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Human Placenta Expresses and Secretes NKG2D Ligands via Exosomes that Down-Modulate the Cognate Receptor Expression: Evidence for Immunosuppressive Function

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During mammalian pregnancy maternal-fetal tolerance involves a number of immunosuppressive factors produced by placenta. Recently, placenta-derived exosomes have emerged as new immune regulators in the maternal immune tolerance. Exosomes are membrane nanovesicles with defined morphology, which are secreted from endosomal multivesicular bodies (MVB) upon fusion with the plasma membrane. Previously, we reported that the MHC class I chain-related (MIC) proteins A and B, human ligands of the activating NK cell receptor NKG2D, are expressed by placenta, sorted to MVB of syncytiotrophoblast and probably released via MIC-bearing exosomes. In this report, we show that the second family of human NKG2D ligands, the UL-16 binding proteins (ULBP), is also expressed by placenta. Importantly, this expression was not due to placental CMV infection. Immunoelectron microscopy disclosed that ULBPs are produced and retained in MVB of the syncytiotrophoblast on microvesicles/exosomes. Using human placenta explant cultures and different assays, we demonstrate that exosomes bearing NKG2D ligands are released by human placenta. Isolated placental exosomes that down-modulate the cognate receptor Fas (FasL)3 by exo-

somates that could induce suppression of intracellular signaling and apoptosis of T cells (4, 7–9). Exosomes are 30- to 100-nm-sized microvesicles with a defined morphology and phenotype that are formed and released through the late endosomal compartment of a variety of cells (10), including syncytiotrophoblast (STB) of human placenta (2, 8). Exosomes can carry membrane-bound and cytosolic proteins, mRNA, and bioactive lipids to target cells and also transfer infectious agents between cells. Depending on their cell type origin, exosomes play a role in different physiological and pathological processes serving as a novel form of intercellular communication. Exosomes from dendritic cells possess immune-activating capacity (11), whereas exosomes derived from other cells such as intestinal epithelia and tumors have a suppressive effect on the immune responses (12). Thus, exosomal secretion of bioactive molecules by human placenta proven by us and others seems to be another mechanism promoting the fetal allograft survival (2, 4, 7–9, 13).

In search of novel placenta-mediated molecular factors responsible for the maternal immune tolerance to the fetus we have chosen to study the potential role of the NKG2D receptor-ligand system in human normal pregnancy for two main reasons: 1) The interaction of the activating NK cell receptor NKG2D with its inducible autologous ligands, the MHC class I chain-related (MIC) proteins A and B, and the UL-16 binding proteins (ULBPs), is a central perforin-mediated cytotoxic pathway by which damaged, transformed, or infected cells are eliminated (14, 15); and 2) in cancer patients, the soluble form of MIC, shed in the circulation by tumor proteases, can bind to NKG2D and systemically down-regulate its expression on the cytotoxic T cells and NK cells, providing a mechanism for tumor immune escape (14). In this respect, the soluble NKG2D ligands can be regarded as true immunosuppressive molecules of the cytotoxic response.


Pregnancy is a unique immunomodulatory state that is characterized by mild systemic immunosuppression and inflammation (1, 2). Many hypothetical systemic and local mechanisms have been proposed to explain the transient maternal tolerance to fetal histoincompatibility (1, 3). Pregnancy success has been associated with suppression of cell-mediated immune responses (for review see Ref. 4). Thus, understanding the fetal escape from a potential cytotoxic attack by maternal NK and T cells holds a central position in elucidating the immune privilege of the fetus.

In mammalian pregnancy the contact of the mother with the fetus is mediated by the placenta. The placenta is not only a source of nourishment and pregnancy hormones but also actively produces and releases various immunomodulatory factors to prevent rejection of the fetal semiallograft by the innate and adaptive maternal immune system (5, 6). In previous reports, we and other researchers have shown that human placenta releases bioactive Fas ligand (FasL)3 by exo-

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3 Abbreviations used in this paper: FasL, Fas ligand; MIC, MHC class I chain-related; STB, syncytiotrophoblast; ULBP, UL-16 binding protein; RAET, retinoic acid early transcript; MVB, multivesicular body; MFI, mean fluorescence intensity.

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Human placenta can be viewed as a tumor because it is rapidly growing and uses similar molecular and cellular mechanisms for growth and survival, such as angiogenesis, tissue invasion, capacity to evade apoptosis, and high proliferation rate (16, 17). In a recent study we investigated MIC expression in human placenta and reported constitutive MIC production and release of the soluble form of MIC by STB in pregnancy sera, probably in exosomal form. Placenta-derived soluble MIC was able to down-regulate the NKG2D receptor on peripheral blood NK cells and T cells and impair the NKG2D-mediated cytotoxicity (13).

The second family of NKG2D ligands is the ULBPs, also known as the retinoic acid early transcript (RAET)1 molecules. The ULBP molecules were identified by the ability of some, but not all, family members to bind the UL-16 protein encoded by human CMV. They comprise five glycoproteins that bind to and activate the NKG2D receptor. The ULBP and MIC families exhibit 23–26% similarity between each other. Like MIC proteins, the ULBPs are distantly related to the MHC class I molecules but they are not β2-microglobulin-dependent for cell surface expression, and they do not present peptides (18). ULBP1–3 share 55–60% amino acid identity and link to the cell membrane via GPI anchor, whereas ULBP4 (RAET1E) and ULBP5 (RAET1G) have transmembrane-bound and soluble isoforms of the proteins (18–20). mRNA transcripts of ULBPs have been found in many tissues and cell lines; however, not all transcripts have been shown to correlate with protein expression on the cell surface (18). In tumor cells, DNA damage response induces the expression of MIC and ULBP1–3. However, in contrast to MIC that can also be induced by heat shock, numerous forms of cell stress, including heat shock, failed to induce expression of ULBPs suggesting that MIC and ULBP gene transcription is governed by different mechanisms (21). The ULBP expression is up-regulated in many tumor cell lines of epithelial and hematopoietic origin suggesting a potential role in tumor immune surveillance. Recent reports have shown that ULBP1–3 can be cleaved, similar to MIC from tumor cell lines, in an uncharacterized soluble form that is able to down-regulate the NKG2D receptor and thus promote tumor escape from NKG2D recognition (22, 23).

Our previous results on the immunosuppressive role of the soluble form of MIC, released by human placenta (13) and the fact that the expression of ULBP/RAET1 molecules has not been studied in pregnancy before, prompted us to investigate whether these proteins are also expressed by human placenta and what putative role they might play in normal pregnancy.

