Evidence for Functional Glycosylation of Human Trophoblasts

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Abbreviations

BBSCT  biantennary bisected type
CFG    Consortium for Functional Glycomics
CTB    cytotrophoblasts
dNK    decidual natural killer
EVT    extravillous cytotrophoblasts
HLA    human leukocyte antigen
LacNAc N-acetyllactosamine
NK     natural killer
PAEP   progestagen-associated endometrial protein
PMMA  partially methylated alditol acetate
STB    syncytiotrophoblast
Summary

Human placental villi are surfaced by the syncytiotrophoblast (STB), with a layer of cytotrophoblasts (CTB) positioned just beneath the STB. STB in normal term pregnancies is exposed to maternal immune cells in the placental intervillous space. STB and CTB do not trigger histocompatibility based responses likely because they do not present any human leukocyte antigens (HLA) on their surface. This lack of HLA class I molecules could make these trophoblasts more sensitive to natural killer (NK) cell-mediated responses. However, STB and CTB are highly resistant to NK cells isolated from the decidua and peripheral blood in vitro. Glycomic analysis of these trophoblasts has revealed the expression of elevated levels of biantennary bisecting type N-glycans that have previously been implicated in the suppression of NK cell responses. Galectin-1 binds to biantennary bisecting type N-glycans. Galectins mediate many immunomodulatory activities in vitro, including the suppression of NK cell responses and promotion of the proliferation and function of inducible regulatory T cells (iT\textsubscript{reg}). These specific modifications of both adaptive and innate immune responses are considered to be essential for the development of the tolerant state in the pregnant human uterus. Extravillous cytotrophoblasts (EVT) express a paternal HLA class Ia molecule (HLA-C) on their surface, suggesting that these trophoblasts could trigger a major histocompatibility-based alloimmune response. Glycomic analysis has also confirmed the enhanced expression of carbohydrate ligands for galectins on EVT, suggesting that these trophoblasts could also sequester these immunomodulatory proteins to their surface. In summary, the specific presentation of glycans on trophoblast subpopulations supports the concept that functional glycosylation could play a role in the induction of tolerance during human pregnancy.
**Introduction**

Human development *in utero* requires the formation of a functional placenta that mediates the transport of nutrients, the exchange of gases, and the production of hormones that are required to maintain fetal viability. The placenta also serves as the immune interface between the mother and her histoincompatible fetus. Human trophoblasts are specialized placental cells that come into direct physical contact with maternal immune cells at both the villous surface and within the maternal decidua and myometrium. The syncytiotrophoblast (STB) layer is localized to the villous surface, with subsyncytial cytotrophoblasts (CTB) lying just beneath the STB. Regions of the syncytium can become damaged, which occurs at an increased frequency in many complicated pregnancies, and this damage can be repaired by division and fusion of cytotrophoblasts. It is likely that CTB in these damaged regions are at least transiently in direct contact with circulating maternal immune cells (1, 2).

Extravillous cytotrophoblasts (EVT) derived from placental stem cells penetrate through the tips of the anchoring villi and differentiate into invasive trophoblasts that migrate into the decidua, myometrium and spiral arteries (3). Trophoblasts that enter the spiral arteries differentiate into endovascular cytotrophoblasts. These cytotrophoblasts act in concert with maternal NK cells to remodel these arteries into flaccid conduits that support a 3-4 fold increase in the rate of maternal blood flow into the placental intervillous space at a reduced pressure compared to the maternal circulation (4-6).

Interactions between villous trophoblasts and maternal immune cells would normally be expected to trigger major histocompatibility responses due to the expression of paternal human leukocyte antigens (HLA). However, STB and CTB do not express the HLA class I molecules that are necessary to evoke such responses (7, 8). This absence of HLA molecules on STB and
CTB could make these trophoblasts potential targets for lysis by NK cells (9). However, STB and CTB are highly resistant to lysis by peripheral blood NK cells in vitro (10, 11).

