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Characterization of the fetal blood transcriptome and proteome in maternal anti-fetal rejection: evidence of a distinct and novel type of human fetal systemic inflammatory response

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Abstract

Background—The human fetus is able to mount a systemic inflammatory response when exposed to microorganisms. This stereotypic response has been termed the “fetal inflammatory response syndrome” (FIRS), defined as an elevation of fetal plasma interleukin-6 (IL-6). FIRS is frequently observed in patients who delivered preterm associated with intra-amniotic infection (IAI), acute inflammatory lesions in the placenta, and a high rate of neonatal morbidity. Recently, a novel form of fetal systemic inflammation, characterized by an elevation of fetal plasma CXCL10, has been identified in patients with placental lesions suggestive of “maternal anti-fetal rejection”. These lesions include chronic chorioamnionitis, plasma cell deciduitis and villitis of unknown etiology (VUE). In addition, a seropositivity for HLA panel-reactive antibodies (PRA) in maternal sera can also be used as an index of suspicious for “maternal anti-fetal rejection”. The purpose of this study was to determine: 1) the frequency of pathologic evidence of “maternal anti-fetal rejection” in term and spontaneous preterm births; 2) the fetal serum concentration of CXCL10 in patients with and without evidence of maternal anti-fetal rejection; and 3) the fetal blood transcriptome and proteome in pregnancy with evidence of fetal inflammatory response associated with maternal anti-fetal rejection.

Methods—Maternal and fetal sera were obtained from normal term birth (*N*=150) and spontaneous preterm births (*N*=150). Fetal inflammatory response associated with maternal anti-fetal rejection was diagnosed when the patients met two or more of the following criteria: 1)

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Conflict of Interest
The authors have no financial conflicts of interest.

presence of chronic placental inflammation; 2) ≥80% of maternal HLA class I panel-reactive antibody (PRA) seropositivity; and 3) fetal serum CXCL10 concentration > 75th percentile of normal. Maternal HLA PRA was analyzed by flow cytometry. The concentration of fetal CXCL10 and IL-6 were determined by ELISA. Transcriptome analysis was undertaken after extraction of total RNA from white blood cells with a whole-genome DASL assay. Proteomic analysis of fetal serum was conducted by two-dimensional difference gel electrophoresis. Differential gene expression was considered significant when there was a $p<0.01$ and a fold-change >1.5.

Results—1) The frequency of placental lesions consistent with maternal anti-fetal rejection was higher in patients with preterm delivery than in those with term delivery (56% vs. 32%; $P<0.001$); 2) patients with spontaneous preterm births had a higher rate of maternal HLA PRA class I positivity than those who delivered at term (50% vs. 32%; $P=0.002$); 3) fetuses who were born to mothers with positive maternal HLA PRA results had a higher median serum CXCL10 concentration than in those with negative HLA PRA results ($P<0.001$); 4) the median serum CXCL10 concentration (but not IL-6) was higher in fetuses with placental lesions associated with maternal anti-fetal rejection than in those without such lesions ($P<0.001$); 5) a whole-genome DASL assay of fetal blood RNA demonstrated differential expression of 128 genes between fetuses with and without fetal inflammatory response associated with maternal anti-fetal rejection; and 6) comparison of the fetal serum proteome demonstrated 20 proteins whose abundance differed between fetuses with and without fetal inflammatory response associated with maternal anti-fetal rejection.

Conclusions—We describe systemic inflammatory response in the fetus born to mothers with evidence of maternal anti-fetal rejection. Using high-dimensional biology techniques, the transcriptome and proteome of this novel type of fetal inflammatory response demonstrated the distinct profile from FIRS type I (which is associated with acute infection). This information is crucial to gain a mechanistic understanding of the syndrome as well as to identify biomarkers for this condition.

Keywords

anti-HLA panel-reactive antibody; apolipoprotein C-III; CD34; CXCL10; chronic placental inflammation; pregnancy; proteome; transcriptome

Introduction

Pregnancy is a unique immunologic state in which the maternal adaptive and innate components of the immune system support the establishment and maintenance of pregnancy, and provide defense mechanisms against microbial pathogens.[1,2] The fetus is a semi-allograft, and active maternal immune tolerance mechanisms are fundamental for a tolerogenic state of paternal antigens and the prevention of the rejection of the fetus. [1,3–25]

The diagnosis of maternal anti-fetal rejection has been a challenge to clinical obstetrics and surgical pathology. We have recently reported a series of studies demonstrating that maternal anti-fetal rejection can be a mechanism of disease associated with spontaneous preterm birth and can be diagnosed by the identification of chronic chorioamnionitis, a lesion characterized by maternal T-cell infiltration of the chorioamniotic membranes.[26–29] Other pathologic lesions reflecting maternal anti-fetal rejection included chronic deciduitis with plasma cells and villitis of unknown etiology (VUE).[30]

Given the unique anatomical relationship between the mother and fetus, maternal anti-fetal cellular rejection and antibody-mediated rejection can affect the fetus by mechanisms operative in graft-versus-host disease (GVHD) and alloimmune reactions.[27,30] Specifically,

maternal antibodies against paternal antigens can cross the placenta to activate complement and elicit a fetal inflammatory response.[27]

We have previously reported that the fetal plasma concentration of CXCL10 is higher for cases in which the placenta has VUE.[30] Therefore, we hypothesized that maternal anti-fetal rejection is linked to a stereotypical derangement of the systemic fetal chemokine milieu, specifically CXCL10, just as intra-amniotic infection/inflammation is associated with an elevation of the fetal plasma concentration of IL-6.[31–35] The latter condition observed in human fetuses of patients with preterm labor and preterm prelabor rupture of membranes (PPROM) has been termed the "fetal inflammatory response syndrome" (FIRS),[31–33,36–44] and has been associated with a higher rate of adverse neonatal outcome,[31–36,45–50] a short interval to delivery, and multi-systemic involvement.[32,42–44,47,51–118] We have recently provided evidence that an elevation of amniotic fluid CXCL10 concentration during the mid-trimester is a risk factor for preterm delivery after 32 weeks of gestation,[119] while an elevation of amniotic fluid IL-6 concentration is associated with preterm delivery before 32 weeks of gestation.[119] This observation suggests that there is heterogeneity in the nature of the intra-amniotic inflammatory response during pregnancy.[119] Typically, an elevation of amniotic fluid IL-6 is observed in cases of intra-amniotic infection associated with acute chorioamnionitis and funisitis [33,120–126]. CXCL10 is a T-cell chemokine which is elevated in the amniotic fluid of patients with chronic chorioamnionitis.[26,27,127] In this lesion, maternal T cells invade the chorioamniotic membranes,[26] presumably because of the chemotactic gradient generated in the amniotic cavity by T-cell chemokines including CXCL10.[26,27,127]

This study was conducted to determine: 1) the frequency of pathologic evidence of cellular and humoral maternal anti-fetal rejection in term and spontaneous preterm births; 2) the fetal plasma concentration of CXCL10 in patients with and without evidence of maternal anti-fetal rejection; and 3) the fetal blood transcriptome and proteome in patients with fetal inflammatory response associated with maternal anti-fetal rejection.

