < 0.01$  vs untreated (two-way repeated measures ANOVA and Student-Neuman-Keuls tests). +, Same genetic amniotic fluid sample used as positive internal control.

the sTLR2 immunoreactivity by 78% (Fig. 6A). Next, we tested the ability of the depleted amniotic fluid to counteract the Pam3Cys agonistic effect. We found that the sTLR2-depleted fluid lost its inhibitory property (Fig. 6B) to a point where the IL-8 levels were no different from those seen with Pam3Cys alone. The nondepleted amniotic fluid (native) retained its ability to significantly lower the IL-8 response after preincubation with Pam3Cys. In all of our ex-

periments the LDH levels in placental explant medium remained unchanged between treated and untreated groups, indicating adequate tissue viability during the time course studied.

#### Monensin but not cycloheximide affects the release of sTLR2 in vitro

To further understand the mechanisms of sTLR2 generation (protein synthesis vs secretion) we studied the production of sTLR2 from placental villous explants in vitro. Monensin is a Na<sup>+</sup> ionophore that blocks posttranslational processing events that take place in internal cellular compartments without affecting activities at the cell surface (32). Conversely, cycloheximide is an antibiotic that inhibits de novo protein synthesis by blocking translation of RNA (33). We found that during the course of 24-h incubation, sTLR2 accumulated in the explant medium villous tissue. The kinetics of sTLR2 accumulation did not change in response to Pam3Cys (Fig. 7A). Treatment of villous explants with monensin (Fig. 7B) but not cycloheximide (Fig. 7C) significantly diminished sTLR2 immunoreactivity in the incubation medium at the 18 and 24 h time points (Fig. 7, C and D). These observations suggest that at least part of the sTLR2 originates from an intracellular processing of TLR2 that does not require protein synthesis. Monensin or cycloheximide treatments did not significantly increase LDH activity levels in placental explant medium, indicating adequate tissue viability during the time course studied.

#### Discussion

In this study we sought to determine the presence and functional role of sTLR2, a soluble innate immune receptor, in the amniotic fluid. The key findings of this study are that human amniotic fluid contains two sTLR2 isoforms that are both immunoreactive with an Ab against the extracellular domain of the TLR2 pattern recognition receptor. Furthermore, we showed that the presence and levels of amniotic fluid sTLR2 are GA, but not infection, regulated. However, the strength of the intraamniotic inflammatory response, as reflected by both IL-6 and IL-8 amniotic fluid levels, was lower in the setting of Gram-positive, compared with Gram-negative, bacterial infection. Therefore, the critical question was whether this finding was only circumstantial evidence or if sTLR2 in the amniotic fluid is indeed able to modulate the function of the TLR2 receptor. The biological relevance of sTLR2 was supported by immunodepletion experiments that abrogated the natural anti-inflammatory effect of the amniotic fluid. Lastly, our results support the conclusion that presence of sTLR2 in human amniotic fluid is not the result of TLR2 activation by bacterial PAMPs, but rather is the consequence of an intracellular processing of the receptor, which does not require protein synthesis.

Inflammation is a highly orchestrated process developed by mammals to fight infection and prevent tissue injury (34). In normal instances, an adequate inflammatory response during pregnancy assures intact survival of the mother and fetus. However, discrimination between self (host) and bacteria occurs primarily on the basis of specific chemical modifications and structural features that are unique to the bacterial wall (35). Unfortunately, in humans, the discriminatory ability of the innate immune system is not always optimal, and inappropriate control of the inflammatory course can lead to premature birth, tissue damage, or even maternal and fetal death (36, 37, 38). Various controlling mechanisms have been established to prevent and minimize excessive cytokine production and TLR activation. One of these protective mechanisms acts through the presence and functional engagement of naturally occurring soluble cytokines and soluble TLRs.

Many cytokines (IL-6, IL-8, IL-10, TNF- $\alpha$ ) and antimicrobial peptides (defensins, calgranulins, ubiquitin, lactoferrin) have been

detected in amniotic fluid, placenta, and decidua in both normal and abnormal pregnancies (20, 39–44). Traditionally, activation of the inflammatory response occurs as a result of direct binding of cytokines and PAMPs to their specific membrane cytokine receptors or TLRs, respectively. However, recent experimental data suggest that many of the biological activities assigned to cytokines, PAMPs, and TLRs are mediated via naturally occurring soluble receptor factors for IL-6 (sIL-6R), TNF- $\alpha$  (sTNFR-1, sTNFR-2), CD14 (sCD14), LPS (LPS-binding protein), ubiquitin (amniotic fluid peptide-1), receptor for advanced glycation end-products (sRAGE), TLR4 (sTLR4), and TLR2 (sTLR2) (14, 15, 45–48).