More recent findings confirm that the presence or absence of HLA class I markers is not the only factor involved in NK cell activation. These lymphocytes express a vast array of activating and inhibitory receptors that recognize specific ligands on the surface of potential target cells (12). Engagement of these ligands by NK cell receptors can potentiate tolerance or cytolysis. Ligands for activating receptors are often expressed on tumor cells and normal cells undergoing stress, tipping the response toward cytolysis of these cells (12). The possibility exists that trophoblasts subjected to stress could express activating receptors for NK cell lysis. Such trophoblasts could escape cytolysis if they also presented a ligand for an inhibitory receptor that potently blocked NK cell responses. Such a fail-safe system of NK cell recognition could promote fetal viability under adverse uterine conditions.

Unlike CTB and STB, EVT express on their cell surface a classical paternal HLA class Ia molecule designated HLA-C (7, 8, 13). This presentation means that EVT are semiallogeneic during natural pregnancies and therefore potentially subject to powerful histocompatibility-based immune responses. However, the activation of such responses in a manner that harms the fetus and placenta does not typically occur during normal pregnancies. How EVT evade immune recognition in the gravid human uterus remains a major enigma. In contrast to STB or CTB, EVT are susceptible to lysis by purified decidual NK (dNK) cells that have been exogenously stimulated with IL-2 (14). Also, other groups have reported that dNK cells can lyse this trophoblast subpopulation (15, 16). Since EVT are precursors of endovascular trophoblasts that remodel the spiral arteries (17), increased lysis of EVT by activated dNK cells could decrease the
number of endovascular trophoblasts, contributing to deficient spiral artery remodeling and inadequate circulation in the intervillous space.

Progestagen-associated endometrial protein (PAEP), mucin 16 (MUC16) and uromodulin (UMOD) are three glycoproteins that are specifically elevated in the pregnant human female (18-20). PAEP and MUC16 are uterine glycoproteins that are often referred to as glycodelin-A and CA125, respectively (21, 22). These glycoproteins modify maternal immune responses \textit{in vitro} in a manner that likely promotes tolerance of the semiallogeneic fetus (21, 23-25). Compelling data indicate that their carbohydrate sequences serve as functional groups that enable these glycoproteins to manifest these immune deviating activities (21, 23-27). Glycomic analyses of glycoproteins, whole cells, tissues, and extracellular matrices have been useful for defining potential carbohydrate functional groups and their diverse roles in many cellular processes (21, 23-25, 28-30). Glycomic analysis of STB, CTB and EVT was performed in this study to determine if the N-glycans presented on the surface of these trophoblasts could contribute to the induction of tolerance to the histoincompatible human fetus.

\textbf{Experimental Procedures}

\textit{Isolation of CTB and STB}

The protocol for obtaining placentas used for this portion of this study was approved by the Institutional Review Board of Washington University School of Medicine. Term, singleton placentas from uncomplicated pregnancies were obtained, and CTB were isolated as described previously (31). All reagents were obtained from Sigma (USA) except for DNAase, which was from Roche Diagnostics (GmbH, USA). In brief, villous tissue was isolated and digested with dispase, trypsin, and DNAse and CTB were isolated on a continuous gradient of Percoll. Over 85\% of the cells stained positive for cytokeratin 7, a TB specific marker. CTB were obtained
after 24 h of culture in DMEM/10% FBS in 5% CO₂/air. To obtain STB, culture was continued for an additional 48 h with daily changes of medium. During this time, spontaneous differentiation and fusion occurs, with over 70% of the nuclei being present in multinucleated syncyitia after 72 h of culture (31). We observed an ~50-fold increase of hCG expression from 24 to 72 h of culture, confirming efficient differentiation.