Materials and Methods

Patients and definitions

The patient population comprised Hispanic women who were enrolled and delivered at the Sótero del Río Hospital, Santiago, Chile. Sera and tissue samples from the patients and their singleton neonates were retrieved from the Bank of Biological Materials of the Sótero del Río Hospital and the Perinatology Research Branch, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U. S. Department of Health and Human Services.

Patients included women who delivered (1) with a normal pregnancy outcome at term (*N*=150) and (2) before 37 completed weeks of gestation after preterm labor with intact membranes or PPROM (*N*=150). Pregnancies with a fetal congenital anomaly and small-for-gestational-age neonate were ineligible to participate. Placental tissues and fetal cord blood samples were collected at the time of delivery. We selected maternal blood samples which were collected within seven days before and after delivery to maintain a meaningful temporal relationship between placental histopathologic findings and concentrations of CXCL10 and IL-6 in maternal sera. Samples were stored at −80°C until use. All patients provided written informed consent at the Sótero del Río Hospital. The Institutional Review Boards of the participating institutions approved the collection and use of biological materials and clinical data for research purposes.

Preterm labor was defined as the presence of regular uterine contractions occurring at a frequency of at least two every 10 minutes associated with cervical dilatation, followed by delivery before 37 completed weeks of gestation. PPROM was diagnosed by sterile speculum examination when pooling of amniotic fluid in the vagina occurred or when positive nitrazine and ferning tests, conducted when necessary, were confirmed before 37 completed weeks of gestation in the absence of labor.

Placental Pathology

Placental histopathologic changes were defined according to diagnostic criteria proposed by the Perinatal Section of the Society for Pediatric Pathology and included lesions consistent with amniotic fluid infection, maternal vascular underperfusion, and fetal vascular thrombo-occlusive disease.[128] The diagnosis of VUE was based on histologic criteria previously defined,[30,129] and chronic chorioamnionitis was diagnosed when lymphocytic infiltration into the chorionic trophoblast layer or chorioamniotic connective tissue was present as previously described. [26–28,127,130,131] Chronic deciduitis with plasma cells was defined as the presence of lymphoplasmacytic infiltration into the decidua of the basal plate.[132] Chronic placental inflammation was defined upon observation of one or more findings among chronic chorioamnionitis, VUE, and chronic deciduitis with plasma cells.

Flow Cytometry for HLA Panel-Reactive Antibodies

Flow cytometric analyses of HLA class I and class II PRA in maternal sera were conducted using the FlowPRA®-I Screening Test and the FlowPRA®-II Screening Test (One Lambda, Inc., Canoga Park, CA, USA), according to the manufacturer's instructions. HLA class I or class II microbeads were mixed with 20 μL of serum, followed by incubation for 30 min at room temperature with gentle rotation. After the microbeads were washed 3 times with 1 mL of FlowPRA® Wash Buffer by centrifugation at 9,000xg for 2 min, they were incubated with 100 μL of FITC-conjugated F(ab)2 fragment of Fcγ fragment specific goat anti-human IgG for 30 min. Thereafter, the microbeads were washed twice with 1 mL of wash buffer, and 0.5 mL of fixing solution (PBS with 0.5% formaldehyde) was added. The FL1 fluorescence of 5,000 events was analyzed using the BD™ LSR II Flow Cytometer (BD Biosciences, San Jose, CA, USA). A sample with panel-reactivity of 10% or more was considered PRA-positive.[133,134]

Enzyme-Linked Immunosorbent Assays for IL-6 and CXCL10

Serum concentrations of IL-6 (Human IL-6 Quantikine® HS ELISA Kit, R&D Systems, Minneapolis, MN, USA) and of CXCL10 (Human CXCL10/IP-10 Quantikine® ELISA Kit, R&D Systems) were measured with specific immunoassays, according to the manufacturer's instructions.

Whole-Genome DASL Assay

To characterize the fetal blood transcriptome in patients with evidence of fetal inflammatory response associated with maternal anti-fetal rejection, the Whole-Genome DASL® Assay (cDNA-mediated Annealing, Selection, Extension, and Ligation: Illumina, Inc., San Diego, CA, USA) was performed using fetal blood samples from cases with (*N*=9) and without (*N*=15) evidence of fetal inflammatory response associated with maternal anti-fetal rejection which was defined as the presence of two or more of the following criteria: 1) chronic placental inflammation (villitis of unknown etiology, chronic chorioamnionitis or chronic deciduitis with plasma cells), 2) ≥80% of maternal HLA class I PRA seropositivity; and 3) fetal serum CXCL10 concentration > 75th percentile. Group 1 comprised cases with evidence of fetal inflammatory response associated with maternal anti-fetal rejection, and nine neonates (five term and four preterm births) met the criteria. In Group 2 (cases without

evidence of fetal inflammatory response associated with maternal anti-fetal rejection), 15 neonates (seven term and eight preterm births) had no chronic placental inflammation, negative maternal HLA class I PRA (<10% of panel-reactivity), and fetal CXCL10 concentration less than the 25th percentile.

Fetal cord blood samples were collected into PAXgene™ Blood RNA collection tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland). Blood tubes were kept at room temperature for 24 h and then frozen at −70°C until assay. Total blood RNA was isolated using the PAXgene™ Blood RNA Kit (Qiagen, Valencia, CA, USA) with DNase I treatment. The quantity and quality of RNA were evaluated by the Dropsense96® Microplate Spectrophotometer (Trinean, Gentbrugge, Belgium) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively.

Gene expression of whole blood RNA was measured using the Whole-Genome DASL® Assay. One hundred nanograms of total RNA were reverse-transcribed with biotin-labeled oligo-dT and random primers. Biotinylated cDNAs were annealed to assay-specific oligonucleotides [DASL® Assay Pool (DAP) probe groups]. The mixtures were then bound on streptavidin-conjugated paramagnetic particles to select the cDNA/oligo complexes. PCR amplification was completed with fluorescently labeled primers, and the amplified PCR products were hybridized overnight onto the BeadChips (Illumina). The intensities of fluorescence were measured using the iScan™ System (Illumina).

Raw gene expression levels were normalized using the quantile normalization method.[135] A linear model was used to fit gene expression levels as a function of disease status (cases with and without evidence of fetal inflammatory response associated with maternal anti-fetal rejection), gestational age at delivery (term or preterm), and gender of the fetus. Coefficients were calculated using moderated t-tests.[136] Differential gene expression was considered significant based upon two criteria: a) the *P* value of <0.01 and b) the magnitude of change (fold-change >1.5).[137] Gene Ontology analysis was conducted using an over-representation approach previously described[138] and implemented in the GOstats package.[139]

The DASL® Assay data used in this study were submitted to the Gene Expression Omnibus (GEO). Interested readers can use the following link to access the data: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=fpwjrqimaqgeehi&acc=GSE28387.

The quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) assay was conducted to confirm DASL® Assay results for genes of interest using the Biomark™ System (Fluidigm, South San Francisco, CA, USA) with specific TaqMan® assays (Applied Biosystems®, Life Technologies Corporation, Foster City, CA, USA), according to the manufacturers' instructions (Supplemental Table I).

Two-dimensional Difference Gel Electrophoresis (2D-DIGE)

An equal amount of fetal serum samples obtained from cases with (*N*=10) and without (*N*=10) evidence of fetal inflammatory response associated with maternal anti-fetal rejection was pooled to compare their proteome. The presence and absence of fetal inflammatory response associated with maternal anti-fetal rejection were defined by the same criteria used in the Whole-Genome DASL® assay; fetal serum CXCL10 concentration for only one case with evidence of fetal inflammatory response associated with maternal anti-fetal rejection was higher than the 50th but less than the 75th percentile. For each sample, 5 μL of lysis buffer [30 mM Tris-HCl (pH 8.8), 7 M urea, 2 M thiourea, 4% CHAPS] were added to 1 μL of serum, followed by labeling with Cy3 or Cy5. The labeling reaction was stopped by adding 1 μL of 10 mM Lysine to each pooled sample, followed by incubation on ice in the dark for an additional 15 min. Labeled samples were then mixed with 2X sample buffer [8

M urea, 4% CHAPS, 20 mg/mL dithiothreitol (DTT), 2% pharmalytes] and 100 μL of DeStreak Rehydration Solution (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/mL DTT, 1% pharmalytes; GE Healthcare Life Sciences, Piscataway, NJ, USA) for a total volume of 250 μL. The samples were mixed, spun, and then loaded into a strip holder. After isoelectric focusing (pH 3-10), IPG strips were incubated in equilibration buffer-1 (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 10 mg/mL DTT) for 15 min with gentle shaking, and rinsed in equilibration buffer-2 [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 45 mg/mL DTT] for 10 min with gentle shaking. Following electrophoresis in a 12% SDS-polyacrylamide gel at 15°C, the gel was scanned using Typhoon Trio™ (GE Healthcare Life Sciences). Scanned images were then analyzed by ImageQuant TL software version 6.0 (GE Healthcare Life Sciences), followed by differential in-gel analysis using DeCyder™ 2D Software Version 6.5 (GE Healthcare Life Sciences), to obtain the fold-changes of protein expression.

Mass Spectrometry

Twenty spots of interest were picked up by the Ettan™ Spot Picker (GE Healthcare Life Sciences) and digested in gel with modified porcine trypsin protease (Trypsin Gold; Promega, Madison, WI, USA). Digested tryptic peptides were desalted by Zip-tip C18 (Millipore Corporation, Billerica, MA, USA). Peptides were eluted from the ZipTips® with 0.5 μL of matrix solution (5 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid, and 25 mM of ammonium bicarbonate) and spotted on the MALDI plate. MALDI-TOF and TOF/TOF mass spectrometry were performed with an AB SCIEX TOF/TOF™ 5800 System (AB SCIEX, Framingham, MA, USA). MALDI-TOF mass spectra were acquired in the reflectron-positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF mass spectrometry fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample. Both the resulting peptide mass and the associated fragmentation spectra were submitted to a GPS Explorer™ Workstation equipped with a MASCOT search engine (Matrix Science Ltd., London, UK) to search the redundant database of the National Center for Biotechnology Information (NCBI). Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters. Candidates with either a protein score of C.I.% or Ion C.I.% >95 were considered significant.

To confirm 2D-DIGE results for proteins of interest, serum concentrations of apolipoprotein E and apolipoprotein C-III were measured with specific immunoassays (Human Apolipoprotein E ELISA Kit, Kamiya Biomedical Company, Seattle, WA, USA; AssayMax Human Apolipoprotein C-III ELISA Kit, AssayPro LLC, St. Charles, MO, USA), according to the manufacturers' instructions.

Statistical Analysis

To obtain statistical significance for continuous variables, distributions were examined for normality using the Kolmogorov-Smirnov test. When data were far from normality, the Kruskal-Wallis one-way analysis of variance and the Mann-Whitney U tests were performed. When there was normality of continuous variables, the one-way ANOVA test and unpaired t-tests were used to compare differences. To assess the categorical variables, proportions were compared with Fisher's exact test or the χ^2 test. Medians and inter-quartile ranges were reported for continuous variables whereas frequencies and percentages were calculated for categorical variables. The Jonckheere-Terpstra test was used to compare continuous variables among multiple-ordered groups, and the linear-by-linear association analysis was used for categorical variables. Statistical analyses were performed using the

SPSS Version 15.0 (SPSS, Inc., Chicago, IL, USA). All *P* values were two-sided, with $P<0.05$ considered statistically significant.

Results

Demographics of the Study Population

Table I showed the clinical characteristic and pathologic findings of the placenta and HLA PRA positivity in patients who delivered at term as well as those who had spontaneous preterm births. Histological evidence of maternal anti-fetal cellular rejection in the placenta (chronic chorioamnionitis, VUE, or chronic deciduitis with plasma cells) was more common in patients with spontaneous preterm delivery than in those who delivered at term [56% (84/150) of spontaneous preterm and 32% (48/150) of term births; $P<0.001$]. Maternal HLA class I PRA positivity was more common in spontaneous preterm births than in term deliveries [50% (75/150) versus 32% (48/150); $P=0.002$].

Anti-fetal Cellular Rejection and Fetal Blood CXCL10 Concentration

The median fetal serum CXCL10 concentration was higher in cases with anti-fetal cellular rejection than in those without cellular rejection (median 99.9 pg/mL, interquartile range [IQR] 75.2–147.5 pg/mL versus median 77.7 pg/mL, IQR 59.6–105.4 pg/mL $P<0.001$), while there was no difference in fetal serum CXCL10 concentration in the presence or absence of acute chorioamnionitis (Fig. 1A). Differences in fetal serum CXCL10 concentration according to the presence or absence of each type of anti-fetal cellular rejection remained significant (for chronic chorioamnionitis: median 99.0 pg/mL, IQR 75.7–147.5 pg/mL versus median 81.2 pg/mL, IQR 62.0–115.8 pg/mL; for VUE: median 128.5 pg/mL, IQR 93.9–181.2 pg/mL versus median 80.6 pg/mL, IQR 61.7–112.2 pg/mL; for chronic deciduitis with plasma cells: median 115.8 pg/mL, IQR 82.2–161.2 pg/mL versus median 81.2 pg/mL, IQR 63.3–114.3 pg/mL) ($P<0.01$, for each). In contrast, median fetal serum IL-6 concentrations were different between cases with and without acute chorioamnionitis ($P<0.001$), while there was a tendency toward higher fetal serum IL-6 concentration in those with anti-fetal cellular rejection ($P=0.06$) (Fig. 1B). The fetal serum concentration CXCL10 was correlated to the extent of the cellular rejection (aggregate number of pathologic lesions consistent with maternal anti-fetal rejection) ($P<0.001$; Fig. 1C).