The scientific interest has been primarily concentrated on the role of amniotic fluid sTNFR, sCD14, LPS-binding protein, and ubiquitin in preventing an exaggerated inflammatory response secondary to microbial invasion of the uterine cavity (18, 23, 44, 46). Presence of TLR2 in the human placenta, deciduas, and amniochorion led us to question whether sTLR2 (14, 15) is present in human amniotic fluid and modulates the level of TLR2 activation. Herein, we report for the first time that two sTLR2 polypeptides are detected in human amniotic fluid and placental villous explant supernatant. The possible sources of amniotic fluid sTLR2 are trophoblasts and amnion epithelium. Western blot analysis of fetal urine retrieved in utero from pregnancies affected by bladder-outlet obstruction (data not shown) did not show sTLR2 immunoreactive bands. This calls into question the extent to which the fetus participates and contributes to the amniotic fluid's sTLR2. Certainly, the fetal contribution to the pool of amniotic fluid sTLR2 should be better defined in the future.

By using two anti-TLR2 Abs against either the N terminus (extracellular domain: anti-N17) or C terminus (intracellular domain: anti-C19) we demonstrated that the amniotic fluid sTLR2 system includes two polypeptides (42 and 30 kDa) that correspond to the extracellular domain of the full-length TLR2 receptor (98 kDa). Our results are slightly different from the previous reports, which communicate that the sTLR2 system is comprised of six polypeptides (83, 66, 50, 40, 38 and 25 kDa) in human breast milk and plasma (15), and three polypeptides (55, 40, and 27 kDa) in the human parotid saliva (14). In our study we showed that the 98-kDa polypeptide corresponds to the full-length transmembrane TLR2 by using as control placental villous tissue, which lacks the two amniotic fluid isoforms attributable to sTLR2. The abundance of the 42-kDa isoform in the human amniotic fluid indicates that this is the main sTLR2 polypeptide released in this compartment. The specificity of the 42- and 30-kDa sTLR2 amniotic fluid detection was confirmed by peptide competition experiments. Given that the anti-TLR2 Abs used in these three studies are common, several possibilities must be considered to explain the differences. First, it is possible that the 66-, 50-, 27-, and 25-kDa signals reflect non-specific binding of the anti-TLR2 polyclonal Ab to polypeptides present constitutively in the human breast milk, plasma, and saliva, but not in amniotic fluid. A closer look at the data suggests that the 66-, 50-, 40-, and 25-kDa polypeptides continue to be detectable in human plasma even after preincubation of the anti-TLR2 polyclonal Ab with the blocking peptide (15). Similar findings are applicable to the 55- and 27-kDa polypeptides in human saliva (14). Second, cleavage and processing of the TLR2 full-length receptor may occur differently in amniotic fluid compared with human breast milk, plasma, or saliva. This requires further exploration and evidence. Third, the discrepancy between the uterine and other biological compartments raises questions about the stability of sTLR2 isoforms in amniotic fluid. Our results suggest that this probably cannot account for such differences since the 42- and 30-kDa were the only two sTLR2-specific polypeptides consistently detected in the human amniotic fluid.

The results of our study suggest that sTLR2 is present constitutively in the human amniotic fluid. The levels of expression for both 42- and 30-kDa sTLR2 isoforms are GA but not infection regulated. These results confirm the complexity of the protective innate immune system given that several soluble amniotic fluid factors (sIL-6, sCD14, LPS-binding protein, sRAGE) vary with GA while others (sTNFR-1, sTNFR-2) do not (18, 23, 45, 46). One possible explanation may rest with evolutionary development. The innate immune system predates adaptive immunity in phylogeny. It is likely that in the context of widespread presence of more virulent bacteria, mammals had to develop more complex innate systems to assure their defense against infection. Our placental villous explant data suggest that similar to human saliva and breast milk, amniotic fluid sTLR2 may originate through an intracellular processing of the full-length TLR2 receptor (14, 15). This process may be viewed as elementary compared with other systems, as it does not require new protein synthesis. For example, presence and function of the sTNFR immune system are dependent on the proteolytic cleavage of the TNF- $\alpha$  membrane receptor via the action of TNF- $\alpha$  converting enzyme (TACE/ADAM-17) (49). This suggests an added level of complexity but also a higher chance for failure compared with the sTLR2 system. At this time it is not clear why processing of the full-length TLR2 receptor would vary with GA. Our observation that TLR2 (probable source of sTLR2) expression in the placenta and amniochorion is decreased at term vs early gestation is consistent with that of other studies (50) and may provide a cause for the decrease in sTLR2 amniotic fluid level after 30 wk of gestation. Further testing will be needed to determine the mechanisms controlling the physiological down-regulation of sTLR2 in the human amniotic fluid.