*Isolation of EVT*

All human tissue collection necessary for this study was approved by the local ethics committee of the Geneva University Hospital. All patients provided their informed written consent prior to their inclusion in the study. Placental tissue was obtained from patients undergoing an elective termination of pregnancy during the first trimester (8–12 weeks of gestation). The procedure for the isolation of EVT was performed as previously described (32). Placental tissue specimens were isolated and washed several times in sterile Hanks balanced salt solution. The tissue samples were subjected to enzymatic digestion five times for 20 min at 37°C (0.25% trypsin, 0.25 mg/ml Dnase I; Roche, Diagnostics GmbH, USA). After this incubation, fetal bovine serum (FBS) was added to neutralize the trypsin cocktail. The cells were suspended in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Switzerland). This cell suspension was filtered through a 50-μm mesh and the resulting supernatant was applied onto tubes containing a Percoll gradient (70–5% Percoll diluted with HBSS). The samples were centrifuged for 25 min at 1200 g. The 30–45% Percoll layer was collected, and the trophoblast cells were harvested, washed, and resuspended in DMEM containing 10% fetal bovine serum.

The cells were transferred to Petri dishes and incubated for 15 min at 37°C. Trophoblasts in the culture supernatants were collected by centrifugation and resuspended in culture medium.
They were transferred to 6-well plates (4 × 10^6 cells/well) and in 96-well plates (1 × 10^5 cells/well). After 48 h of culture, 95% of the cells were: (i) negative for vimentin; and (ii) positive for cytokeratin 7 and HLA-G.

**Processing of trophoblasts to acquire N- and O-glycans**

All cell samples were subjected to a standard protocol (33). Briefly, cells were suspended in lysis buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA and 1% CHAPS (v/v), pH 7.4) before homogenization and sonication were performed. The homogenates were subsequently dialysed against a 50 mM ammonia bicarbonate buffer, pH 7.5, after which the samples were lyophilized. Cell/tissue extracts were reduced and carboxymethylated and then treated with trypsin. The treated samples were purified using a C18 cartridge (Oasis HLB Plus Waters) prior to the release of N-glycans by PNGase F (recombinant from *Escherichia coli*, Roche Applied Science) digestion. Released N-glycans were permethylated and then purified using a Sep-Pak C18 cartridge (Waters) prior to MS analysis.

Purified, underivatized N-glycans were incubated with sialidase S (recombinant from *Streptococcus pneumoniae*, Prozyme Glyko) or β1,4-galactosyltransferase (from bovine milk, Merck) separately. Sialidase S digestion was carried out using sialidase S in 50 mM sodium acetate, pH 5.5. The β1,4-galactosyltransferase reaction was performed using β1,4-galactosyltransferase in 50 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) containing 45 μM UDP-Gal, pH 7.4. The resulting enzyme-treated samples were lyophilized and permethylated prior to MS analysis.

**Mass spectrometric analysis**
MS data were obtained by using a Voyager-DE™ STR MALDI-TOF (Applied Biosystems) mass spectrometer. Purified permethylated glycans were dissolved in 10 µl methanol and 1 µl of the sample was mixed with 1 µl of matrix, 20 mg/ml 2,5-dihydroxybenzoic acid (DHB) in 70% (v/v) aqueous methanol and loaded on to a metal target plate. The instrument was run in the reflectron positive ion mode. The accelerating voltage was 20kV. MS/MS data were acquired using a 4800 MALDI-TOF/TOF mass spectrometer (AB SCIEX). In the MS/MS experiment, the dissolved sample was dried and then re-dissolved in 10 µl methanol. 1 µl of the sample was mixed with 1 µl of matrix, 10 mg/ml diaminobenzophenone (DABP) in 70% (v/v) aqueous acetonitrile and loaded on to a metal target plate. The instrument was run in the reflectron positive ion mode. The collision energy was set to 1 kV with argon as the collision gas. The 4700 calibration standard (mass standards kit for the 4700 proteomics analyzer, Applied Biosystems) was used as the external calibrant for the MS and MS/MS modes.

