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Pregnancy-Associated Exosomes and Their Modulation of T Cell Signaling¹

Douglas D. Taylor,² Sibel Akyol, and Cicek Gerçel-Taylor

Exosome release by viable cells is a feature of activated cell types, including tumors, fetal cells, and cells of the immune system. Exosomes critically regulate immune activation, by mediating activation-induced cell death. Fetal cells may mimic these events to selectively delete reactive lymphocytes. In this study the presence and composition of placenta-derived exosomes are demonstrated in the maternal circulation along with their consequences on T cell activation markers. For all pregnant patients, exosomes were isolated from sera obtained between 28 and 30 wk gestation. For pregnant women, subsequently delivering at term, circulating levels of placental exosomes were 1.8 times greater than those delivering preterm ($p < 0.0001$). Exosomes isolated from pregnancies subsequently delivering at term expressed significantly higher levels of biologically active components, including Fas ligand (FasL) and HLA-DR, than those from pregnancies delivering preterm. Standardizing for protein concentrations, exosomes from term-delivering pregnancies exhibited greater suppression of CD3- ζ and JAK3 than those delivering preterm. The suppression of CD3- ζ and JAK3 correlated with exosome expression levels of FasL ($r^2 = 0.92$ and $r^2 = 0.938$, respectively). Fractionation of exosomes from term-delivering pregnancies by continuously eluting electrophoresis indicated that intact 42kD FasL and an unidentified 24-kDa protein were associated with CD3- ζ suppression. Our results demonstrated that exosomes from pregnancies ultimately delivering at term are present at significantly greater concentrations than those from pregnancies delivering preterm; however, exosomes from term-delivering pregnancies also exhibit significantly greater suppression of CD3- ζ and JAK3. *The Journal of Immunology*, 2006, 176: 1534–1542.

In most developed countries, the incidence of preterm births is ~5–7% of all live births, and its incidence appears to be increasing (1, 2). However, in the United States, preterm births account for 11.9% of all live births and result in 70–80% of neonatal deaths among genetically normal neonates (3). Most preterm births follow spontaneous, unexplained preterm labor or spontaneous preterm rupture of the amniotic membranes. Although the cause of spontaneous or idiopathic preterm birth remains unclear, multiple risks factors have been identified including anemia, abruption placentas, drug use, low maternal weight, placenta previa, polyhydramnion, premature rupture of membranes, smoking stress, and infections.

The most common and most investigated risk factor for preterm birth has been infections. Approximately 30–40% of preterm deliveries have been postulated to result from or to be associated with infections (4). Recent studies of the role of infections have focused on the association of periodontal disease and preterm birth (5, 6). Current investigations have correlated periodontal disease with increased risk of preterm birth; however, only a small percentage of pregnant women with even severe periodontal disease experienced preterm delivery, and this correlation was only statistically significant in gestational ages <28 wk (7, 8) in a prospective study of

3738 women from the United Kingdom addressing all preterm births, there was no correlation between periodontal disease and preterm birth or low birth weight (7). Recent studies have focused on the alternative hypothesis that suggests a genetically defined abnormal immune response may be a primary etiology of preterm birth (9, 10). A correlation was demonstrated between preterm birth and polymorphisms in cytokine genes (9). Polymorphisms in TNF- α , IL-1 β , and IL-6 have been the most consistently linked alterations in preterm birth (9, 10). In certain populations, maternal polymorphisms in TLRs, primarily TLR-4, have also been associated with preterm birth (10). Thus, rather than a causative factor, the presence of periodontal disease might reflect aberrant immunoregulation within a subpopulation of pregnant patients. Together, these data suggest that aberrant immune regulation exists in a subpopulation of pregnant women that could lead to preterm labor and birth, and such aberrations might also increase the risk of infections, including the development of periodontal disease.

Previous studies of the maternal immunophenotypic profile in normal term pregnancies and pregnancies delivering preterm demonstrated that the suppressive immunophenotypic profiles observed in term pregnancies were absent in preterm births (11–13). Successful pregnancy is associated with decreased cell-mediated responses and an increased rate of Ab production, suggesting an elevated Th2/Th1 immune response (14, 15). A key pathway mediating a state of immune tolerance is activation-induced cell death (AICD)³ (16, 17). Lymphoid cells appear to release membrane vesicles or exosomes after activation, and these appear to play an essential role in immunoregulation, preventing excessive immune responses and the development of autoimmunity (18, 19). It was

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³ Abbreviations used in this paper: AICD, activation-induced cell death; FasL, Fas ligand; IDO, indoleamine 2,3-dioxygenase; PLAP, placental-type alkaline phosphatase; TSG, tumor susceptibility gene.

postulated that microvesicle shedding by tumor cells was a reexpression of the fetal cell shedding, and that both constituted pathways to circumvent immunosurveillance (20, 21).

The induction of anergy at the maternal-fetal interface may be the result of reduced expression and altered function of intracellular signaling molecules within T lymphoid populations (22). One pathway linked to reduced lymphocyte proliferation after antigenic challenge and reduced cytokine production is the decreased expression of the signal-transducing molecule, CD3- ζ (21, 23). Suppression of the signal-transducing ζ -chain has been observed in both decidual T cells and peripheral blood T lymphocytes of pregnant women (23). It has been postulated that the fetoplacental unit redirects maternal immunity away from cell-mediated immunity to favor humoral immunity by secreting Th2 cytokines while downregulating Th1 cytokines (24). One mechanism capable of producing decreased T cell proliferation and cytokine production is the decreased expression of the ζ -chain. The ζ -chain plays a critical role in determining the TCR repertoire *in vivo*, with the negative selection of specific reactive lymphocyte populations within the decidua occurring by apoptosis. A key regulatory link between CD3- ζ expression and apoptosis is the expression and activation of JAK3, which also serves to activate signaling by many cytokine receptors (25, 26).

This study investigates the suppression of CD3- ζ and JAK3 as contributors to the maternal immunosuppression associated with pregnancy success. The importance of the components examined in this study is that they transcend the Th1/Th2 paradigm. Modulation of the JAK3 pathway is critical for specific type 1 cytokine receptors, which possess common γ -chains and can modulate CD3- ζ -mediated activation, leading to T cell anergy. We propose that the loss of signal-transducing molecule suppression results in a failure to impair maternal lymphoid activation, leading to spontaneous preterm labor and birth.