Antibody-mediated Rejection and Fetal Blood CXCL10 Concentration

The median fetal serum CXCL10 (but not IL-6) concentration was higher in maternal HLA class I PRA-positive cases than in PRA-negative cases (median 111.5 pg/mL, IQR 80.3–157.2 pg/mL versus median 76.6 pg/mL, IQR 59.5–100.1 pg/mL, $P<0.001$, Fig. 2A and 2B). A similar difference was also found with HLA class II PRA positivity (median 122.5 pg/mL, IQR 85.5–177.6 pg/mL versus median 81.1 pg/mL, IQR 62.4–113.6 pg/mL, $P<0.001$). When cases were graded as negative (PRA<10%), mildly sensitized (PRA≥10% and <80%), and highly sensitized (PRA≥80%) according to the reactivity of maternal HLA class I PRA,[134,140,141] there was a significant correlation between fetal serum CXCL10 concentration and the degree of maternal sensitization (Fig. 2C). Similar differences in fetal serum CXCL10 concentration were also found in accord with maternal HLA class II PRA positivity.

Whole-Genome DASL Assay of the Blood Transcriptome

To characterize the blood transcriptome in cases with fetal inflammatory response associated with maternal anti-fetal rejection, Whole-Genome DASL® Assay was performed using fetal blood samples from patients with evidence of fetal inflammatory response associated with

maternal anti-fetal rejection (fetal inflammatory response associated with maternal anti-fetal rejection: two or more parameters of cellular rejection, maternal HLA class I PRA ≥80%, and fetal serum CXCL10 concentration >75th percentile) and those without evidence of fetal inflammatory response associated with maternal anti-fetal rejection (no cellular rejection, negative maternal HLA class I and class II PRA, and fetal serum CXCL10 concentration <25th percentile) (Fig. 3A and 3B). A total of 128 genes were differentially expressed in the WBCs of fetuses with and without evidence of fetal inflammatory response associated with maternal anti-fetal rejection (Table II). *CD34*, *BAALC* (brain and acute leukemia, cytoplasmic), *PRTN3* (proteinase 3), *AZU1* (azurocidin 1), *CTSG* (cathepsin G), *MPO* (myeloperoxidase), and *RNASE3* (ribonuclease, RNase A family, 3) were among the 98 genes whose expression was decreased in cases with evidence of fetal inflammatory response associated with maternal anti-fetal rejection. Differential expression of these genes was confirmed by qRT-PCR along with the decreased mRNA expression of CD66b (but not of CD3, CD4, CD8, CD14, CD16a, CD19, CD23, CD56, CD64, and CD68) in the blood of fetuses with evidence of fetal inflammatory response associated with maternal anti-fetal rejection (Fig. 3C and 3D). Gene Ontology analysis of differentially expressed genes showed enrichment of 24 biological processes such as ‘response to other organism’ and ‘killing by host of symbiont cells’ (Table III).

When we compared differentially expressed genes in cases with evidence of fetal inflammatory response associated with maternal anti-fetal rejection (*N*=128) with those found to be linked with FIRS-associated intra-amniotic infection (*N*=448),[84] only 14 genes (*RETN*, *LCN2*, *TCN1*, *RNASE2*, *CEBPE*, *FOXM1*, *CEP55*, *C12orf59*, *CAPN3*, *TP53I3*, *TYMS*, *GINS2*, *ID3*, and *FCER2*) were common to both conditions, but all were inversely correlated, demonstrating a clear difference between these two conditions (Fig. 3E).

2D-DIGE of Fetal Serum

Fig. 4A shows the 2-D electrophoresis gel with 30 spots displaying differentially expressed proteins (more than a 1.5 fold-change) between cases with and without evidence of fetal inflammatory response associated with maternal anti-fetal rejection. Table IV shows the list of 20 differentially expressed proteins identified from 30 spots in the 2D-DIGE analysis of pooled fetal serum samples from each group (cases with and without evidence of fetal inflammatory response associated with maternal anti-fetal rejection). Serum albumin and hemoglobin were decreased in cases with evidence of fetal inflammatory response associated with maternal anti-fetal rejection. Interestingly, several apolipoproteins were found to be differentially abundant between the two groups: apolipoprotein E precursor, apolipoprotein J precursor, and apolipoprotein E3 fragment were decreased, and apolipoprotein C-III was increased, in cases with evidence of fetal inflammatory response associated with maternal anti-fetal rejection.

To confirm the results of proteins of interest in 2D-DIGE, serum concentrations of apolipoprotein C-III and apolipoprotein E were measured with specific immunoassays. A higher serum concentration of apolipoprotein C-III in cases with evidence of fetal inflammatory response associated with maternal anti-fetal rejection than in those without it was confirmed (fetal inflammatory response associated with maternal anti-fetal rejection: median 63.0 μg/mL, IQR 47.4–69.9 μg/mL versus no evidence of fetal inflammatory response associated with maternal anti-fetal rejection: median 38.1 μg/mL, IQR 29.8–53.4 μg/mL, *P*=0.013; Fig. 4C), while serum apolipoprotein E concentration was not significantly different between the two groups (evidence of fetal inflammatory response associated with maternal anti-fetal rejection: median 148.6 μg/mL, IQR 137.2–180.2 μg/mL versus no evidence of fetal inflammatory response associated with maternal anti-fetal rejection: median 119.9 μg/mL, IQR 111.6–165.6 μg/mL, *P* =0.199).

Discussion

Principal findings of this study

1) The frequency of placental lesions consistent with maternal anti-fetal rejection was higher in patients with spontaneous preterm delivery than in those with term delivery; 2) patients with spontaneous preterm births had a higher rate of maternal HLA PRA class I positivity than those who delivered at term; 3) fetuses born of pregnancies with evidence of maternal anti-fetal rejection had a higher fetal serum CXCL10 than those without this process; and 4) the WBC transcriptome and serum proteome were different in those with and without evidence of fetal inflammatory response associated with maternal anti-fetal rejection, suggesting the existence of a distinct form of a systemic inflammatory response in fetuses that were immunologically rejected by their mothers.