**GC/MS linkage analysis**

GC-MS linkage analysis of partially methylated alditol acetates (PMAAs) was carried out using a PerkinElmer Life Sciences Clarus 500 instrument fitted with RTX-5MS column (30 m × 0.32 mm internal diameter, Restek Corp.). The PMAAs were prepared from permethylated N-glycans as described previously (34). The permethylated glycans were hydrolyzed with 2 M trifluoroacetic acid at 121 °C for 2 hours, and then reduced with 10 mg/ml sodium borodeuteride in 2 M aqueous ammonium hydroxide at room temperature for 2 hours, and acetylated with acetic anhydride at 100 °C for 1 h. The sample was dissolved in hexanes and injected onto the column after the oven temperature reached 60 °C. The column was maintained at this
temperature for 1 min and then heated to 300°C at a rate of 8°C/min. Running a blank was required before every sample analysis.

**Experimental Design**

*CTB N-glycans* — 1) All N-glycans were presumed to have a Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc core structure based on knowledge of the N-glycan biosynthetic pathway and PNGase F specificity. 2) The composition (numbers of Hex, Fuc, etc.) of the glycans derived from MALDI-TOF MS in positive ion mode were manually interpreted. 3) MALDI-TOF/TOF MS/MS analyses were carried out on the following ions: m/z 2592, 2635, 2837, 2850, 3141, 3212, 3300 and 3416. All MS/MS spectra were annotated manually with the assistance of a glycobioinformatics tool, GlycoWorkBench (version 1.0.3353). 4) GC-MS linkage analysis was performed on partially methylated alditol acetates (PMAAs). 5) Sialidase S digestion was followed by manual assignment of the composition (numbers of Hex, Fuc, etc.) of the glycans derived from MALDI-TOF MS in positive ion mode. 6) Sialidase S digestion was followed by MALDI-TOF/TOF MS/MS analyses of the following ions: m/z 3143 and 4939. All MS/MS spectra were annotated manually with the assistance of a glycobioinformatics tool, GlycoWorkBench (version 1.0.3353). 7) The β1,4-galactosyltransferase reaction was followed by manual assignment of the composition (numbers of Hex, Fuc, etc.) of the glycans derived from MALDI-TOF MS in positive ion mode.

*STB N-glycans* — 1) All N-glycans were presumed to have a Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc core structure based on knowledge of the N-glycan biosynthetic pathway and PNGase F specificity. 2) The composition (numbers of Hex, Fuc, etc.) of the glycans derived from MALDI-TOF MS in positive ion mode were manually interpreted. 3) MALDI-
TOF/TOF MS/MS analyses were carried out on the following ions: m/z 2592, 2635, 2837, 2850, 3141, 3212, 3300 and 3416. All MS/MS spectra were annotated manually with the assistance of a glycobioinformatics tool, GlycoWorkBench (version 1.0.3353). 4) GC-MS linkage analysis was performed using partially methylated alditol acetates (PMAAs). 5) Sialidase S digestion was followed by manual assignment of the composition (numbers of Hex, Fuc, etc.) of the glycans derived from MALDI-TOF MS in positive ion mode. 6) Sialidase S digestion was followed by MALDI-TOF/TOF MS/MS analyses of following ions: m/z 3143 and 4939. All MS/MS spectra were annotated manually with the assistance of a glycobioinformatics tool, GlycoWorkBench (version 1.0.3353). 7) The β1,4-galactosyltransferase reaction was followed by manual assignment of the composition (numbers of Hex, Fuc, etc.) of the glycans derived from MALDI-TOF MS in positive ion mode.