Materials and Methods

Patient-derived materials

Blood specimens (10 ml) were obtained from women who had never been pregnant ($n = 21$; designated group 3, control), from pregnant women ultimately delivering without complications at term (≥ 37 wk gestation; $n = 27$; group 1), and from pregnant women delivering preterm (≤ 34 wk gestation; $n = 19$; group 2). Samples were obtained under an informed consent protocol approved by the institutional review board at University of Louisville. For group 1, venous blood samples were obtained at routine visits from asymptomatic pregnant women who were 28–30 wk estimated gestation at the time of the blood draw, and serum was isolated after clotting. For group 2, blood specimens were obtained from women with threatened or imminent preterm labor (regular uterine contractions occurring with a minimum frequency of two every 10 min and associated changes in either cervical effacement or dilation) in wk 28–37 of gestation and were included if they proceeded to deliver preterm. For group 3, blood samples were obtained from healthy nonpregnant female volunteers in private gynecology offices. Serum samples were stored at -70°C until use, and for pregnant patients, their delivery outcome was determined. All women included in this study were free of obvious infections within the uterus or amniotic cavity, and all pregnancies were singletons. Clinical variables potentially affecting neonatal outcome were abstracted from the maternal and infant medical records, including indications for delivery, estimated gestational age, use of intrapartum antibiotics, use of antenatal steroids, and maternal demographics. The mean age for pregnant women in group 1 was 26.4 ± 6.5 years; for group 2, the mean age was 24.1 ± 5.7 years; and for group 3 (control nonpregnant women), the mean age was 26.1 ± 5.3 years.

Isolation of circulating exosomes

Using sera from the three groups, exosomes were isolated by a two-step procedure using size exclusion chromatography and density gradient centrifugation. Serum samples (2.5 ml) were applied to a Sepharose 2B column (2.5×16 cm) and eluted with PBS, and 2-ml fractions were collected, monitoring elution at 280 nm. The void volume fractions were pooled and centrifuged at $100,000 \times g$ for 1 h. Flootation of exosomes on a discon-

tinuous sucrose gradient was then performed at 4°C as previously described (27, 28). Exosomes were then centrifuged at $100,000 \times g$ for 1 h at 4°C , exosomes were resuspended in PBS, and the quantity of protein was determined by the Bradford microassay method (Bio-Rad) using BSA as a standard.

To examine the distributions of proteins in exosomes, SDS-PAGE using an 8–15% acrylamide gel was performed (29), followed by silver staining (Bio-Rad). The stained gels were analyzed by a 1D Image Analysis software (Eastman Kodak).

Western immunoblots analyses of exosome protein expression

Western immunoblotting was performed to analyze the presence of specific proteins, including placental-type alkaline phosphatase (PLAP), tumor susceptibility gene 101 (TSG101), and Fas ligand (FasL). Proteins from each membrane vesicle isolate (25 $\mu\text{g}/\text{lane}$) were applied on a 4–20% SDS-PAGE gel. The proteins were electrophoretically separated by the method of Laemmli (29) and analyzed by Western immunoblot as previously described (30), probing overnight at 4°C with rabbit polyclonal anti-FasL (1 $\mu\text{g}/\text{ml}$; clone G247-4; Calbiochem), rabbit polyclonal anti-PLAP (1 $\mu\text{g}/\text{ml}$; Abcam), mouse monoclonal anti-TSG101 (1 $\mu\text{g}/\text{ml}$; Abcam), rabbit polyclonal anti-furin (1 $\mu\text{g}/\text{ml}$; Santa Cruz Biotechnology), mouse monoclonal anti-HLA-G (1 $\mu\text{g}/\text{ml}$; Santa Cruz Biotechnology), or rabbit polyclonal anti-HLA-DR (1 $\mu\text{g}/\text{ml}$; Santa Cruz Biotechnology), as the primary Abs and peroxidase-conjugated anti-rabbit, -goat, or -mouse Ig as the secondary Ab. The bound immune complexes were visualized by ECL (Amersham Bioscience) and quantitated by densitometry (Un-Scan-it software; Silk Scientific).

Expression of signaling proteins, CD3- ζ and JAK3

Jurkat E-61 cells, a human T cell lymphoma, was obtained from American Type Culture Collection. These cells were used as an *in vitro* assay for lymphocyte modulation by serum-derived exosomes. This T cell line was grown in RPMI 1640 medium supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 200 mM L-glutamate, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 IU/ml penicillin in a humidified 5% CO_2 chamber at 37°C . Cell viability was evaluated by trypan blue exclusion. All cultures used for this study were $>95\%$ viable.

For bioassay of CD3- ζ expression, viable Jurkat cells (10^6 cells/ml) were incubated in a medium supplemented with 400 $\mu\text{g}/\text{ml}$ isolated exosomes for 48 h and were compared with unexposed Jurkat cells or Jurkat cells exposed to the analogous gradient fractions from control sera. After 2 days, the cells were centrifuged, and the cell pellet was washed and used for protein analysis. To assess CD3- ζ protein, the cell pellet was lysed using 50 mM HEPES (pH 7.2), 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 2.5% Triton X-100, 200 $\mu\text{g}/\text{ml}$ trypsin/chymotrypsin inhibitor, 200 $\mu\text{g}/\text{ml}$ chymostatin, and 2 mM PMSF. The cell lysate was assayed for protein by the Bio-Rad protein assay. The modulation of signaling proteins was analyzed by Western immunoblot using a 15% SDS-PAGE gel, as described above, with mouse monoclonal anti-CD3- ζ and mouse anti-JAK3 Abs (Santa Cruz Biotechnology) as the primary Abs. As an additional loading control, blots were probed using rabbit polyclonal anti- β -actin (Santa Cruz Biotechnology).

Induction of IL2 production

To assess the role of pregnancy-associated exosomes on the suppression of T cell function, Jurkat cells (10^6 cells/ml) were incubated with or without 1 $\mu\text{g}/\text{ml}$ PHA and 1 ng/ml PMA in the presence of medium alone; medium with 100, 200, and 400 $\mu\text{g}/\text{ml}$ isolated exosomes from normal term pregnancies ($n = 8$) or from pregnancies delivering preterm ($n = 8$); or analogous fractions from nonpregnant controls ($n = 8$) for 48 h in 96-well, flat-bottom microtiter plates. After 48 h, the plates were centrifuged, and 100 μl of medium was removed from triplicate wells for analysis of induced IL-2 production using a commercial ELISA kit to assay IL-2 levels (Chemicon International). A standard curve was generated according to the manufacturer's instructions, and samples were measured by spectrophotometry at 490 nm. For each sample, the mean and SD were calculated from duplicate assays of triplicate wells.