In this study we report to our knowledge for the first time that all five ULBPs are constitutively transcribed and stored intracellularly as proteins, and packed on intraluminal microvesicles/exosomes of the multivesicular late endosomal compartment of human early STB. Moreover, placental explants actively release ULBP- and MIC-expressing exosomes that can down-regulate the NKG2D receptor on PBLs. Taken together, our results strongly suggest that endosomal formation and release of ULBP- and MIC-expressing exosomes by the placenta is a new form of intracellular NKG2D ligand storage. Moreover, it is a novel mechanism for generation of soluble NKG2D ligands in form of secreted nanovesicles that are able to reduce the NKG2D surface expression on cytotoxic effector cells and thus contribute to the immune escape of the fetus.

Materials and Methods

**Samples and Abs**

Healthy women undergoing elective termination of pregnancy (8–16 wk of gestation) and at normal delivery donated placental specimens after ethical committee approval and informed consent. The clones and specificities of the Abs and the isotype-matched Ab controls used in this study are as follows: mAbs against CD56, perforin, NKG2D (blocking clone 1D11), pan MIC (clone 6D4; BD Biosciences); mAbs against CD3, CD8, CD45, CD69, placentar alkaline phosphatase, and isotype-matched control mAbs IgG1, IgG2a, IgG2b, normal rabbit Ig (DakoCytomation); mAb against TCRγδ (Endogen); mAb against CD63 (clone CLB-gran/12,435; Fitzgerald); FITC-conjugated mAb against CD63 (Immunotech); mAb against human pan TCRγδ (Pierce); mAb against NKG2D (clone 149810), MICA (clone 159227), ULBP1 (clone 170818), ULBP2 (clone 165903), ULBP3 (clone 166510), goat anti-human ULBP2 (R&D Systems); goat anti-human GRP78, goat anti-human TSG101, rabbit anti-human Abs against ULBP1 (H-46), ULBP2 (H-48), ULBP3 (H-45), goat anti-human MIC/A/B (E16, G20) and mAb against ULBP4 (clone 6E6), ULBPs (clone 6D10), normal rabbit IgG (Santa Cruz Biotechnology); and normal goat serum (Vector Laboratories).

**Immunohistochemistry**

Placenta samples were fixed in 4% paraformaldehyde for 2 h, washed in PBS supplemented with 3.5% sucrose and 1% fish gelatin and snap frozen in liquid nitrogen. Cryosections (8 μm) were stained using polyclonal rabbit Abs against ULBP1, ULBP2, and ULBP3 as previously described (8). PBS supplemented with 0.1 M glycine was used for blocking of aldehyde groups and 0.03% H2O2 for extinguishing the endogenous peroxidase activity. One percent of normal human serum (AB+) and 0.5% BSA in PBS was used to block Fcγ receptors on STB and in all blocking steps (24). Enhancement of the secondary Ab signal was done by ImmPRESS anti-human IgG1, IgG2a, IgG2b, normal rabbit Ig (DakoCytomation). For negative isotype-matched control, sections were stained with affinity purified normal rabbit IgG instead of specific Abs in the first step.

**Immunoelectron microscopy of chorion villi**

Placental samples were fixed in 4% paraformaldehyde (Merck) in 0.1 M phosphate buffer for 4 h, washed in the same buffer containing 7% sucrose and 0.05% saponin (Merck) at 4°C overnight, and snap frozen. Cryosections were processed by indirect immunoperoxidase method previously described (8) using Abs against ULBP1–5, CD63, and TSG101. One percent normal human serum (AB+) and 0.5% BSA in PBS was used to block Fcγ receptors on STB in all blocking steps. Anti-rabbit or anti-mouse ImmPRESS reagents were used as secondary Abs. Ultrathin sections were examined without additional staining. Sections incubated with appropriate isotype-matched control Abs, or affinity purified normal rabbit IgG instead of specific mAbs or specific polyclonal Ab were used as negative controls.

**Isolation of PBMC, subpopulations of PBMC and villous trophoblast cells from first trimester placenta**

PBMC were isolated from nonpregnant healthy donors by Lymphoprep (Nycomed) gradient centrifugation. Subpopulations of NK, CD8+ and γδ T lymphocytes were purified from isolated PBMC using negative selection and Dynabeads (Dynal Biotech) according to the manufacturer’s instructions. The purity, checked by flow cytometry, was >96% (data not shown). First trimester villous trophoblast cells were isolated following the procedure as described (25). The isolated villous trophoblast or the PBMC and the lymphocyte subpopulations were used in immunofluow cytometry, Western blot, functional experiments, and for total RNA extraction.

**Light capture microdissection**

To obtain STB as unaffected by isolation procedure as possible, laser capture microdissection was used. Placental samples from normal early and term placenta were snap frozen in liquid nitrogen. Frozen sections (5 μm) were prepared and stained with HistoGene LCM Frozen Section Staining kit. The STB was laser microdissected using CapSure HS LCM Caps with the PixCell II LCM System. Total RNA was isolated with Picopure RNA Isolation Kit and used in qPCR and RT-PCR analyses. All materials for the microdissection and RNA isolation were purchased from Arcturus LCM Instruments (Molecular Devices).

**Short-term cultures of explants from early normal placenta**

Short-term placental explant cultures were established as previously described (13). In brief, small pieces of chorion villi of ~5–10 mg of wet weight were cultured in RPMI 1640 supplemented with 0.5% BSA (Sigma-Aldrich) or 10% FCS, ultracentrifuged for 5 h at 110,000 × g, and antibiotics. The cultures were maintained at 37°C in 5% CO2 and humidified air. Supernatants were harvested after 24 h of culture and used for exosome isolation.