The clinical significance of an elevation of CXCL10

CXCL10, a ligand for CXCR3, is chemotactic for activated T cells, macrophages, and NK cells.[142–144] Notably, CXCL10 is one of the most commonly expressed chemokines during allograft rejection and GVHD.[145–148] An elevated intra-graft CXCL10 expression is associated with renal, lung, and cardiac allograft rejection.[149–157] Additionally, an elevated serum CXCL10 concentration before organ transplantation is predictive of poor allograft outcome.[151,153,154,158] Our study shows that maternal anti-fetal cellular rejection and antibody-mediated rejection are associated with increased systemic fetal chemokine CXCL10 concentration, as intra-amniotic infection is linked to an elevation of the systemic fetal cytokine IL-6 concentration.[31,33] Further, we also demonstrated that maternal anti-fetal rejection shares common features with allograft rejection. Indeed, the current study demonstrates that fetuses with evidence of maternal anti-fetal rejection have elements of an inflammatory response which is quite distinct from that observed in FIRS-associated intra-amniotic infection and acute inflammatory lesions[31–33]. We proposed the term “fetal inflammatory response syndrome type II” for this condition. The bases for the proposal are that: 1) fetal serum CXCL10 (but not IL-6) concentration is associated with anti-fetal cellular rejection and antibody-mediated rejection, and 2) there are no overlapping changes in the fetal blood transcriptome between fetal inflammatory response associated with maternal anti-fetal rejection and FIRS associated intra-amniotic infection and acute inflammatory lesions, which we will refer to henceforth as “FIRS type I”.

We conducted comprehensive analyses of the fetal blood transcriptome and proteome to characterize fetal systemic changes associated with fetal inflammatory response associated with maternal anti-fetal rejection, and found biologically meaningful changes. *BAALC* is expressed in CD34+ hematopoietic progenitor cells from bone marrow, and it is a poor prognostic factor in acute myeloid leukemia.[159] *PRTN3*, *AZU1*, *CTSG*, *MPO*, and *RNASE3* can also be expressed in CD34+ hematopoietic progenitor cells which are essential for the function of mature neutrophils and eosinophils.[160] Universal down-regulation of mRNA expression of the genes for neutrophil granule proteins and the polymorphonuclear leukocyte surface marker (CD66b) is consistent with earlier observations of neonatal alloimmune neutropenia induced by maternal HLA antibodies.[161,162] Overall changes in the fetal blood transcriptome strongly suggested the presence of an alloimmune reaction in the fetus probably caused by the deleterious effect of maternal anti-HLA antibodies which cross the placenta and activate complement in the endothelium of the umbilical cord vein.

Among changes in the serum proteins, we found an overexpression of apolipoprotein C-III which was confirmed by immunoassay. Apolipoprotein C-III in plasma has been shown to be associated with coronary heart disease, atherosclerosis, and metabolic syndromes such as obesity, hypertriglyceridemia, and type 2 diabetes.[163,164] Therefore, the increase in serum

apolipoprotein C-III in fetuses with evidence of fetal inflammatory response associated with maternal anti-fetal rejection is intriguing, and raises the need for further studies about potential long-term consequences of prenatal exposure to maternal immunological rejection. Several studies have clearly indicated that an abnormal intrauterine environment can affect lifelong fetal well-being in the form of abnormal fetal programming.[165–167] Individuals exposed to the Dutch famine during pregnancy had a higher frequency of coronary heart disease,[168] and also display an atherogenic blood lipid profile as a consequence of metabolic stress *in utero*.[169] Although fetal inflammatory response associated with maternal anti-fetal rejection does not have a direct relationship to maternal nutritional intake, changes in blood lipid profiles strongly suggest that fetal inflammatory response associated with maternal anti-fetal rejection could alter fetal programming.

In organ transplantation, humoral antibody-mediated allograft rejection occurs by different mechanisms from T-cell-mediated rejection.[170,171] However, both cell-mediated and antibody-mediated rejections begin with recognition as a common starting point and are followed by: 1) CD4+ and CD8+ T cell cytotoxicity, 2) CD4+ and CD8+ derived IFN-γ production and delayed-type hypersensitivity, and 3) complement activation or antibody-dependent cell-mediated cytotoxicity by antibodies reactive to donor MHC molecules;[171] and these three phenomena are closely related to each other. Our previous studies demonstrated a robust association between anti-fetal cellular rejection (chronic chorioamnionitis) and anti-fetal antibody-mediated rejection (both positive maternal HLA PRA and C4d deposition on umbilical vein endothelium),[27] and fetal HLA specificity of maternal HLA antibodies.[29] The findings in this study also support the hypothesis that anti-fetal antibody-mediated rejection has biological consequences similar to cellular rejection, sharing the feature of an increased CXCL10 concentration in fetal sera.

Strengths and limitations

We described a novel form of fetal systemic inflammation in the context of maternal anti-fetal rejection. The limitations of this study include: 1) we did not define the cut-off value of fetal serum CXCL10 concentration to predict evidence of fetal inflammatory response associated with maternal anti-fetal rejection. This has been defined as FIRS type I with a fetal plasma IL6 concentration above 11 pg/ml in blood obtained by cordocentesis.[31,33,48] Future studies are required to identify a cut-off value and also the short- and long-term consequences of this inflammatory process; and 2) the leukocyte counts of cord blood at the time of delivery were not analyzed. As retrospective analysis of cord blood is impossible, we instead compared mRNA expression levels of genes encoding cell-type-specific surface markers of leukocytes.

Conclusions

Collectively, the findings reported herein link maternal anti-fetal rejection with a systemic inflammatory response in the fetus. This inflammatory response is characterized with changes in the fetal blood transcriptome and proteome which are different from FIRS type I associated with acute inflammatory lesions of the placenta. Future studies are required to define pragmatic diagnostic criteria as well as short- and long-term consequences of this condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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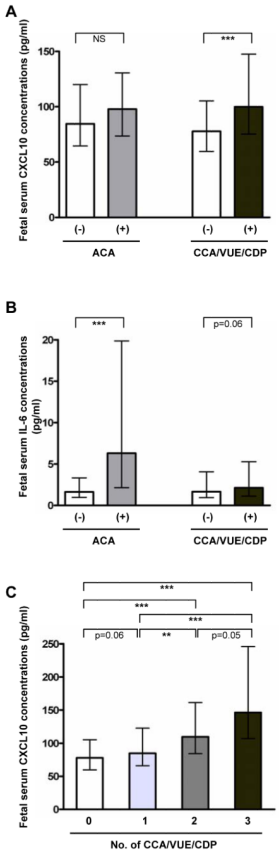

Figure 1. Fetal serum CXCL10 and IL-6 concentrations according to the presence or absence of maternal anti-fetal cellular rejection

(A) Fetal serum CXCL10 concentration was higher in cases with anti-fetal cellular rejection (chronic placental inflammation) than in those without (P<0.001), while fetal serum CXCL10 concentration was not different according to the presence or absence of acute chorioamnionitis. (B) Cases with acute chorioamnionitis had higher median fetal serum IL-6 concentration than those without (P<0.001), and fetal serum IL-6 concentration tended to be higher in cases with anti-fetal cellular rejection than in those without (P=0.06). (C) The upward trend of blood CXCL10 concentration correlates with the extent of cellular rejection (P<0.001 by the Jonckheere-Terpstra test). Fetal serum CXCL10 and IL-6 concentrations were shown as median and inter-quartile ranges. *P<0.05; **P<0.01; ***P<0.001 (by the Mann-Whitney U test for comparison between the two groups). *ACA*, acute chorioamnionitis; *CCA*, chronic chorioamnionitis; *CDP*, chronic deciduitis with plasma cells; *NS*, not significant; *VUE*, villitis of unknown etiology.