EVT N-glycans — 1) All N-glycans were presumed to have a Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc core structure based on knowledge of the N-glycan biosynthetic pathway and PNGase F specificity. 2) The composition (numbers of Hex, Fuc, etc.) of the glycans derived from MALDI-TOF MS in positive ion mode were manually interpreted. 3) MALDI-TOF/TOF MS/MS analyses were carried out on the following ions: m/z 2519, 2850, 3141, 3212, 3415, 3964 and 4041. All MS/MS spectra were annotated manually with the assistance of a glycobioinformatics tool, GlycoWorkBench (version 1.0.3353). 4) Sialidase S digestion was followed by manual assignment of the composition (numbers of Hex, Fuc, etc.) of the glycans derived from MALDI-TOF MS in positive ion mode. 5) Sialidase S digestion was followed by MALDI-TOF/TOF MS/MS analyses of following ions: m/z 2489, 2519, 2693, 2968, 3142, 3387, 4040 and 4489. All MS/MS spectra were annotated manually with the assistance of a glycobioinformatics tool, GlycoWorkBench (version 1.0.3353).
The symbolic nomenclature used in the all the spectra annotations (CTB, STB and EVT) is the same as the one used by the Consortium for Functional Glycomics (CFG) (http://www.functionalglycomics.org/) and the Essentials for Glycobiology on-line textbook (http://www.ncbi.nlm.nih.gov/books/NBK1931/figure/ch1.f5/?report=objectonly).

Results

Analysis of N-glycans associated with CTB

A modification of the Kliman procedure was employed to isolate samples of CTB from eight term placentas (31, 35). Each sample was subjected to glycomic profiling exactly as described in a previous study (28). High quality MALDI data were obtained for the N-glycans of all the CTB samples. There were no apparent differences in the spectra between eight individuals. A representative MALDI spectrum of N-glycans derived from CTB from one patient is shown in Fig. 1. The theoretical and observed m/z values of the glycans and the manual interpretation of the composition of these glycans are shown in supplementary Table 1. Signals for high mannose type N-glycans (Man$_5$-$\beta$GlcNAc$_2$) were abundant at m/z 1579.8, 1783.9, 1988.0, 2192.1, and 2396.1. In addition numerous signals for complex-type biantennary, triantennary and tetraantennary N-glycans were observed, and their structures are shown in the cartoon annotations in Fig 1. To decrease the complexity of all the MALDI spectrum figures, the cartoon annotations for minor signals less than m/z 3250 are shown in supplementary Table 2. Nearly all the complex-type glycans carry core $\alpha$1-6 linked fucose, consistent with their localization to the plasma membrane, and sialic acid is the major capping sugar on their N-acetyllactosamine antennae. The most abundant complex-type structures give signals at m/z 2431.1 (monosialylated biantennary), 2489.2 (non-sialylated, core fucosylated biantennary), 2850.3 (mono-
sialylated, core fucosylated biantennary), 2966.3 (disialylated, core fucosylated biantennary) and 3211.4 (disialylated, core fucosylated biantennary). Interestingly three of these glycans have masses consistent with biantennary bisected type (BBSCT) structures ($m/z$ 2489.2, 2850.3, and 3211.4). Minor signals for N-glycans bearing Lewis$^x$ ($m/z$ 2592.2, 2837.3, 3024.3) and sialyl-Lewis$^x$ type antenna ($m/z$ 3140.3) were also detected. Evidence for N-glycans bearing polylactosamine type sequences was indicated by the signal at $m/z$ 3299.5.