TLR2 is expressed in the human placenta and fetal membranes (3). Its presence is noted in the trophoblast cells and also in decidual and amnion cells (3, 4). This is important, as the placenta and membranes are critical components of the mechanisms set in place to protect the fetal compartment. Along with other TLRs, TLR2 acts as costimulatory receptor to enhance proliferation and cytokine production of neutrophils (51). In the amniotic fluid, neutrophils rapidly initiate microbicidal functions, including production of antimicrobial defensins, calgranulins, and proinflammatory cytokines (20). Collectively, these findings point to a potential key role played by sTLR2 in controlling the host immune response secondary to microbial invasion of the amniotic fluid cavity from early gestation. We speculate that the high levels of endogenous sTLR2 before 30 wk GA reflect the need for a tighter control of the inflammatory response early in gestation. Importantly, this is the gestational time characterized by a higher incidence of infection-related preterm deliveries and increased vulnerability of the fetus (52).

Fetal insult with a Gram-negative organism is accompanied by a significant increase in neonatal morbidity and mortality when compared with pregnancies affected by Gram-positive organisms (53, 54). Neonates with early-onset sepsis with Gram-negative bacteria also have lower survival rates (53, 54). Our results showing a greater cytokine and chemokine response in intraamniotic infections with Gram-negative vs Gram-positive pathogens make sense when the overall role of sTLR2 is considered. sTLR2 may either homodimerize with cell surface TLR2 receptors or bind to the microbial wall components. As a result, the efficacy of TLR2 signaling is significantly reduced and the sTLR2 antiinflammatory role achieved. At this time, however, it is unknown how the functions of sTLR2 are impacted by whole bacteria, other TLR2 ligands, PAMPs, or other TLRs in general. This is especially relevant given the possible interactions that the individual TLRs may

have with one another with regard to recognition and signaling. Further studies are required for future elucidation (55, 56).

The function of sTLR2 is thought to be immunomodulatory (14, 15). Our results in vivo and in vitro are provocative. We demonstrated that the amniotic fluid sTLR2 system does not appear to vary with the infectious process although it seems to play an active role in modulating the host inflammatory response. The immunodepletion experiments demonstrated that native amniotic fluid carries antiinflammatory properties and sTLR2 is at least partially responsible for this effect. Thus, a contradiction seems to exist, as intraamniotic infection and inflammation are closely interrelated processes. Perhaps one of the most instructive aspects of our study is the description of yet another noninducible immunomodulatory mechanism in the human amniotic fluid. This is in sharp contrast with the existence of other "acute" phase innate immune systems (defensins, calgranulins, amniotic fluid peptide-1, lactoferrin, bactericidal permeability increasing protein) that seem to be suppressed in the absence of infection (20, 43, 44, 57, 58). When one considers the role of the innate immune system as microbes make their way into the amniotic cavity, the value of a temporal reaction becomes obvious. The immune system is capable of marshaling a complex network of acute responses that can take time to activate. Having such soluble defense systems ominously present in amniotic fluid would confer survival advantage in the context of low virulence bacteria by decreasing the risk and occurrence for unnecessary instances of preterm birth, thereby producing a healthier offspring. Moreover, the constitutive presence of sTLR2 makes sense given that the most common bacterial species that normally populate the vaginal environment are Gram-positive in nature (59). In this context, amniotic fluid sTLR2 may serve as an endogenous antiinflammatory mediator by preventing ascending Gram-positive bacterial ligand TLR2 engagement. The benefit for the mother and her fetus is obvious given that a robust inflammatory reaction may lead to preterm birth and fetal tissue damage. Recently, we have provided evidence that the spectrum of microorganisms associated with intraamniotic inflammation includes a number of uncultivated and difficult to cultivate bacterial species (60). Therefore, our finding that sTLR2 is able to modulate the intensity of the inflammatory process triggered subsequent to invasion of the amniotic fluid cavity by Gram-positive bacteria is of significant clinical relevance, particularly when the true prevalence of intraamniotic infection in women who are delivering preterm is underestimated.

## Acknowledgments

We are indebted to the nurses, residents, and Maternal-Fetal Medicine physicians and fellows at Yale New Haven Hospital, Department of Obstetrics, Gynecology, and Reproductive Sciences, and to all patients who participated in the study.

## Disclosures

The authors have no financial conflicts of interest.

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