Isolation of exosome-associated inhibitory components

Using a continuously eluting electrophoresis system (model 490; Bio-Rad), the components of exosomes mediating suppression of CD3- ζ were analyzed. Exosomes (500 μg each) from two group 1 pregnant patients were applied to 12.5% acrylamide preparative column gels, and each gel was run

at 100 V. Fractions (1 ml) were eluted from the bottom of the gel, monitoring at 280 nm. Proteins from aliquots (200 μ l) of each protein-containing fraction were precipitated using a two-dimensional cleanup kit to remove SDS (Amersham Biosciences). The precipitated proteins were resuspended in PBS by sonication and assayed for ζ suppression in the Jurkat cell bioassay. Fractions from the peaks exhibiting ζ suppression were pooled, concentrated, and analyzed by SDS-PAGE on a 12.5% gel with silver staining and subsequently by Western immunoblotting with anti-FasL Ab (1 μ g/ml; clone G247-4; Santa Cruz Biotechnology).

Modulation of CD3- ζ suppression by Fas blockage

The exosome modulation of CD3- ζ expression was performed as described above, except to assess the role of FasL in mediating this effect, cultures of Jurkat cells (2×10^5 cells/ml) were initially incubated with anti-Fas neutralizing mAb, ZB4 (10 μ g/ml), or IgG1 isotype control (10 μ g/ml; Upstate Biotechnology), followed by medium alone or treatment with 200 μ g/ml group 1 exosomes (term pregnancies; $n = 5$) for 48 h. Cd3- ζ expression was analyzed as previously described, and expression was quantified by densitometry.

Statistical analysis

Western blot analyses of TSG101, HLA-G and -DR, PLAP, FasL, CD3- ζ , and JAK3 were performed at least twice. Densitometric quantitation of bands on each gel was standardized to a control lane included on that gel and compared by the Kruskal-Wallis test. Correlations between the level of FasL on the exosomes (defined as pixels) and their suppression of CD3- ζ and JAK3 in the Jurkat bioassay were calculated by linear regression analysis. In the remainder of the experimental data, all relative absorbance determinations were performed at least twice, and the mean \pm SEM for each sample were calculated. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using Instat (GraphPad).

Results

Exosomes from sera of pregnant patients associated with preterm and term births

In the two-step isolation procedure, serum samples were applied to a Sepharose 2B column, monitoring elution at 280 nm (see Fig. 1 for representative profiles). The fractions labeled Exos represent the >50 million-Da fractions, void volume containing exosomes. These fractions were then subjected to density gradient centrifugation using floatation of membranes on a discontinuous sucrose gradient performed at 4°C. The level of exosomal protein in sera from group 1 pregnant patients was 1.92 ± 0.69 mg/ml initial serum, whereas exosomes from group 2 (delivering preterm) consisted of

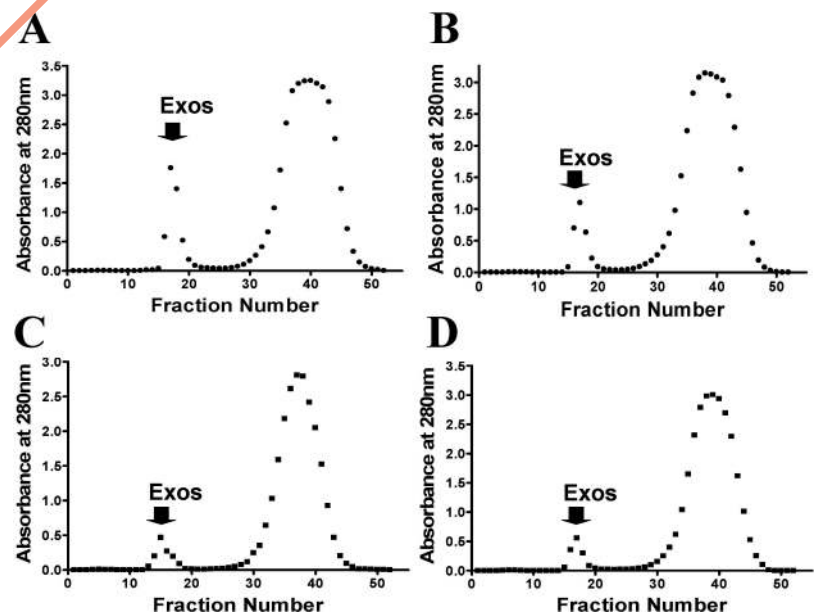
1.05 ± 0.34 mg/ml protein ($p < 0.001$). In sera from group 3 (nonpregnant controls), exosomes were detected at <0.25 mg/ml.

To compare the distributions of proteins in exosomes isolated from group 1 vs group 2, SDS-PAGE analysis was performed. After standardizing for protein content, the exosomes isolated from both groups 1 and 2 were electrophoretically separated, and the protein compositions were compared (Fig. 2 presents representative samples). Based on general protein composition, although group 2 patients exhibited decreased quantities of released exosomes, both groups appeared to release similar components qualitatively. Exosomes derived from group 3 sera exhibited no discrete bands on SDS-PAGE.

Comparison of exosome compositions

Despite the similarity observed in general protein staining, to determine whether differences existed in specific protein components between exosomes isolated from groups 1 and 2, Western immunoblot analysis of the isolated exosomes was performed (Fig. 3). Because the expression of TSG101 has been demonstrated as a marker of exosomes, its presence on the exosomes from both pregnant groups was analyzed. TSG101 was present in all exosomes preparations, and although individual differences were seen, based on densitometric analysis, no significant differences in the expression of TSG101 between groups 1 and 2 were observed ($69,614 \pm 5,264$ vs $69,636 \pm 10,420$ pixels; $p = 0.997$; Fig. 3A). To define the origin of these exosomes, exosome-associated PLAP, a plasma membrane enzyme isoform specifically produced by the placenta, was also analyzed (Fig. 3B). All exosome isolates stained positively for PLAP, confirming the placental origin of these membranous materials, and based on densitometric analyses of all samples, no significant differences were observed between PLAP expression on exosomes isolated from groups 1 and 2 ($129,776 \pm 19,770$ vs $111,275 \pm 9,577$ pixels; $p = 0.105$). Previous work demonstrated the presence of proteolytic activities associated with exosomes, including matrix metalloproteinases, and the presence of the proteolytic convertase, furin, was examined on exosomes (Fig. 3C). The expression of furin was significantly increased in exosomes isolated from group 2 compared with those from group 1 ($110,649 \pm 29,870$ vs $28,598 \pm 17,583$ pixels; $p = 0.0013$). Because placental expression of FasL appears to play an important

FIGURE 1. Representative chromatographic profiles of sera from pregnant women subsequently delivering at term (A and B) and preterm (C and D). Serum samples (1 ml) were applied to a Sepharose 2B column (2.5 \times 16 cm), eluted with PBS, and 2-ml fractions were collected, monitoring elution at 280 nm. The fractions labeled Exos represent those containing exosomes.