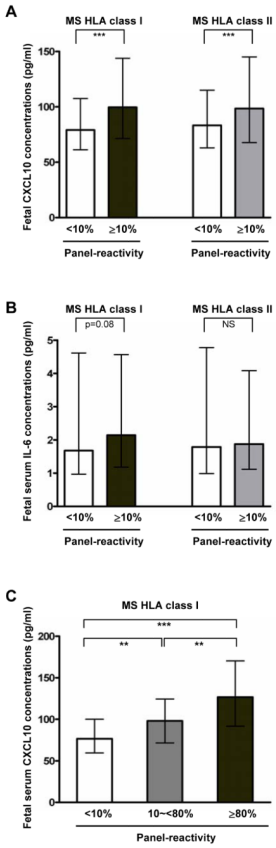

Figure 2. Fetal serum CXCL10 and IL-6 concentrations according to the presence or absence of maternal HLA PRA

(A) Median fetal serum CXCL10 concentration is higher in maternal HLA class I PRA-positive cases than in PRA-negative cases ($P<0.001$). Similar findings were shown between maternal HLA class II PRA-positive and PRA-negative cases. (B) Fetal serum IL-6 concentration was not different according to maternal HLA class I or class II PRA positivity. (C) There was a significant upward trend in fetal serum CXCL10 concentration associated with the degree of maternal HLA sensitization ($P<0.001$ by the Jonckheere-Terpstra test). Fetal serum CXCL10 and IL-6 concentrations were shown as median and inter-quartile ranges. *$P<0.05$; **$P<0.01$; ***$P<0.001$ (by the Mann-Whitney U test for comparison between the two groups). *HLA*, human leukocyte antigen; *MS*, maternal serum; *NS*, not significant; PRA, panel-reactive antibodies.

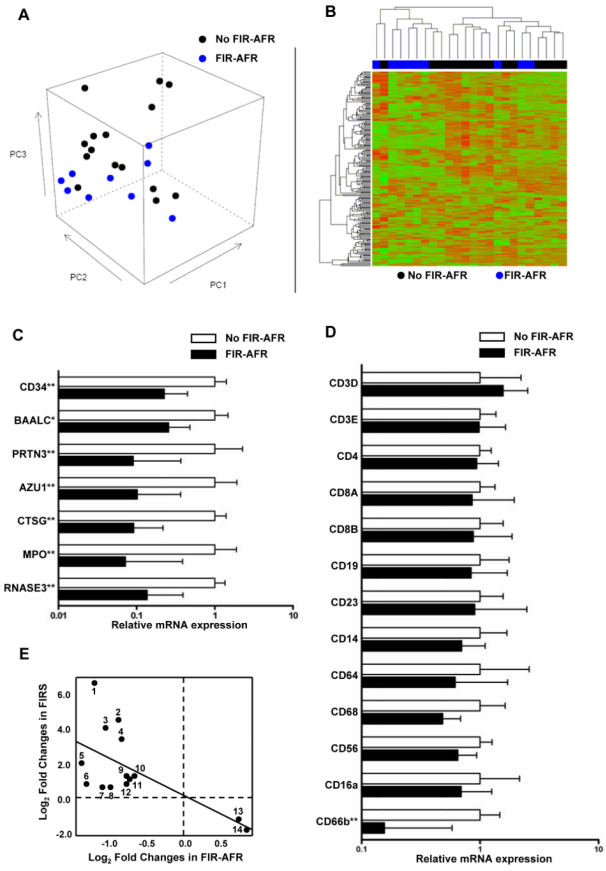

Figure 3. Transcriptome analysis of fetal blood using whole genome DASL® assay according to the presence or absence of fetal inflammatory response associated with maternal anti-fetal rejection

(A) An unsupervised Principal Component Analysis based on expression of all genes on the array shows that samples of the group without fetal inflammatory response associated with maternal anti-fetal rejection tend to have higher PC3 and PC1 coordinates than samples of the fetal inflammatory response associated with maternal anti-fetal rejection group. (B) A clustered heat map based on the top 200 most varying genes shows two main clusters: one dominated by samples of the fetal inflammatory response associated with maternal anti-fetal rejection group (left) and one dominated by samples of the group without fetal inflammatory response associated with maternal anti-fetal rejection (right). (C) Quantitative RT-PCR results confirm differential expression of genes of interest: mRNA expression of *CD34*, *BAALC* (brain and acute leukemia, cytoplasmic), *PRTN3* (proteinase 3), *AZU1* (azurocidin 1), *CTSG* (cathepsin G), *MPO* (myeloperoxidase), and *RNASE3* (ribonuclease, RNase A family, 3) was decreased in cases with fetal inflammatory response associated with maternal anti-fetal rejection ($P<0.05$, for each). (D) Quantitative RT-PCR of leukocyte marker genes demonstrates that mRNA expression of CD66b (a marker for polymorphonuclear leukocyte) was decreased in the blood of cases with fetal inflammatory response associated with maternal anti-fetal rejection ($P<0.01$). However, there was no difference in mRNA expression of T cell markers (CD3D, CD3E, CD4, CD8A, and CD8B), B cell markers (CD19 and CD23), monocyte markers (CD14 and CD64), and natural killer cell or macrophage markers (CD56 and CD68). (E) Comparison of differentially expressed genes between fetal inflammatory response syndrome to intra-amniotic infection (FIRS) shown in a previous study by Madsen-Bouterse et al.[6] Fetal inflammatory response associated with

maternal anti-fetal rejection cases showed only 14 genes common to both conditions – *RETN*, *LCN2*, *TCN1*, *RNASE2*, *CEBPE*, *FOXM1*, *CEP55*, *C12orf59*, *CAPN3*, *TP53I3*, *TYMS*, *GINS2*, *ID3*, and *FCER2* – and all were inversely correlated. Relative mRNA expressions were shown as median and inter-quartile ranges.
**P* < 0.05; ***P* < 0.01; ****P* < 0.001 (by the Mann-Whitney U test). *FIR-AFR*, fetal inflammatory response syndrome associated with maternal anti-fetal rejection; *FIRS*, fetal inflammatory response syndrome.