**Isolation of exosomes from supernatants of placental explant cultures**

Culture supernatants were collected after 24 h and were cleared of cell debris by sequential centrifugations at 4000 × g for 30 min and 17,000 × g for 30 min. Then, the supernatants were centrifuged at 100,000 × g for 1 h. The exosome-containing supernatants were collected and subjected to density gradient ultracentrifugation as previously described (26). The pellets were resuspended in PBS and analyzed by immunoblotting. The exosome sample was aliquoted and stored at −80°C. For flow cytometry, exosomes were stained with anti-human CD63 (Immunotech), mAb against CD8, CD69, placental alkaline phosphatase, and isotype-matched control mAbs IgG1, IgG2a, IgG2b, normal rabbit Ig (DakoCytomation); mAb against TCRγδ (Endogen); mAb against CD63 (clone CLB-gran/12,435; Fitzgerald); FITC-conjugated mAb against CD63 (Immunotech); mAb against human pan TCRγδ (Pierce); mAb against NKG2D (clone 149810), MICA (clone 159227), ULBP1 (clone 170818), ULBP2 (clone 165903), ULBP3 (clone 166510), goat anti-human ULBP2 (R&D Systems); goat anti-human GRP78, goat anti-human TSG101, rabbit anti-human Abs against ULBP1 (H-46), ULBP2 (H-48), ULBP3 (H-45), goat anti-human MIC/A/B (E16, G20) and mAb against ULBP4 (clone 6E6), ULBPs (clone 6D10), normal rabbit IgG (Santa Cruz Biotechnology); and normal goat serum (Vector Laboratories).
FIGURE 1. ULBPs are constitutively expressed by STB of human normal pregnancy placenta. A. Quantitative real-time RT-PCR expression of ULBP1–5 in laser microdissected STB (n = 6). PMA/ionomycin stimulated normal PBMC served as normal control. All samples were normalized to 18 S rRNA as indicated in Materials and Methods. *, p < 0.05 for statistical significance. B, Immunohistochemical staining of early normal placenta showing the presence of ULBP1–3 in STB. Rabbit anti-human Abs against ULBP1–3 and affinity-purified normal rabbit IgG as a negative control were used. Original magnification is ×400 and ×800 (inset).

C, Immunoflow cytometry of isolated villous trophoblast cells stained with mAbs against ULBP1–3 and pan-MIC. Note that the ULBP1–3 molecules are only intracellularly expressed, whereas MIC is expressed both intracellularly and on the cell surface. Shaded histograms represent negative controls performed with irrelevant, isotype-matched murine mAbs.

Western blot analysis
Exosomes purified from placental explant culture supernatants, frozen sections of placenta, isolated villous trophoblast, and cultured K562 cells were solubilized with RIPA buffer and separated by SDS-PAGE on 12% polyacrylamide gels. The proteins were transferred to poly(vinylidene difluoride) membrane (GE Healthcare) and the membranes were blocked for 1 h at room temperature in 3–5% w/v nonfat milk and 0.05% Tween 2% Tween 20 in PBS. Primary rabbit anti-ULBP Abs and MIC mAbs in appropriate concentrations were applied overnight at 4°C and after three 5-min washes in PBS-Tween 20, secondary peroxidase-conjugated Abs were applied for 1 h, followed by three 5-min washes in PBS-Tween 20 and three 5-min washes in MilliQ water. The corresponding bands were detected by Amersham ECL plus Western blotting detection system (GE Healthcare).

Immune electron microscopy of protein expression on the surface of isolated placental exosomes
To maximally concentrate the exosome preparations, 15-µl drops of isolated exosomes in PBS were placed on 2% agarose. Formvar/carbon-coated nickel grids were placed on top of exosome drops and allowed to stand for 5–10 min to absorb the excess fluid. The grids with adherent exosomes were transferred to several 50-µl drops of PBS and fixed with 2% paraformaldehyde in PBS for 15 min to block Fc receptors. Thereafter, 50,000–100,000 trophoblast cells per well were plated in round-bottom microtiter plates and incubated with 25 µl of appropriate concentrations of primary anti-ULBP1–3 mAbs (R&D Systems) for 45 min with constant shaking. After the incubation, the cells were washed and the beads were analyzed on FACScan (BD Biosciences) according to the manufacturer’s instructions. After blocking of uncoupled sites and washing of the Ab-coated beads, purified exosomes were revealed by FITC-coupled anti-CD63 mAbs. Anti-MIC (Santa Cruz Biotechnology) and anti-ULBP1–3 (R&D Systems) Abs were used in the coating procedure that was performed according to the manufacturer’s instructions. After blocking of uncoupled sites and washing of the Ab-coated beads, purified exosomes (30 µg/ml) were added and incubated for 2 h at 4°C with end-to-end rotation. After four washes, a 30-min incubation with FITC-conjugated anti-CD63 Abs and more washing, the beads were analyzed on FACScan (BD Biosciences) using CellQuest software. In the negative controls (as superimposed in Fig. 5B), isotype-matched Abs were used.

Immunoflow cytometry of isolated trophoblast, PBMC and lymphocyte subpopulations
Surfactant-free ultra-clean 4-µm sulfated latex microbeads (Interfacial Dynamics) were used for analysis of NK2G2 ligand surface expression on isolated exosomes as previously described (26). Principally, anti-NKG2D ligand Ab- and isotype-matched control Ab-coating of the beads was used and the bead-bound exosomes were revealed by FITC-coupled anti-CD63 mAbs. Anti-MIC (Santa Cruz Biotechnology) and anti-ULBP1–3 (R&D Systems) Abs were used in the coating procedure that was performed according to the manufacturer’s instructions. After blocking of uncoupled sites and washing of the Ab-coated beads, purified exosomes (30 µg/ml) were added and incubated for 2 h at 4°C with end-to-end rotation. After four washes, a 30-min incubation with FITC-conjugated anti-CD63 Abs and more washing, the beads were analyzed on FACScan (BD Biosciences) using CellQuest software. In the negative controls (as superimposed in Fig. 5B), isotype-matched Abs were used.

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centrifuged through a layer of 50 μl of FCS at 400 g. After two more washes, FITC-conjugated secondary Abs were applied for 45 min in darkness with constant shaking followed by washing steps. Isotype-matched irrelevant mAbs were used as negative controls. For intracellular staining, the cells were fixed in 2% paraformaldehyde in PBS for 20 min at room temperature and after washing, incubated for 15 min in 50 mM glycine to block free aldehyde groups. The cells were then washed and permeabilized in PBS supplemented with 0.1% saponin and stained as described.