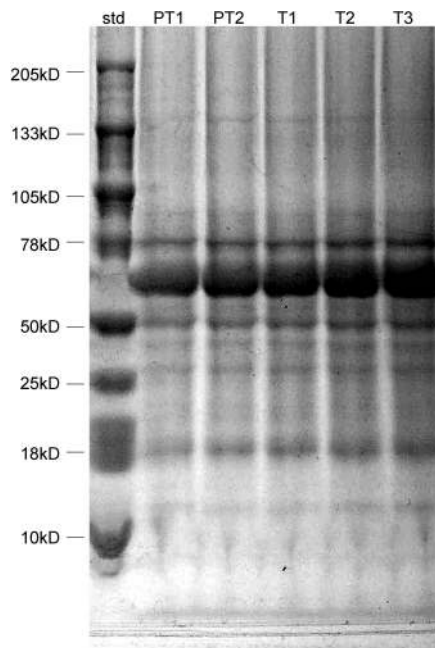


FIGURE 2. Representative SDS-PAGE of exosomes derived from the sera of pregnant women from group 1 (subsequently delivering at term T1-T3) or group 2 (delivering preterm, PT1-2). Exosome protein concentrations were standardized, and 40 μ g of protein was loaded into each well. Exosome-associated proteins were visualized using Imperial purple stain.

role in establishing an immune-privileged site, exosomes were assessed for the expression of FasL (Fig. 3D). Although all group 1 samples were positive for the intact 42-kDa form and the 27-kDa cleaved form of FasL, the exosomes isolated from group 2 were negative or only weakly positive for either form of FasL. The expression of intact 42-kDa FasL on group 1-derived exosomes was $91,569 \pm 18,892$ pixels. The analogous gradient fractions from group 3 (controls) were analyzed for the presence of TSG101, PLAP, furin, and FasL, and were found to be negative (data not shown).

Association of histocompatibility Ags with circulating exosomes

Previous proteomic analysis of various exosome populations indicated their expression of major histocompatibility Ags, both class I and II. The expression of class I (HLA-G) and class II (HLA-DR α) MHC components on exosomes isolated from groups 1 and 2 was analyzed by Western immunoblot (Fig. 4). Exosomes appeared to express both class I and II components. No significant differences were observed in the expression of HLA-G between exosomes from groups 1 and 2 (Fig. 4A; $121,469 \pm 24,632$ vs $106,613 \pm 23,413$ pixels; $p = 0.184$). Variable expression of HLA-DR α was observed; however, exosomes isolated from group 1 exhibited 6.9-fold higher levels of HLA-DR α than group 2 (Fig. 4B; $87,529 \pm 12,143$ vs $12,596 \pm 4,289$ pixels; $p < 0.0001$).

Modulation of CD3- ζ and JAK3 expression by pregnancy-derived exosomes

This study addressed the consequences of exosome expression of biologically active molecules, such as FasL. The expressions of CD3- ζ and JAK 3 proteins were determined by Western immunoblot. When group 1-derived exosomes were incubated with T cells (Jurkat cells) for 48 h, suppressions of both CD3- ζ (Fig. 5A) and JAK 3 (Fig. 5B) proteins were observed. When Jurkat cells were treated with group 1-derived exosomes, CD3- ζ expression was only $27.8 \pm 10.7\%$ of the untreated control value, whereas CD3- ζ

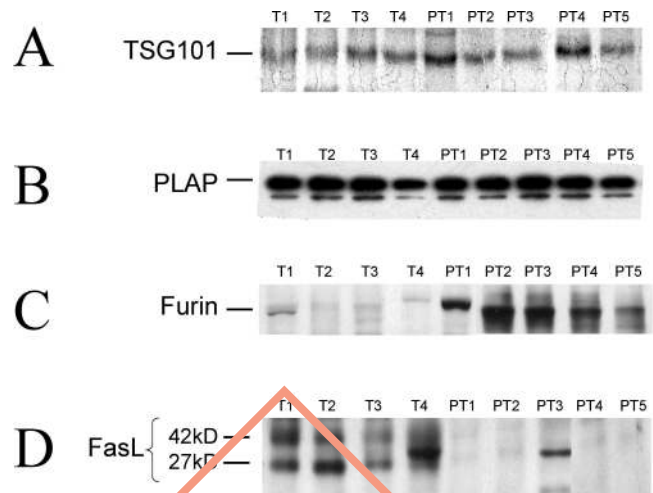


FIGURE 3. Representative Western immunoblots demonstrating the presence of TSG101 (A; marker of exosomes), placental-type alkaline phosphatase (B; marker of placental origin), furin (C; prohormone convertase family), and FasL (D) on isolated exosomes. Exosomes were isolated from the sera of pregnant patients from group 1 (subsequently delivering at term, T1-T4) or group 2 (delivering preterm, PT1-5).

expression by Jurkat cells exposed to group 2 exosomes was $32.8 \pm 18.2\%$ of the untreated control value. JAK 3 expression was decreased in Jurkat cells incubated with group 1-derived exosomes ($39.2 \pm 12.2\%$ of untreated control value), whereas JAK3 expression of group 2-derived exosomes treated Jurkat cells was $76.8 \pm 21.3\%$ of the untreated control value. Jurkat cells treated with the analogous control material from group 3 failed to significantly alter either CD3- ζ or JAK3 expression.

Because FasL has been postulated to play a critical role in the modulation of T cell activation, the expression of FasL on exosomes isolated from group 1 was correlated with Jurkat expression of both CD3- ζ and JAK3. Plotting exosome-FasL expression against Jurkat CD3- ζ after exposure to the same exosome preparation produced a correlation coefficient (r^2) of 0.92, whereas comparison with FasL levels with JAK3 expression yielded a correlation (r^2) of 0.938 (Fig. 6).