Additional experiments were performed to firmly establish the glycan assignments shown in Fig. 1. First, to confirm assignments of BBSCT glycans, the N-glycan mixture was incubated with a β-galactosyltransferase in the presence of UDP-Gal. This enzymatic modification adds galactose to all antennae bearing terminal GlcNAc except for the bisecting GlcNAc which is sterically inaccessible to the enzyme. After this treatment, there was no detectable addition of galactose to putative BBSCT glycans (supplementary Fig. 1). In contrast, the truncated antennae of immature glycans (for example $m/z$ 1835.9) were extended by galactose, confirming that the β-galactosyltransferase was reacting successfully. In addition, GC-MS linkage analysis of the glycan mixture (supplementary Table 3) provided further evidence for the presence of bisecting GlcNAc. The presence of Lewis$^x$ and sialyl-Lewis$^x$ type antenna on these N-glycans was confirmed by MS/MS analysis (supplementary Figs. 2, 3, 4). The glycan mixture was also treated with sialidase S, a bacterial neuraminidase that specifically hydrolyzes α2-3 linked sialic acid. MS analysis of the products indicated that the majority of the sialic acid was eliminated by this digestion. Substantial amounts of sialic acid were released, but signals for sialylated N-glycans remained at $m/z$ 2431.2, 2676.3, 2880.3 and 3241.4, indicating that they were likely terminated with α2-6 linked sialic acid.
The N-glycans associated with CTB were treated with a neuraminidase (sialidase S) that specifically removes terminal α2-3 linked sialic acid. This enzymatic step removed nearly all of the sialic acid from these glycans, confirming that only a minor amount of sialic acid was α2-6 linked on glycoproteins associated with this cell type (Fig. 2). The major N-glycans that were detected after digestion were high mannose type N-glycans and the BBSCT N-glycan. This desialylation step also revealed minor amounts of N-glycans bearing polylactosamine sequences at m/z 3591.6, 3765.6, 3836.6, 4040.6, 4214.8, 4489.6 and 4938.6 (Fig. 2). MS/MS analysis of the peak at m/z 4938.6 (4939) showed that the maximum number of the LacNAc unit observed is five (Fig. S5).

*Glycomic Analysis of Human STB*

These trophoblasts were obtained after *in vitro* differentiation of primary CTB as described previously (31, 35). They were subjected to the same procedure that was employed to analyze the CTB N-glycome (28). The theoretical and observed m/z values of the glycans and the manual interpretations of the composition of these glycans are shown in supplementary Table 4. There were no apparent differences in the spectra between individuals. A representative MALDI spectrum is shown in Fig. 3. There were essentially no differences in glycan expression between STB and CTB. This same overlap was also observed after sialidase S digestion of N-glycans derived from STB (Fig. 2). The major signals were for the high mannose type N-glycans (Man₅₋₉GlcNAc₂) and the BBSCT N-glycan (m/z 2489.1).

*Glycomic Analysis of Human EVT*
This subpopulation of trophoblasts was subjected to glycomic analysis to determine if there were any differences between them and other types of trophoblasts. The theoretical and observed m/z values of the glycans and the manual interpretations of the composition of these glycans are shown in supplementary Table 5. Although the N-glycans linked to EVT were very similar to those associated with CTB and STB, there were major differences in levels of expression (Fig. 4). The larger complex-type N-glycans were far more prevalent in EVT, which can be most readily observed via the comparison of glycans after sialidase S digestion (Fig. 2). The most abundant peak, after the removal of α2-3 linked sialic acid, was m/z 3141.7, which corresponds to a complex type N-glycan with four LacNAc units in its antennae. This peak was accompanied by a prominent series of N-glycans with extended polylactosamine antennae containing additional LacNAc moieties (m/z 3590.6, 4039.6, 4488.4 and 4937.3). These glycans were far more abundant in EVT than in either CTB or STB. As a consequence, the relative abundance of the BBSCT N-glycan approximately m/z 2489 became less prominent in the overall spectrum of EVT compared to the other complex N-glycans. Another important difference was the absence of any detectable sialylated glycans after digestion with sialidase S digestion. This result confirms that all of the sialic acid linked to EVT is α2-3 linked.