For blocking experiments of NKG2D receptor expression, PBMC or subpopulations of NK, CD8+, and γδ T lymphocytes from healthy donors were incubated in 5% CO₂ at 37°C for 24 h in the absence or presence of increasing concentrations of isolated placental exosomes before and after treatment with a mix of Abs against ULBP1–5 or a mix of anti-MIC and anti-ULBP1–5 Abs. To exclude that the results are due to steric hindrance by the Ab mixture, in the experiments with lymphocyte subpopulations, anti-CD63 mAbs were used to block the exosomes. Anti-CD45, anti-CD3, and anti-CD69 staining was used as a control of the selectivity of the NKG2D receptor blocking in the PBMC experiments and to estimate the activation status of the lymphocyte subpopulations respectively. After incubation, the cells were stained for

**FIGURE 2.** Immunoelectron microscopic localization of ULBP1–5 in STB of human early placenta. A, Low-power micrograph of STB stained for ULBP1. The electron-dense reaction product labels numerous vacuolar- and tubule-like structures in the syncytioplasm (arrows). Note that the apical microvillus surface (AMS) of STB is ULBP1-negative. B, High-power micrograph of apically located ULBP-1 positive MVB, which exhibits staining of the limiting membrane (arrow) and intraluminal microvesicles (arrowheads), and forms an opening to the intervillous space (asterisk). C, Micrograph of ULBP1-positive MVB in perinuclear location filled with tightly packed and intensely stained intraluminal microvesicles (arrowheads). D, Low-power micrograph of ULBP2-positive tubular structures (arrows) in STB. E, High-power micrograph of apically located ULBP2-positive MVB shown in D (inset). Note the intensive staining of the limiting membrane (arrow) and intraluminal vesicles (arrowhead) that seem to be released in the intervillous space (asterisk). F, Micrograph of ULBP3-positive MVB in perinuclear position, demonstrating the stained limiting membrane (arrow) and polymorphic intraluminal content with positively labeled microvesicles (arrowhead). G, Micrograph of ULBP3-positive MVB located in the apical part of STB. Arrow shows the stained limiting membrane and arrowheads indicate clusters of intraluminal vesicles. H, Low-power micrograph of two ULBP4-positive MVB. One of them is located in the perinuclear area (thick arrow), the other is contiguous with the apical plasma membrane (thin arrow). Arrowhead points at the stained limiting membrane of MVB. I, High-power micrograph demonstrating ULBP5-positive MVB. Rabbit anti-ULBP1–3 and murine mAbs against ULBP4 and ULBP5 were used. N, Nucleus; AMS, apical microvillus surface. Magnification is at ×5000 (A and D), at ×20,000 (B, F, and I), at ×19,000 (C and G), at ×21,000 (E), and at ×4000 (H).
NKG2D as described. A part of the cells incubated with native exosomes were fixed and permeabilized before staining for NKG2D, and for perforin. After the staining, the cells were analyzed in FACScan (BD Biosciences) using CellQuest software.

**Cytotoxicity assay**

Cell-mediated cytotoxicity was measured by CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) based on assessment of the total release of cytoplasmic lactate dehydrogenase into the cultured medium as a consequence of damaged cell membranes. K562 cells, known to lack Fas and MHC class I expression and express NKG2D ligands, were used as target cells and were mixed with the effector cells at the E:T ratio 10:1, 20:1, and 40:1 and incubated at 37°C for 4 h alone or in the presence of various concentrations of native (20, 40, or 60 μg/ml) exosomes or Ab-blocked exosomes of the same concentrations. The assay was performed according to the manufacturer’s instructions. For blocking of the NKG2D receptor the effector cells were incubated with either native exosomes as described or with blocking anti-NKG2D Abs (clone ID11, 10 μg/ml). For blocking of the NKG2D ligands a mix of ULBPI–5 and MICA/B or with anti-CD63 Abs were used. The specific lysis was calculated by a standard formula according to the manufacturer’s instructions. The unstimulated PBMC used in the experiments were analyzed for Fas expression and expressed <3% Fas (data not shown) ensuring that the apoptotic Fas-FasL pathway was not activated by the exosomes in this assay.

**Analysis of the effect of NKG2D ligand-expressing placental exosomes on perforin mRNA expression by NK, CD8+ (CTL), and γδ T lymphocytes**

Subpopulations of NKG2D-expressing NK, CD8+, and γδ T cells were cultured for 24 h alone, incubated with 60 μg/ml exosomes or with exosomes pretreated with anti-CD63 mAbs or a mix of Abs against the NKG2D ligands. After incubation, total RNA was isolated and cDNA transcribed as previously described (27). Real-time quantitative RT-PCR was performed with specific primers and probes for perforin as described below.

**DNA and total RNA extraction and quantitative real-time PCR and RT-PCR**

To exclude CMV infection of the placental samples, DNA was extracted and subjected to quantitative PCR as described (28). Total RNA and cDNA were obtained from laser microdissected and isolated STB and from purified lymphocyte subpopulations incubated in the presence or absence of exosomes. ULBP1–5 were amplified from STB cDNA and perforin from NK, CD8+, and γδ T cell cDNA on ABI PRISM 7700 by TaqMan Gene Expression Plate (I) protocol (PE Applied Biosystems) as described (27). Primer and probe sequences, detecting all five ULBP members, were as follows: ULBP1/RAET1I (forward) 5’TGCTTCATCCTCTCGCTTGATCTGA; ULBP2/RAET1N (forward) 5’TGGCACACCATCCTAGTCCTCG; ULBP3/RAET1H (forward) 5’CTCCACCTGGATGTTTGCTCA; ULBP4/RAT1E (forward) 5’TGCTTCATCCTCTCGCTTGATCTGA; ULBP5/RAET1F (forward) 5’TGGCACACCATCCTAGTCCTCG; ULBP2/RAET1M (forward) 5’CTCCACCTGGATGTTTGCTCA; ULBP3/RAET1H (forward) 5’CTCCACCTGGATGTTTGCTCA. The amplicons for ULBP1–5 are presented as relative quantities related to a reference (1) of the corresponding mRNA expression in PMA-ionomycin stimulated PBMC.

**Results**

**Constitutive expression of NKG2D ligands by human placenta**

We assessed ULBP expression in human placenta on the mRNA and protein level using quantitative RT-PCR, immunohistochemistry and immunofluorescence cytometry. Total RNA was obtained from placental samples by laser microdissection of STB or by isolation of villous trophoblast. Fig. 1A shows the overall ULBP1–5 mRNA expression in six individual samples. ULBP2 mRNA was expressed at a higher relative expression level compared with ULBP5 (p < 0.05). No statistically significant differences in expression levels were observed between the rest of the ULBP family members. There was no difference in the expression levels between laser microdissected and isolated STB (n = 6, data not shown) implicating that the STB isolation procedure did not affect the ULBP mRNA expression. To confirm the ULBP gene expression at protein level we stained placenta samples with Ab to ULBP1–3. A positive staining (Fig. 1B) of STB was seen in all samples (n = 6) and seemed to be localized inside the syncytiotrophoblast cells, stained with mAbs against ULBP1–3 and MIC (Fig. 1C). As can be seen, nonpermeabilized villous trophoblast cells do not show staining. A positive staining (Fig. 1B) of STB was seen in all samples (n = 6) and seemed to be localized inside the syncytiotrophoblast cells, stained with mAbs against ULBP1–3 and MIC (Fig. 1C). As can be seen, nonpermeabilized villous trophoblast cells do not show staining.
not express ULBPs on their surface. Upon permeabilization, however, intracellular ULBPs were revealed. In contrast, MIC was expressed both on the cell surface and intracellularly as previously described (13). No staining was observed in negative controls performed with isotype-matched mAbs (Fig. 1C, gray histograms).