Suppression of IL-2 production

Because during normal pregnancy decreased production of Th1 cytokines is observed, the effects of group 1 and 2 serum-derived exosomes or the analogous material isolated from group 3 non-pregnant controls were addressed on T cell production of IL-2. IL-2 production was induced in Jurkat cells (10^6 cells/ml) by incubation with 1 μ g/ml PHA and 1 ng/ml PMA for 48 h. When material from group 3 female controls was added to the T cells during activation, IL-2 secretion was not significantly inhibited compared with the medium control at any of the concentrations assayed ($p > 0.05$). The induction of IL-2 secretion was slightly suppressed by material from preterm group 2 compared with the medium control ($p = 0.048$ at 200 mg/ml and $p < 0.01$ at 400 mg/ml). In contrast, exosomes isolated from group 1 (delivering at term without complications) exhibited significant differences at all concentrations ($p < 0.001$) from that of the medium or nonpregnant control groups (Fig. 7).

Exosome-associated inhibitory components

Two exosome preparations from group 1 pregnancies were fractionated by continuously eluting electrophoresis. Each of the eluted fractions of exosome components was analyzed for suppression of CD3- ζ in the Jurkat bioassay (Fig. 8A shows patient T1 as

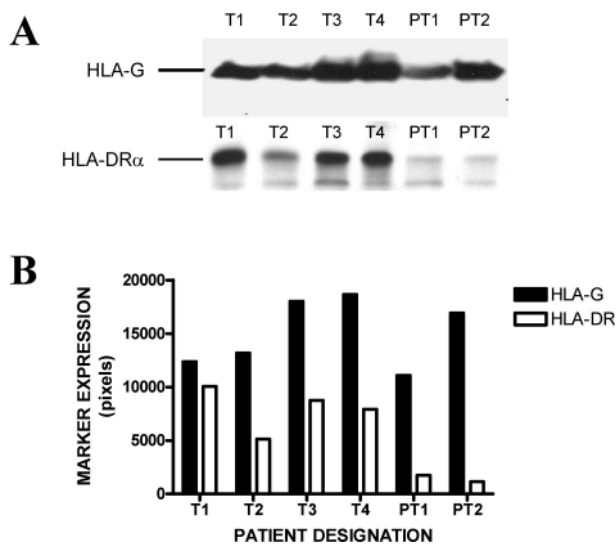


FIGURE 4. A, Representative Western immunoblots demonstrating the presence of class I MHC (HLA-G) and class II MHC components (HLA-DR α) on isolated exosomes from the sera of pregnant women from group 1 (T1-T3) or group 2 (PT1-2). B, Densitometric data comparing class I and II Ags.

representative). For both patient exosome preparations, two peaks of CD3- ζ suppressive activity were observed at fractions 75/76 and 86/87. For both patients, these four fractions (75, 76, 86, and 87) were pooled and concentrated, and the components were visualized by silver staining on standard SDS-PAGE gels. Two major components were observed; one component appeared at 24 kDa, and the second at 42 kDa (Fig. 8B). Separate aliquots of these pooled fractions were analyzed by Western immunoblotting with anti-FasL Ab, because it has been implicated in the suppression of ζ expression and exhibits M_r forms at these M_r ranges. By Western immunoblotting, only the 42 kDa component stained positively for FasL (Fig. 8C). Because the anti-FasL Ab used recognizes an epitope present in the intact and cleaved forms, the absence of reactivity indicated that the 24-kDa protein is not FasL.

Blocking of FasL activity associated with exosomes

To determine whether exosome-associated FasL is responsible for the suppression of Jurkat T cell CD3- ζ expression, blockage of exosome-induced effects was analyzed in these cells using anti-Fas (ZB4) Ab (Fig. 9). Digitization of Western immunoblot data indicated that exposure of Jurkat cells to normal pregnancy-derived exosomes induced a $53.5 \pm 11.2\%$ suppression of CD3- ζ , whereas CD3- ζ expression in Jurkat cells incubated with the Fas-blocking Ab (ZB4) exhibited only a $23.4 \pm 9.6\%$ reduction ($p = 0.0015$ for paired samples with and without ZB4). Although this represents a significant reversal of exosome-induced apoptosis, it is not a complete reversal of the exosome effect.

Discussion

Exosomes appear to play a central role in communication between lymphocytes and dendritic cells, mediating the development of cellular immune responses or suppressing excessive activation, as in AICD (16-19). The phenomenon of shedding exosomes has also been demonstrated with other hemopoietic cells, such as mast cells and platelets, and it is thought to play an important role in mediating their actions (16, 18, 27, 28, 31, 32). Recent studies have isolated exosomes from tumor cells in vivo and in vitro and have demonstrated the presence of tumor-associated Ags and class I

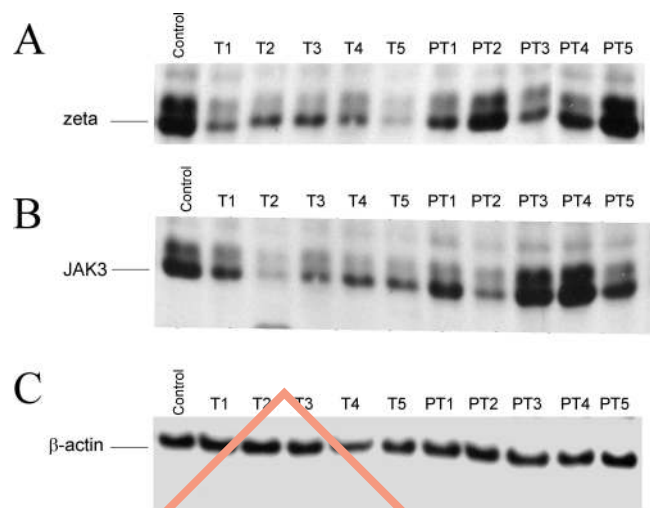


FIGURE 5. Western immunoblots indicating the expression of CD3 ζ (A) and JAK3 (B) by Jurkat cells after a 4-day incubation with exosomes (at 200 μ g/ml) obtained from pregnant women from group 1 (subsequently delivering at term, T1-T5) or group 2 (delivering preterm, PT1-5). C, β -Actin staining of the same samples as the loading control.