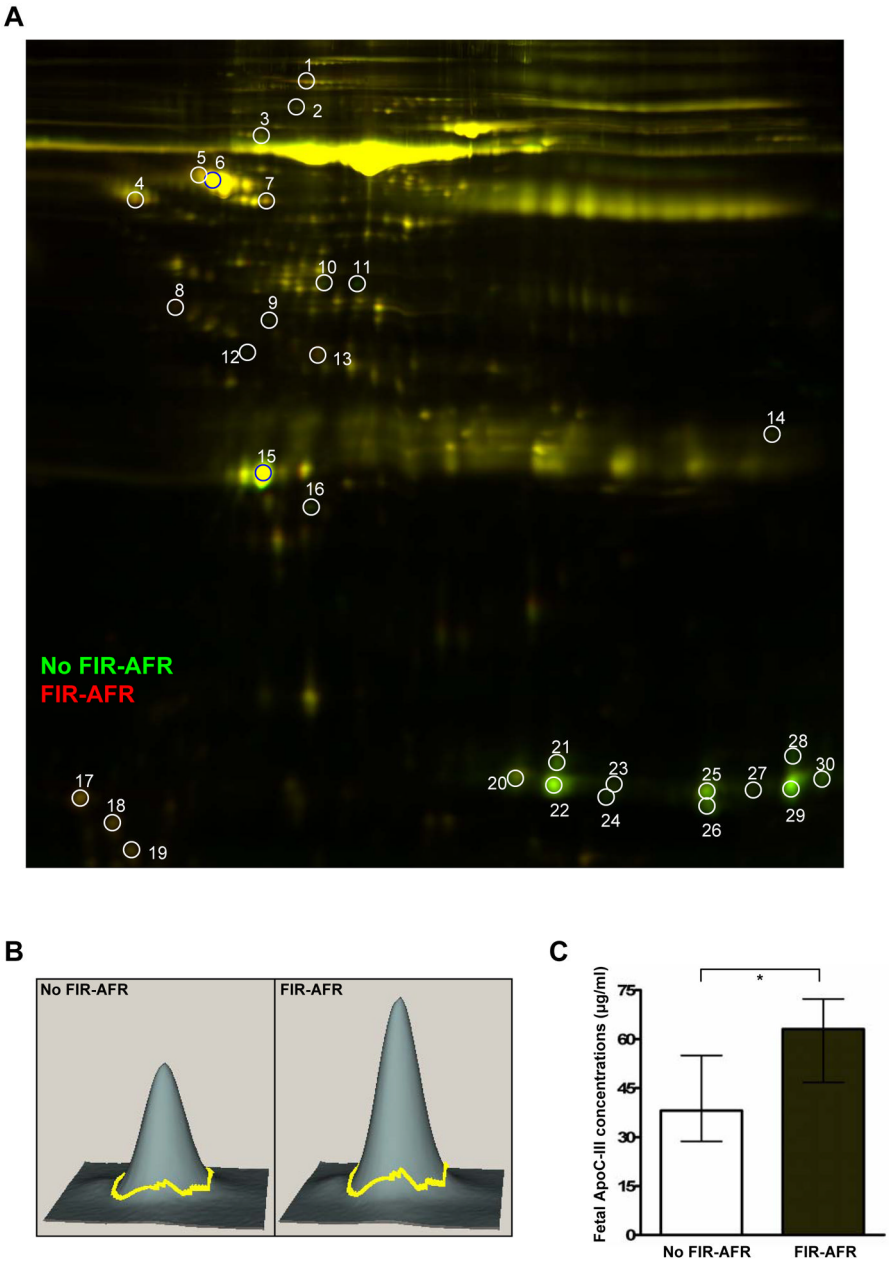

Figure 4. Comparison of the fetal blood proteome between cases with and without fetal inflammatory response associated with maternal anti-fetal rejection

(A) Two-dimensional difference gel electrophoresis (2D-DIGE) images show differentially abundant proteins between cases with (Cy5: red) and without (Cy3: green) Fetal inflammatory response associated with maternal anti-fetal rejection. The spots are labeled by number. (B) Three-dimensional images from DeCyder software analysis for spot 17 marked in the 2D-DIGE image (A), which is increased in cases with fetal inflammatory response associated with maternal anti-fetal rejection. The spot was identified as apolipoprotein C-III by MALDI-TOF-MS/MS. (C) The difference in apolipoprotein C-III concentration in fetal serum samples between the cases with and without fetal inflammatory response associated with maternal anti-fetal rejection was confirmed by specific immunoassay (P=0.013). *P<0.05 (by the Mann-Whitney U test)

ApoC-III, Apolipoprotein C-III; *FIR-AFR*, fetal inflammatory response syndrome associated with maternal anti-fetal rejection.

Table I

Demographics and clinical characteristics of the study population

	Term delivery	Spontaneous preterm delivery	
	***n*=150**	***n*=150**	
Maternal age (year)*	27 (17–43)	25 (15–44)	NS
Gestational age at delivery (weeks)*	39.5 (37.0–41.6)	34.8 (22.9–36.9)	<0.001
Birth weight (g)*	3440 (2650–4110)	2460 (530–3900)	<0.001
Baby gender (male, %)	54.7 (82/150)	66.7 (100/150)	0.033
Cesarean delivery (%)	50.0 (75/150)	22.0 (33/150)	<0.001
Primigravida (%)	16.0 (24/150)	38.7 (58/150)	<0.001
Nullipara (%)	18.0 (27/150)	42.7 (64/150)	<0.001
Cellular rejection			
CCA (%)	14.0 (21/150)	44.0 (66/150)	<0.001
VUE (%)	16.7 (25/150)	22.0 (33/150)	NS
CDP (%)	14.7 (22/150)	26.0 (39/150)	0.015
One or more of CCA/VUE/CDP (%)	32.0 (48/150)	56.0 (84/150)	<0.001
Severity of chronic inflammation (%)			<0.001
None of CCA/VUE/CDP (%)	68.0 (102/150)	44.0 (66/150)	
One of CCA/VUE/CDP (%)	22.7 (34/150)	28.0 (42/150)	
Two of CCA/VUE/CDP (%)	5.3 (8/150)	20.0 (30/150)	
All of CCA/VUE/CDP (%)	4.0 (6/150)	8.0 (12/150)	
Humoral rejection			
Maternal HLA class I PRA positive (%)†	32.0 (48/150)	50.0 (75/150)	0.002
Maternal HLA class II PRA positive (%)†	18.0 (27/150)	18.7 (28/150)	NS
Fetal HLA class I PRA positive (%)†	18.0 (27/150)	22.7 (34/150)	NS
Fetal HLA class II PRA positive (%)†	8.7 (13/150)	2.7 (4/150)	0.025

*Median (range).

†Positive HLA PRA is defined as 10% or more of reactivity of HLA panel-reactive antibodies.

ACA, acute chorioamnionitis; *CCA*, chronic chorioamnionitis; *CDP*, chronic deciduitis with plasma cells; *HLA*, human leukocyte antigen; *NS*, not significant; *PRA*, panel-reactive antibodies; *VUE*, villitis of unknown etiology.