From these experiments we conclude that the ULBP genes are constitutively transcribed and expressed as intracellular proteins in STB of human placenta.

The placental ULBP expression is not associated with human CMV infection

To exclude the possibility that the detected ULBP gene expression was due to undiagnosed CMV infection, the samples were analyzed for CMV gene expression using a quantitative PCR method (27). None of the analyzed samples expressed the human CMV genome (data not shown) indicating that the constitutive ULBP gene transcription that we found in human placenta was not CMV-induced.

**Immunoelectron microscopy reveals presence of ULBPs in the multivesicular late endosomes of STB**

We used an indirect immunoperoxidase staining and electron microscopy to determine the precise location of ULBPs in chorionic villi. Three early placenta samples were examined. No significant differences in the staining pattern of ULBPs were observed between the individual samples or between the individual ULBP members. The intracellular localization of ULBPs is shown in Fig. 2, which illustrates a summary of representative staining of all five proteins. The presence of ULBPs was restricted to STB. The apical microvillous and the basal surfaces of STB were not stained, except for ULBP2, which sometimes exhibited a weak patchy labeling of the apical or basal membranes (data not shown). Instead, the ULBP staining was concentrated to the membrane of numerous cytoplasmic vacuoles and tubule-like structures that were heterogeneous in appearance. They had variable intracytoplasmic locations, as shown in Fig. 2A, which is a low power micrograph of
indicating their endosomal nature. Fig. 3 and the intraluminal vesicles were positively stained for TSG101, the limiting membrane of perinuclear MVB A marker of late multivesicular endosomes (32) was performed. As endosomes, placenta staining with mAb against the common en-
The MVB were located at different levels in the syncytioplasm, some were located at the perinuclear area (Fig. 2, A, F, and H), whereas others were fused with the apical microvillous membrane, opening to the apical intervillous space and releasing their microvesicular content (Fig. 2, B, E, and G). The limiting membrane of the MVB was also positively stained for ULBPs (Fig. 2), suggesting that the intraluminal microvesicles were formed by inward budding from the limiting membrane of the MVB. To further confirm that the ULBP-positive MVB are late endosomes, placenta staining with mAb against the common endosomal marker protein TSG101 (31) and the tetraspanin CD63, a marker of late multivesicular endosomes (32) was performed. As can be seen in Fig. 3A, the limiting membrane of perinuclear MVB and the intraluminal vesicles were positively stained for TSG101 indicating their endosomal nature. Fig. 3B shows a strong CD63 labeling of the apical STB surface and two perinuclear MVB that contain positively stained internal vesicles illustrating a probable recirculation of CD63 between the apical surface and the late endosomal compartment (32). Negative controls, as described in Materials and Methods, revealed no detectable staining of STB (data not shown).

In summary, the immune electron microscopy of STB from human early placenta showed that exosome-containing MVB are the major storage site of ULBP1–5 and that ULBP-bearing exosomes of MVB seem to be released from STB to the maternal blood.

Explants from human early placenta release NKG2D ligand-bearing exosomes

As already shown the ULBP-positive MVB were in contact with the apical membrane of STB implying secretion of exosomes into the intervillous space. To confirm this suggestion, we cultured explants from early placenta and isolated exosomes from the collected supernatant. The exosomes were stained for surface expression of MIC, ULBP1–5, placental alkaline phosphatase, and CD63 using immunogold electron microscopy. No staining of exosomes was observed when specific Abs were replaced by isotype-matched control Abs or affinity purified rabbit IgG (data not shown). Staining for TSG101, a molecule present inside the exosome, was negative (data not shown), ensuring that our staining method was only revealing exosomal surface-expressed proteins. Fig. 4 summarizes the results of these experiments (n = 5). In Fig. 4A, an overview of microvesicles released from human placental explants is presented showing that they were heterogeneous in size, measuring between 40 and 90 nm in diameter and had the typical cup-shaped morphology of exosomes (33). They were dispersed one by one or clustered (Fig. 4B, A, F, and G). Besides the morphological characteristics, the exosomal nature of these ve-
cles was confirmed by anti-CD63 staining (Fig. 4B) and their STB origin by anti-placental alkaline phosphatase staining (Fig. 4C). Fig. 4, D–I, illustrates staining of the exosomes with Ab against MIC (Fig. 4D) and ULBP1–5 (Fig. 4, E–I, respectively). As can be seen, both families of NKG2D ligands were expressed on the placent exoplant exosomes. At present, we could not visualize whether several of these molecules are coexpressed on the same exosome because we could not find commercial Abs from different species suitable for double labeling. We strongly suspect that se-

However, some of these molecules might be present on separate exosomes. In our NKG2D receptor blocking experiments described below, we could not completely block the NKG2D receptor using

FIGURE 5. The expression of the NKG2D ligands on exosomes was assessed. A, Western blot analysis of NKG2D ligand protein expression by exosomes isolated from supernatant of placenta explant cultures from two donors (20 and 50 μg/well). CD63 was used to confirm the exosomal origin and the endoplasmic reticulum marker GRP78 to estimate the purity of the exosomal preparations. Whole cell lysate from placenta and isolated villous trophoblast (VT) were used for comparison to the exosomal fractions and the erythroid cell line K562, a target in the cytotoxic experiments, was used as a positive control. Rabbit anti-ULBP1–3 and goat anti-GRP78 Abs and mAbs against MICA and CD63 were used. B, Immunoflow cytometry of purified exosomes captured by latex microbeads coated with mAbs or Abs against NKG2D ligands. The exosomes were revealed by fluorescence staining with mAbs against CD63. Superimposed shaded histogram repre-
sents negative controls in which isotype-matched Abs were used.
only anti-ULBP or only anti-MIC Ab but a mix of both. These indirect observations suggest that some NKG2D ligands could be expressed on different exosome subpopulations.