MHC, suggesting that tumor-derived exosomes can serve as direct vaccines or processed by autologous dendritic cells (33-35).

In a recent report (36), cytoplasmic microvesicular forms of FasL were demonstrated in syncytiotrophoblasts, and although not able to demonstrate release, these investigators postulated the release of these intracellular microvesicles in the form of exosomes. Their hypothesis was supported by the demonstration of secreted full-length, biologically active FasL in microvesicular form in first and third trimester pregnancies (21, 37). Although not specifically assessed, these previous studies proposed that exosome-associated FasL could promote an immune-privileged state. In the current report, the presence, composition, and biologic activities of exosomes circulating in pregnant women were analyzed, and their consequences on T cell activation signaling pathways were directly examined.

Significant differences in maternal immunity, in terms of lymphoid cell numbers, subset distributions, and functions, can be demonstrated between pregnancies delivering preterm and those ultimately delivering at term. Investigations have implicated placenta-derived exosomes as a key component in establishing the placenta as an immune-privileged site (36, 37). These past studies have not addressed qualitative, quantitative, or functional characteristics of placental exosomes in normal term pregnancies or in pregnancy complications. In the current study, although exosomes could be demonstrated in all pregnant patients tested, significant quantitative differences in the circulating levels of exosomes (Fig. 1) were observed in specimens from group 1 (women subsequently delivering at term; 1.92 ± 0.69 mg/ml) compared with group 2 (women delivering preterm; 1.05 ± 0.34 mg/ml). Although exosome release can be demonstrated in activated lymphocytes and dendritic cells in vitro, the in vivo accumulation of exosomes from volunteers without pathologic conditions is difficult to demonstrate. Under our experimental conditions, the level of circulating exosomes in controls (group 3) was <0.25 mg/ml, which was approximately background. Although this fraction was used as control material, vesicular components could not be visualized by electron microscopy, and discrete protein bands could not be demonstrated on SDS-PAGE.

Although the quantitative differences in exosomes might be sufficient to cause the differences observed in T cell numbers and

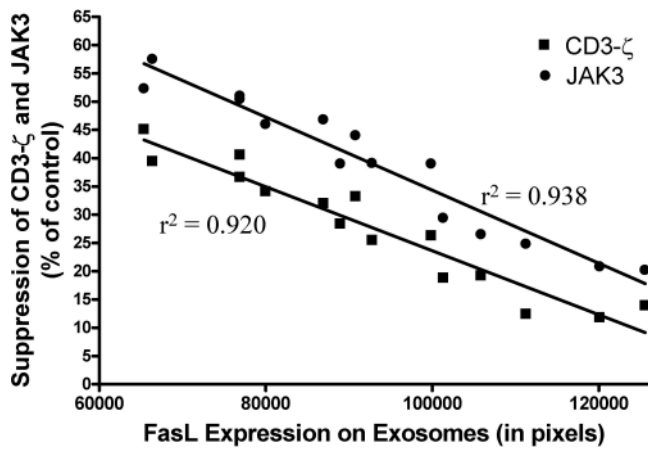


FIGURE 6. Effects of exosomal FasL on expression of ζ and JAK3 in Jurkat cells. A linear, but reverse, correlation between the FasL content of exosomes obtained from group 1 patients ($n = 15$) and expressions of CD3- ζ and JAK3 in Jurkat cells coincubated with these exosomes for 48 h. The FasL content of exosomes and JAK3 and CD3- ζ of Jurkat cells was determined by semiquantitative densitometry as described in *Materials and Methods*.

activation between pregnancies delivering preterm and at term, the presence of qualitative differences was also examined by general protein staining. Based on general protein composition, both populations of exosomes appear to be similar qualitatively; however, this general approach only identifies major protein components. A more detailed assessment of specific components by Western immunoblotting revealed significant differences in specific protein components. An important point of debate is whether the released microvesicles are derived from the plasma membrane or secretory lysosomes. Although many of the components are shared between lysosomes/endosomes and plasma membranes, TSG101, also termed vesicular protein sorting 23, is a marker of endosomes within cells and exosomes extracellularly. In all exosome preparations from our pregnant patients, TSG101 was detected, and no significant differences in the expression between all term and preterm pregnancy patients were observed (Fig. 3A). Because many

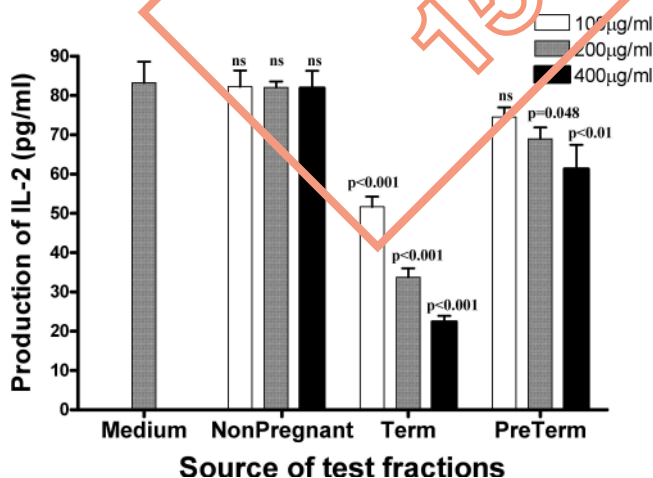


FIGURE 7. Induced IL-2 levels in Jurkat cells after incubation with 100, 200, and 400 $\mu\text{g/ml}$ pregnancy-derived exosomes from pregnant women of group 1 (subsequently delivering at term) and group 2 (delivering preterm) or analogous chromatographic fractions from nonpregnant women. Bars present the mean \pm SD of all patient samples. Differences in IL-2 induction from Jurkat cells coincubated with medium alone are indicated on the graph. ns, not significant.