Table II

Top 25 each of up- and down-regulated genes in fetal inflammatory response associated with maternal anti-fetal rejection

Gene	Fold-change	*P* value	Direction
GCET2	2.02	0.0008	↑
EFEMP1	1.99	0.0071	↑
TCEA3	1.83	0.0000	↑
FCER2	1.79	0.0049	↑
FCRL5	1.75	0.0096	↑
SCARNA21	1.65	0.0090	↑
TTC39B	1.63	0.0057	↑
ID3	1.62	0.0090	↑
PPAPDC1B	1.62	0.0011	↑
GBP1	1.61	0.0019	↑
DKK3	1.59	0.0043	↑
ATPBD4	1.59	0.0033	↑
HAPLN3	1.59	0.0099	↑
AXIN2	1.59	0.0018	↑
GBP1	1.57	0.0027	↑
C6orf105	1.57	0.0023	↑
HPCAL4	1.57	0.0056	↑
NUDT9P1	1.57	0.0034	↑
FAM134B	1.56	0.0059	↑
ZNF391	1.56	0.0030	↑
GNB5	1.55	0.0019	↑
ZNF667	1.54	0.0027	↑
LOC100129902	1.54	0.0036	↑
P2RY10	1.54	0.0073	↑
SOCS1	1.53	0.0062	↑
PRTN3	7.09	0.0000	↓
AZU1	6.71	0.0002	↓
CTSG	4.58	0.0002	↓
MPO	4.35	0.0003	↓
MS4A3	4.05	0.0001	↓
RNASE3	3.96	0.0003	↓
DEFA4	3.74	0.0018	↓
TACSTD2	3.73	0.0021	↓
COL17A1	3.72	0.0008	↓
ELANE	3.58	0.0001	↓
CEACAM6	3.53	0.0005	↓

Gene	Fold-change	*P* value	Direction
TCTEX1D1	3.53	0.0004	↓
LTF	3.37	0.0059	↓
CEACAM8	3.31	0.0007	↓
ABCA13	2.95	0.0067	↓
MS4A3	2.83	0.0005	↓
SERPINB10	2.78	0.0066	↓
SLC2A5	2.72	0.0062	↓
BPI	2.68	0.0017	↓
CD34	2.60	0.0007	↓
CEBPE	2.59	0.0047	↓
MKI67	2.54	0.0070	↓
FIS	2.53	0.0077	↓
FOXM1	2.51	0.0063	↓
CKAP2L	2.45	0.0055	↓

Table III

Top biological processes enriched in fetal inflammatory response associated with maternal anti-fetal rejection

Biological process	No. Differentially Expressed Genes/No. Total Genes	*P* value	False Discovery Rate
Response to bacterium	10/145	0.0000	0.0004
Defense response to bacterium	7/57	0.0000	0.0004
Killing of cells of another organism	4/12	0.0000	0.0011
Defense response to fungus	3/8	0.0000	0.0103
M phase	12/348	0.0001	0.0103
Nuclear division	10/245	0.0001	0.0103
Mitosis	10/245	0.0001	0.0103
Response to other organism	10/251	0.0001	0.0103
M phase of mitotic cell cycle	10/252	0.0001	0.0103
Organelle fission	10/254	0.0001	0.0103
DNA replication	9/210	0.0001	0.0114
Neutrophil mediated cytotoxicity	2/3	0.0002	0.0216
Neutrophil mediated killing of symbiont cell	2/3	0.0002	0.0216
Response to fungus	3/16	0.0004	0.0304
Cell division	10/307	0.0004	0.0321
Disruption by host of symbiont cells	2/4	0.0005	0.0329
Killing by host of symbiont cells	2/4	0.0005	0.0329
Cell cycle process	14/574	0.0005	0.0352
Cell cycle phase	12/443	0.0006	0.0352
Response to biotic stimulus	10/323	0.0006	0.0352
Cell killing	4/44	0.0006	0.0352
Disruption of cells of other organism involved in symbiotic interaction	2/5	0.0008	0.0403
Killing of cells in other organism involved in symbiotic interaction	2/5	0.0008	0.0403
Mitotic spindle organization	3/22	0.0009	0.0469

Table IV

Fetal serum proteins show significant changes in cases with fetal inflammatory response associated with maternal anti-fetal rejection

Spot number	Protein name	Accession number	Molecular weight (Da)	Protein PI
2	Chain A, Crystal Structure of the Ga Module Complexed with Human Serum Albumin	gi|55669910	65178.2	5.6
3	Alpha-1-B glycoprotein [Homo sapiens]	gi|119592981	54238.6	5.6
8	Apolipoprotein J precursor [Homo sapiens]	gi|178855	48772.1	6.3
9	Apolipoprotein E precursor [Homo sapiens]	gi|4557325	36131.8	5.7
11	Chain B, Crystal Structure Of Fibrinogen Fragment D	gi|2781208	37624.7	5.8
12	Chain A, Apolipoprotein E3 22kd Fragment Lys146gln Mutant	gi|15826034	22116.5	5.4
14	C1q B-chain precursor [Homo sapiens]	gi|573114	23925.9	8.9
16	Peroxiredoxin-2 isoform a [Homo sapiens]	gi|32189392	21878.2	5.7
17	Apolipoprotein C-III [Homo sapiens]	gi|521205	10815.5	5.2
20	Chain G, Structure of Human Foetal Deoxyhaemoglobin	gi|157875419	15985.2	6.7
21	Chain B, Human Hemoglobin A Mutant Beta H63w Carbonmonoxy-Form	gi|300508775	15906.3	6.8
22	Chain G, Structure of Human Foetal Deoxyhaemoglobin	gi|157875419	15985.2	6.7
23	Hemoglobin subunit gamma-2 [Homo sapiens]	gi|6715607	16116.3	6.6
24	Chain A, Solution Structure of Human Normal Adult Hemoglobin	gi|157883730	15071.8	8.1
25	Chain A, Crystal Structure of Oxy-Human Hemoglobin Bassett at 2.15 Angstrom	gi|37928140	15072.9	9.1
26	Hemoglobin alpha-1 globin chain [Homo sapiens]	gi|319739573	10776.5	8.1
27	Chain A, Structure of Haemoglobin in the Deoxy Quaternary State with Ligand Bound at the Alpha Haem	gi|229751	15116.9	8.7
28	Chain A, Structure of Haemoglobin in the Deoxy Quaternary State with Ligand Bound at the Alpha Haem	gi|229751	15116.9	8.7
29	Hemoglobin alpha-1 globin chain [Homo sapiens]	gi|319739573	10776.5	8.1
30	Chain A, Structure of Haemoglobin in the Deoxy Quaternary State with Ligand Bound at the Alpha Haem	gi|229751	15116.9	8.7