The expression of the NKG2D ligands on exosomes was further assessed by Western blot analysis. Specific bands for ULBP1–3, MICA and the exosomal marker CD63 were revealed in all so far tested exosomal preparations isolated from the placental explant cultures, exemplified by two such samples in Fig. 5A. The absence of endoplasmic reticulum protein GRP78 in exosome preparations demonstrates lack of contamination by endoplasmic reticulum-containing apoptotic cell debris fragments thus ensuring the purity of the isolated exosomal fraction.

The NKG2D ligand protein expression was also found in lysates of tissue sections from early placenta and from isolated villous

**FIGURE 6.** Placental NKG2D ligand-expressing exosomes down modulate NKG2D expression on PBMC and lymphocyte subpopulations from healthy donors in a dose-dependent manner. A, Down-regulation of the NKG2D receptor on PBMC from one donor by exosomal ligand-induced receptor internalization. Green line histogram represents NKG2D receptor staining of untreated PBMC and shaded histogram represents negative controls. Red line histogram represents PBMC incubated for 24 h with (1) 60 μg/ml native exosomes, (2) exosomes, blocked with a mixture of ULBP1–5 mAb, (3) exosomes, blocked with a mix of ULBP1–5 mAb and MIC mAb, and (4) PBMC incubated with 60 μg/ml native exosomes, fixed and permeabilized before staining. Blue line histograms show anti-CD3 staining. B, NKG2D down-regulation experiments with four individual PBMC donors, showing that the reduction of NKG2D receptor expression, measured by normalized geo MFI, was dose-dependent. The normalization was done by giving the geo MFI of untreated PBMC the value of 1. C, Exosome-mediated NKG2D receptor down-regulation on PBMC (n = 8) and subpopulations of lymphocytes (n = 5). Healthy donor PBMC were incubated for 24 h with PBS, native placental exosomes, or exosomes, treated with a mixture of anti-ULBP1–5 mAbs, a mixture of anti-ULBP and MIC mAbs, anti-CD63 mAb or with supernatant depleted from exosomes. *, p < 0.05.
Additionally, we have analyzed and confirmed the NKG2D ligand expression on the K562 cell line showing that these cells can be lysed through the NKG2D-mediated pathway and are thus suitable targets for the cytotoxic assay (34).

Additionally, the exosomal NKG2D-ligand surface expression was confirmed by experiments with latex beads. Isolated exosomes were captured on anti-ULBP or anti-MIC Ab-coated beads and revealed by anti-CD63 staining (Fig. 5B). In summary, our electron microscopy data, Western blot, and flow cytometric analyses of exosomes coupled to anti-NKG2D-ligand coated latex beads indicate that human placenta releases exosomes expressing NKG2D ligands on their surface.

Placental NKG2D ligand-bearing exosomes selectively down-regulate the surface NKG2D receptor expression on PBMC and lymphocyte subpopulations in a dose-dependent manner

To test whether the placental NKG2D ligand-bearing exosomes were able to selectively down-regulate the cell surface NKG2D expression we isolated PBMC from healthy donors and assessed the NKG2D expression on PBMC and subpopulations of lymphocytes before and after 24 h incubation with placental exosomes. The experimental results are shown in Fig. 6. The down-modulation of the receptor was measured by geo MFI. Fig. 6A illustrates one representative experiment of NKG2D down-regulation on PBMC. As can be seen in Fig. 6A, graph 1, native exosomes induced 5- to 10-fold decrease of geo MFI and the decrease was dose-dependent as illustrated in Fig. 6B, showing experiments with PBMC from four individual donors. When the exosomes were pre-treated with a mixture of anti-ULBP1–5 mAb, the surface staining of NKG2D receptor was restored to approximately two-thirds of the staining intensity before the exosome incubation (Fig. 6A, graph 2). A mix of both anti-ULBP and anti-MIC mAb (Fig. 6A, panel 3) completely restored the staining intensity, suggesting that at least part of MIC might be carried on separate exosomes. To determine whether the NKG2D receptor was internalized or just masked on the cell surface by the exosomes, a part of the PBMC, which was incubated with native exosomes, was fixed, permeabilized,

FIGURE 7. NKG2D ligand-expressing placental exosomes impair the killing ability of PBMC from healthy donors in vitro. A, Cytotoxicity against K562 in an E:T ratio of 40:1 was tested with untreated PBMC, or in the presence of native exosomes, effector cells treated with anti-NKG2D receptor blocking mAb, target cells blocked with a mixture of anti-ULBP1–5 and anti-MIC mAb, and exosomes, blocked with a mixture of anti-ULBP1–5 and anti-MIC mAb or with a single anti-CD63 mAb. Additionally, the effect of supernatant depleted from exosomes was tested. Native exosomes significantly decreased the killing ability (measured by percentage of target cell lysis) of the effector cells to a level comparable to that after blocking the NKG2D receptor on effector cells or blocking the NKG2D ligands on target cells. The suppression by native exosome-expressed cytotoxicity was reversed with a mixture of mAbs against ULBP1–5 and MIC or with a single anti-CD63 mAb arguing against steric hindrance as a cause of the reversion. Used supernatant after exosome isolation did not affect cytotoxicity. Data represent mean values of six experiments. B, Exosomal dose-dependent suppression of cytotoxicity shown in three E:T ratios: 10:1, 20:1, and 40:1. Note that the suppressive effect is reversed when the exosomes are blocked with anti-NKG2D ligand Abs. C and D, Perforin protein (C) and mRNA (D) expression levels in subpopulations of effector lymphocytes incubated for 24 h in the presence or absence of exosomes (n = 3). Note that the perforin protein and mRNA levels remained unaltered, suggesting that the NKG2D ligand-bearing placental exosomes did not affect the perforin-mediated lytic ability of the effector cells.
and analyzed for the presence of intracellular NKG2D. The staining, reflecting the total NKG2D receptor expression, was comparable to that of freshly isolated PBMC, suggesting that the cell surface down-modulation of NKG2D was caused by internalization of the receptor (Fig. 6A, panel 4). To find out whether this down-regulation was NKG2D receptor-specific, CD45, CD3, and CD69 were stained in parallel. The geo MFI of the staining of these parameters remained unchanged. This result is illustrated by showing CD3 expression in Fig. 6A. This effect was consistent with different lymphocyte donors and with different exosome preparations. A summary with statistical comparison is shown in Fig. 6C (n = 6) where staining of PBMC and lymphocyte subpopulations of NK/CD56+, CD8+, and γδ T cells are shown. To exclude the possibility of steric hindrance due to the usage of an Ab mixture, experiments (n = 4) with exosomes blocked with a single anti-CD63 Ab were done and they showed comparable results. The initial NKG2D expression in the NK cell subpopulation was lower compared with the other subpopulations reflecting the low expression of NKG2D on resting NK cells (35). As can be seen, the suppressive effect was found in all lymphocytes subpopulations at 24 h of incubation but was most prominent for CD8+ T cells. This difference is in agreement with the data of Clayton and Tabi (44) who found that tumor exosome-mediated down-modulation of the NKG2D receptor on NK cells was less pronounced compared with CD8+ T cells. In summary, we found that placenta-derived NKG2D ligand-bearing exosomes could selectively reduce the NKG2D receptor cell surface expression on effector lymphocytes in a dose-dependent manner.