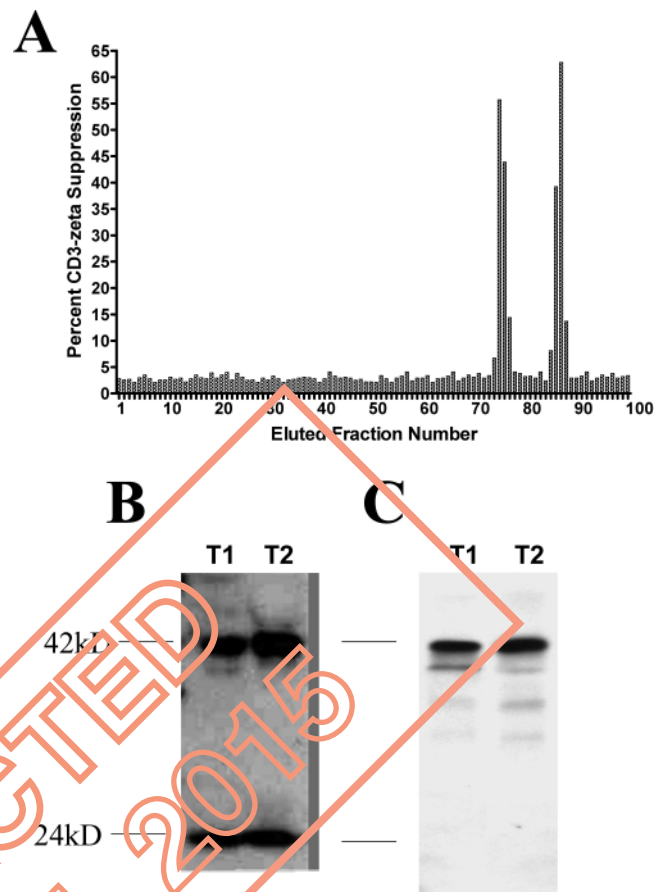


FIGURE 8. Identification of the exosome-associated components inhibiting CD3- ζ expression. Exosomes, isolated from two patients from group 1, were fractionated by continuously eluting electrophoresis, and each fraction was subsequently assayed for ζ suppression in the Jurkat bioassay (A). ζ suppressive fractions (75/76 and 86/87) were pooled, concentrated, and examined by SDS-PAGE with silver staining (B) and by Western immunoblotting with anti-FasL Ab (C). Molecular mass estimates were calculated based on prestained standards run simultaneously.

cell types can release exosomes, the placental origin of these exosomes was defined by the presence of PLAP, a plasma membrane enzyme isoform specifically produced by the placenta. As observed with TSG101 expression, all exosome isolates stained positively for PLAP, confirming the placental origin of these membranous materials, and no significant differences were observed between exosomes isolated from group 1 (subsequently delivering at term) and group 2 (delivering preterm) pregnant patients (Fig. 3B).

In addition to markers of tissue origin and subcellular source, exosomes have been demonstrated to express biologically active components. Previous work with cancer-derived exosomes demonstrated the presence of proteolytic activities associated with exosomes, including matrix metalloproteinases (38). Extracellular matrix homeostasis is critical in maintaining the tensile strength of the amniochorion. Matrix metalloproteinase-9 is the key mediator of the membrane rupture process by degrading the extracellular matrix of the chorioamniotic membranes. Furin is an activator of matrix metalloproteinases, in addition to other prohormones. Furin expression was significantly elevated in exosomes from group 2 preterm pregnancies compared with group 1-derived exosomes (Fig. 3C).

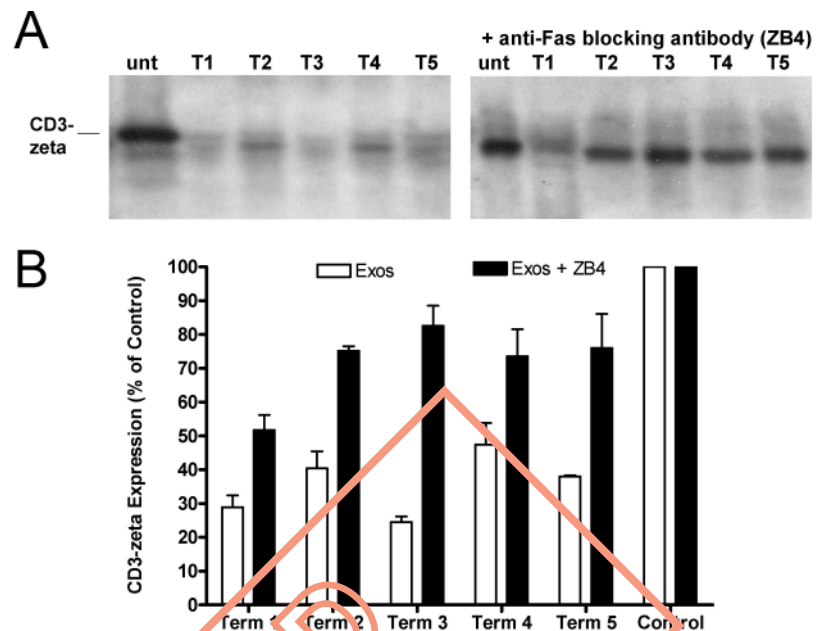


FIGURE 9. Reversal of exosome-induced CD3- ζ expression by blockage of Jurkat-expressed Fas. **A**, Western immunoblots indicating the expression of CD3 ζ by Jurkat cells after a 2-day incubation with exosomes (at 200 $\mu\text{g}/\text{ml}$) obtained from pregnant women from group 1 (subsequently delivering at term; T1-T5) in the presence or the absence of anti-Fas ZB4 Ab. **B**, Densitometric data from the Western immunoblots compared CD3- ζ expression in the presence and absence of Fas-blocking Ab.

Fas signaling is a major mechanism for inducing peripheral clonal deletion of lymphoid cells (39). Using an anterior chamber of the eye model to study induction of systemic tolerance, Griffith et al. (39) demonstrated that apoptosis of T cells via Fas/FasL was essential for induction of immune deviation leading to decreased delayed-type hypersensitivity and IFN- γ production, whereas mechanisms resulting in necrosis failed to elicit tolerance. In pregnancy models addressing maternal responses to fetal H-Y expressed in male fetuses, Jacobitz and Hodes (40) demonstrated that maternal Fas interactions with fetal FasL was “necessary and sufficient for both clonal deletion and induction of hyporesponsiveness of H-Y-reactive CD8⁺ T cells during pregnancy.” All exosomes derived from the peripheral circulation of group 1 (delivering at term) samples were positive for the intact 42- and the 27-kDa cleaved forms of FasL (Fig. 3D). In contrast, exosomes from group 2 were weakly positive or negative for both forms of FasL. Although control sera failed to exhibit exosomes, the analogous gradient fractions were analyzed for the presence of FasL and were found to be negative (data not shown). This absence of exosomal FasL in preterm birth is consistent with general immunologic differences observed between these two pregnancy groups.