Placental NKG2D ligand-bearing exosomes down-regulate the in vitro NKG2D-dependent killing ability of PBMC from healthy donors without affecting perforin protein content or mRNA transcription

Next we assessed whether the NKG2D down-regulation on PBMC by NKG2D ligand-expressing exosomes had functional consequences for their killing ability (Fig. 7). PBMC containing NKG2D receptor expressing CD8+ CTLs, NK cells, and γδ T cells were isolated from healthy donors and used as effector cells. The NKG2D ligand-expressing, Fas-negative and MHC class I molecule-negative human erythroleukemia cell line K562 (34) was used as target cells. The PBMC were used directly or after pretreatment with native exosomes, exosomes treated by Ab mixture against all NKG2D ligands or by anti-CD63. The cytotoxicity was compared with that when the NKG2D receptor on the effector cells was blocked and when the NKG2D ligands on the target cells were blocked. In addition, supernatant depleted of exosomes was tested. As shown in Fig. 7A (n = 6), in the presence of native exosomes, the lysis of target cells was substantially decreased. The reduction of the specific lysis was similar to that seen when the effector cells or the target cells were blocked by anti-NKG2D receptor mAb or anti-NKG2D ligand mAb, respectively. When the NKG2D ligands on the native exosomes were blocked by a mixture of specific mAb, they did not reduce cytotoxicity suggesting that the suppressive effect was due to exosomal NKG2D ligand-NKG2D receptor interactions. When using single mAb against the exosomal marker CD63, the cytotoxicity was restored to the initial levels in a similar way as when the anti-NKG2D ligand Ab mixture was used showing that the suppressive effect was not due to steric Ab hindrance. In the presence of supernatant left after isolation of the exosomes, there was no reduction of the cytotoxicity indicating that the suppressive activity was in the exosome fraction. These results were also confirmed in cytotoxic experiments with three different E:T ratios and with different concentrations of exosomes. The results of these experiments (n = 3) are shown in Fig. 7B. As shown, there was an exosomal dose-dependent suppression of the NKG2D receptor-mediated cytotoxicity that could be reversed by blocking with specific anti-NKG2D ligand Abs. To further analyze whether the exosome-mediated reduction of NKG2D is also combined with impairment of the perforin-mediated lytic machinery of the effector cells (35), perforin protein and mRNA expression was assessed in NK, CD8+ , and γδ T cells using immunoflow cytometry and quantitative RT-PCR before and after incubation with native exosomes or anti-CD63 mAb-blocked exosomes. As shown, the perforin protein (Fig. 7C) and mRNA (Fig. 7D) expression were not affected (n = 3). The specific down-modulation of the NKG2D receptor by placental exosomes suppresses the in vitro killing ability of the effector cells but does not affect their perforin-mediated lytic ability.

Discussion

Data presented can be summarized with the following: 1) ULBP1–5 genes, similar to MIC (13), are constitutively transcribed and expressed as proteins in STB; 2) in contrast to MIC, ULBPs are absent on the cell surface of STB and only present on the membrane of intraluminal vesicles/exosomes of the multivesicular late endosomes/MVB in the syncytiotrophoblast; 3) cultured placental explants release exosomes bearing ULBP and MIC molecules on their surface membrane; 4) the exosome-associated ULBPs and MIC selectively down-regulate the NKG2D receptor on PBMC and purified NK, CD8+, and γδ T cells and impair their cytotoxicity in vitro without altering their activation status and lytic potency, implying that placental exosomes are immune inhibitory rather than activating; and 5) the exosome-mediated release of ULBP and MIC is a novel way to generate soluble form of NKG2D ligands. Taken together, this report and our previous data (8, 13) suggest that exosome-mediated secretion of bioactive molecules might be a mechanism by which the placenta promotes a state of immune privilege of the fetus.

Most studies of NKG2D ligands traditionally consider their expression only on infected or transformed cells. Recent data have revealed that several normal cells and tissues also express these ligands (13, 36). Interestingly, constitutive intracellular presence of ULBP1–4 has been newly described in normal human bronchial epithelium (37) but their subcellular location has not been defined precisely. In the present study, we provide the first report of the intracellular localization of ULBP1–5 in STB, i.e., normal placental epithelium, at the ultrastructural level. Using immunoelectron microscopy we found presence of ULBPs on numerous intraluminal vesicles/exosomes packed in cytoplasmic vacuoles. A multivesicular ultrastructure, high content of intraluminal vesicles (30) and parallel staining for CD63, a marker of the late endosomal compartment, identified these vacuoles as MVB/late endosomes. It seems that newly synthesized ULBPs were directly transported from the trans-Golgi network to the MVB where they were retained on the membrane of intraluminal vesicles/exosomes. Besides intracellular storage, MVB transport cargo proteins to the cell surface. In our ultrastructural analysis we frequently observed MVB opening to the free apical microvillous surface of STB and releasing ULBP-loaded exosomes in the intervillosus space. From these observations we could conclude that the late endosomal compartment of STB is used for posttranslational retention and exosomal release of ULBPs. Thus, our ultrastructural data on placental exosomes are consistent with the existing general knowledge about the biogenesis of exosomes (10). MVB/late endosomes are major protein sorting stations in the cellular endocytotic pathway. Proteins can be targeted to the limiting membrane of MVB where inward budding of this membrane produces protein-carrying intraluminal vesicles called exosomes.
These vesicles are stored, sorted for protein degradation to lysosomes, or released as exosomes into the extracellular milieu. The underlying cellular mechanisms for sorting of proteins for storage, secretion, or degradation are currently not known (38). In some cells, MVB can also serve as temporary protein storage compartments (39). At present, we do not know why the ULBPs in STB are preferably or exclusively sorted to exosomes. Two pathways are known for directing proteins to MVB. The first one is based on protein ubiquitination, which is recognized by the ESCRT machinery (38). The second one is mediated by lipids and tetraspanins in the endosomal-limiting membrane that forms microdomains, so called lipid rafts. These are likely involved in the sorting of proteins and formation of intraluminal vesicles. GPI-linked proteins, such as ULBP1–3, and other proteins in complexes are selectively targeted to the lipid rafts by lipid or protein affinity and appear on the exosomal membrane (38, 40).