Proteomic analysis of various exosome populations have indicated their expression of major histocompatibility Ags, both class I and II, by fetal tissues. Exosomes appeared to express both class I and II components (Fig. 3). No significant differences were observed in the expression of HLA-G between exosomes isolated from group 1 and 2 sera (Fig. 4A). However, variable expressions of HLA-DR α were observed, with exosomes from group 1 pregnancies exhibiting higher levels of HLA-DR α (Fig. 4B). Transplantation studies analyzing donor bone marrow dendritic cell-derived exosomes demonstrated the expression of functional class I and II MHC components and that these exosomes could modulate allograft rejection by decreasing CD4⁺ antidonor responses (41). Other studies examining tolerosomes associated with the development of immunological tolerance to orally administered Ags demonstrated the expression of specific class II Ags: DM and DR (42). Thus, the differential exosomal expression of class II Ags between groups 1 and 2 may also contribute to the suppressive immunophenotype associated with uncomplicated term pregnancies.

This study also addressed the consequences of exosome expression of biologically active FasL. Whiteside (43) has proposed a link between T cell apoptosis and decreased CD3- ζ expression. Her work previously demonstrated that coincubation of T lymphocytes with FasL-expressing ovarian tumor cells resulted in both loss of CD3- ζ and induction of lymphocyte apoptosis (44). Placenta-derived exosomes from group 1, which expressed FasL, suppressed both CD3- ζ and JAK3 proteins, whereas exosomes from group 2 failed to show a statistically significant difference. When the degree of CD3- ζ and JAK3 suppression was correlated with the exosomal level of FasL for group 1 samples, correlation coefficients of >0.9 were obtained, because exosomes with a higher FasL content induced the greatest reduction in ζ or JAK3 expression. The suppression by these exosomes were of sufficient magnitude to significantly reduce the capacity of T cells to produce IL-2 (Fig. 7).

Modulation of the JAK3 pathway is critical for specific type 1 cytokine receptors, which possess common γ -chains and can modulate CD3- ζ -mediated activation, leading to T cell anergy. ζ expression is a key signaling intermediate for receptor signaling in both T and NK cells, and the JAK/STAT pathway is also critical for T regulatory cells and NK cell activation and function. Normal pregnancy is associated with expansion of regulatory T cells, which appear to suppress fetal Ag-specific responses critical in allograft tolerance (45). This expansion results in amelioration of cell-mediated autoimmune disease, and the reappearance of autoimmune symptoms after delivery parallels the postpartum decrease in regulatory T cells (46). Regulatory T cells expressing CTLA-4 can induce dendritic cells to express indoleamine 2,3-dioxygenase (IDO) by interacting with its ligand CD80-CD86, thereby promoting maternal tolerance of the fetal allograft (47). Regardless of lymphoid cell number, pregnancy-associated immunosuppression has been linked with impaired IL-15 signaling, which uses the intermediate, JAK3 (48). Failure to generate an effective regulatory T cell can result in the loss or absence of maternal tolerance, potentially leading to pregnancy loss (47). Although these placenta-derived exosomes may be a key factor in the suppression of activated CD4 and CD8 lymphocytes by modulation of CD3- ζ , its consequences in other pathways might lead to expansion of lymphocytes with suppressive phenotypes, such as

regulatory T cells. Although most investigations of pregnancy have focused on simple Th1/Th2 ratios, these pathways are common to both T and NK cells and may provide an additional layer of understanding of maternal-fetal immune interactions beyond the simple production of Th2 and Th1 cytokines, to the functional capacity of their receptors expressed on maternal lymphoid cells.

The correlation of FasL levels and signaling molecule suppression is suggestive of a causative relationship; however, to define the suppressive exosomal component, the CD3- ζ inhibitory component was isolated by continuously eluting electrophoresis. Each of the fractionated exosome components was analyzed for suppression of CD3- ζ in the Jurkat bioassay, and two peaks of suppressive activity were observed (Fig. 7A). When these components were visualized by silver staining on standard SDS-PAGE gels, one component appeared at 24 kDa, and the second at 42 kDa (Fig. 7B). Because FasL has been shown to be a component of group 1-derived exosomes, and it exhibits M_r forms in this range, the identities of these components were assessed by Western immunoblotting using anti-FasL Ab. Only the 42-kDa component stained positively for FasL (Fig. 7C). Because the anti-FasL Ab used recognized an epitope present in the intact and cleaved forms, the absence of reactivity suggested that the 24-kDa protein is not FasL. To confirm the role of FasL in these exosomal effects, Fas expressed on Jurkat cells was blocked with ZB4 Ab (Fig. 9). This blockage of Fas resulted in a significant reversal of exosome-induced CD3- ζ suppression. However, this did not represent a complete reversal of exosomal suppression, which might result from technical issues (Ab concentration, time of preincubation, or exosome incubation) or may mean that in addition to FasL, other exosome-associated molecules contributed to CD3- ζ suppression.

This study presents data indicating that the placentas of fetuses destined to deliver prematurely exhibit significant quantitative and qualitative differences in released exosomes compared with those from pregnancies subsequently delivering without complications at term. In addition to qualitative differences between exosomes from these two groups, there are significant differences in the levels of components associated with immune regulation. The release of exosomes expressing FasL may be one mechanism by which the placenta promotes a state of immune privilege. The mechanism leading to the absence or diminished levels of FasL on exosomes derived from the sera of women delivering preterm is unclear. Current studies are investigating the synthesis of FasL in the preterm placenta as well as in the mechanism resulting in the overall decreased levels of exosomes associated with preterm birth. Although the underlying mechanisms remain to be defined, because exosomes can play a critical regulatory role in AICD, preventing excessive immune responses that can lead to autoreactive responses, aberrations in exosome production and composition during fetal development might be an indicator of the inability of specific individuals to appropriately regulate cellular immunity, which could result in pathologic conditions during early childhood as well as later in life.

Disclosures

The authors have no financial conflict of interest.

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RETRACTED
15 JUNE, 2015

Letter of Retraction

An institutional research misconduct investigation committee determined multiple figures in the following paper were falsified: “Pregnancy-Associated Exosomes and Their Modulation of T Cell Signaling” by Douglas D. Taylor, Sibel Akyol, and Cicek Gerceel-Taylor, *The Journal of Immunology*, 2006, 176: 1534–1542.

Based on the foregoing, the University of Louisville has requested that the above paper be retracted.