The presence of NKG2D ligands on placental exosomes in situ was further confirmed by our phenotypic analyses of the microvesicles isolated from the supernatant of placental explant cultures. Electron microscopy, Western blot analysis, and FACS analyses showed that the secreted NKG2D ligand-loaded microvesicles displayed morphology, size, and expression of known exosomal markers such as CD63 and TSG101, proving their exosomal nature (40). Thus our results extend and provide a visual proof of our previous suggestion that MIC proteins are exosomally secreted by human placenta (13). In a recent publication Apps et al. (41) showed that mRNA for NKG2D ligands is constitutively expressed in human placental and decidual tissue and that the cytotrophoblast cells do not express NKG2D ligands, findings in concert with our results. However, in contrast to our findings, the authors were unable to reveal these molecules by immunohistochemistry probably due to the usage of anti-NKG2D ligand Abs that do not work in immunohistochemistry (13). Moreover, no attempt was made to look for protein expression by immunoblotting and the NKG2D ligand expressing STB cells were not investigated.

Tumor-shed soluble NKG2D ligands have been shown to impair the cytotoxic antitumor response by down-regulating the NKG2D receptor (42). Protease-mediated cleavage of NKG2D ligands on the cell membrane is considered as a mechanism by which soluble form of the ligands is generated (43). The release of placental exosomes, which mimic cell-cell interactions, represents an alternative mechanism for generation of soluble bioactive NKG2D ligands that was also observed in tumor cell lines (44, 45). It will be important to compare the functional outcome of exosome-mediated NKG2D receptor down-regulation with that by soluble NKG2D ligands.

Our current results raise the question about the physiological function of placental NKG2D ligand-expressing exosomes. Recent studies implicate both an immunostimulatory and immunosuppressive role for exosomes (11, 46). Exosome-associated secretion of FasL from STB induces apoptosis of activated T cells and thus promotes the fetal allograft survival (7, 8). However, exosomal FasL does not appear to be the sole mediator of immune escape of the fetus. We found that the ULBP-1 and MIC-expressing exosomes were able to impair the in vitro cytotoxic response against K562 target cells by down-regulation of the NKG2D receptor on CTls without a change of their activation status. Furthermore, the perforin content as well as the perforin mRNA transcription were unchanged indicating that the cytotoxic potency of the lymphocytes was unaffected by the exosomes. Placental release of NKG2D ligand-bearing exosomes seems to provide a possibility for permanent engagement of the NKG2D. Recent studies have shown that this engagement not only impairs the NKG2D function per se but includes suppression of other, perforin-independent cytotoxicity pathways (47), confirming the finding of Taylor et al. (9) that placental exosomes suppress the CD3-ζ chain and JAK3 expression in T lymphocytes.

It is known that the expression of NKG2D ligands is induced in CMV infected cells (48). Moreover, UL-16 binds selectively to ULBP1, 2 and 5, and retains these molecules intracellularly, inhibiting their cell surface expression to evade NKG2D-mediated immune response (49). Human placenta is susceptible to CMV infection and is the replication site of the virus from which the infection is spread to the fetus with consequences of deleterious pregnancy outcome or severe fetal malformations (50). Thus, it was very important to exclude that the constitutive intracellular ULBP expression we found in human placenta, was not due to CMV infection. Two findings argue against CMV infection. Firstly, no viral transcripts were found in the placental samples. Secondly, we found expression of all ULBP members not only ULBP 1, 2 and 5 (49). Therefore we conclude that the ULBP expression in human placenta is not CMV-associated, but must have another tissue-specific reason for induction.

A logical question is why are the NKG2D ligands expressed in human normal placenta and how are they regulated. The current concept is that NKG2D ligands can be induced in humans and mice by developmental signals, heat shock, DNA damage and infection (14). Despite these findings, the regulation of the NKG2D ligand gene expression still remains unclear. The presence of these ligands in normal tissues, such as placenta, suggests that there must be additional, unique tissue-specific regulation that remains to be discovered. Maybe the expression of NKG2D ligands in human placenta is a result of a normal genetic program and not induction. It is unlikely that the primary role of the NKG2D ligands in STB is to trigger a cytotoxic immune response against it. On the contrary, in normal pregnancy, from the point of view of the fetus, NKG2D ligand expression on the cell surface of STB should be avoided as a potential threat to its existence. We suggest that this is solved by tight regulation of the NKG2D ligand expression on the cell surface and directing these ligands to the MVB compartment of STB to be further released in exosomal form. In this way placenta avoids a direct immune targeting of STB and gains a new pathway of exosome-mediated down-regulation of the cognate receptor. Another possibility maybe that the regulation of these genes in normal tissue is induced through signaling by other danger-detection systems. Recent publications have shown that cell expression of NKG2D ligands was induced by signaling through TLRs (51, 52). There is accumulated evidence that human placenta expresses TLR-2, TLR-3, and TLR-4 (53). One can speculate that signaling through TLR might be the missing link that explains the induction of these molecules in STB on exosomes with capacity to dampen the NKG2D receptor-mediated responses and thus promote immunotolerance.

In summary, this detailed investigation is the first of the ultrastructural location of ULBP1–5 in a normal tissue. Moreover, using human placental explant cultures, we provide the first evidence that exosomes, secreted by human placenta express all so far known NKG2D ligands. Our finding that placental NKG2D ligand-bearing exosomes induce reduction of the cognate receptor and thus impair the cytotoxicity of effector lymphocytes without affecting the perforin-mediated lytic pathway might be a possible fetal immune escape mechanism which supports the current view of placenta as a unique immunosuppressive organ.

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Disclosures
The authors have no financial conflict of interest.

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