**Discussion**

The human placenta is the organ that negotiates the peaceful co-existence of the mother and her histoincompatible fetus during term pregnancy. Specific adaptations have been made that enable 85-90% of women to fully accommodate their fetus until development in utero is complete. Many different models have been presented to explain this induction of tolerance to the fetal allograft during pregnancy (26, 36-41). Trophoblasts are placental cells that encounter maternal immune cells at two distinct interfaces. STB and CTB interact with maternal immune cells at the
villous interface. EVT and their derivatives come into direct physical contact with maternal immune cells during their invasion of the decidua, myometrium and spiral arteries. The current study was undertaken to determine if trophoblasts could employ their carbohydrate sequences as functional groups to promote tolerance at these uteroplacental interfaces.

_Glycosylation of Villous Trophoblasts_  
Our glycomic analyses of CTB and STB reveal that these cells express both high mannose and complex-type glycans. The majority of the latter are core fucosylated, biantennary structures with LacNAc or oligo-LacNAc antennae that are partially capped with α2-3 linked (major) or α2-6 linked (minor) sialic acid. A tiny portion of the glycome carries fucosylated antennae in Lewis^x/y and sialyl-Lewis^x^-type sequences. Significantly, many of the biantennary glycans have a bisecting GlcNAc (BBSCT N-glycans). These N-glycans have previously been implicated in the suppression of NK cell cytotoxicity. HLA class I-negative K562 erythroleukemia cells are routinely targeted cells for in vitro NK cell lytic assays (42). K562 cells become completely resistant to NK cell lysis if plasma membrane levels of BBSCT N-glycans is increased by insertion of a specific glycoprotein carrier for BBSCT N-glycans (glycophorin) or the introduction of a gene for a specific glycosyltransferase (GlcNAcT-III) that attaches the bisecting GlcNAc to biantennary N-glycans (43, 44). Thus, there is likely an undefined, inhibitory lectin-like receptor for BBSCT N-glycans on the surface of NK cells.

Why NK cells would recognize BBSCT N-glycans is a relevant question. BBSCT N-glycans represent 35-92% of the oligosaccharides linked to the only glycosylation site on HLA class Ia molecules (Asn-86) (45). The possibility exists that there is an unidentified lectin-like receptor for BBSCT N-glycans in close proximity to other NK cell receptors that bind to the heavy chain of HLA class I molecules. This type of dual receptor system could enable binding to both the
protein and carbohydrate sequences associated with HLA class Ia molecules, facilitating their rapid recognition. If the heavy chain receptor and the receptor that recognizes the BBSCT N-glycan were coupled to inhibitory pathways, then the net effect of this dual receptor system could yield potent suppression of NK cell cytotoxic response.

In addition to CTB and STB, several other types of somatic cells and germ cells lack HLA class I molecules (46). Erythrocytes are among these cell types, with BBSCT N-glycans being the major type of oligosaccharides linked to glycophorin, the most abundant glycoprotein in erythrocyte plasma membranes (47). Neurons also lack HLA class I molecules (46) and BBSCT N-glycans constitute 60-70% of the total N-glycans associated with human brain and cerebrospinal glycoproteins (48, 49). Human sperm and eggs are also HLA class I negative (46, 50). Human sperm display high levels of BBSCT N-glycans on their plasma membranes (51, 52). The plasma membranes of human eggs also stain intensely with erythroagglutinating phytohemagglutinin (E-PHA), a lectin that binds specifically to bisecting type N-glycans (52). Human villous cytotrophoblasts also stain intensely with E-PHA (53). The presentation of BBSCT N-glycans on surface glycoproteins in HLA class I negative cell types, including CTB and STB, could provide a fail-safe mechanism for blocking NK cell responses.

Expression of Glycans on EVT

Notably, the profile of N-glycans in EVT was different from those observed in villous trophoblasts, with EVT expressing relatively lower levels of BBSCT N-glycans and much higher levels of triantennary and tetraantennary N-glycans. N-glycans decorated with polylactosamine sequences were also considerably higher in EVT than in either CTB or STB. The sialylated N-glycans associated with EVT were exclusively capped with α2-3 linked NeuAc. Our finding of polylactosamine-decorated N-glycans in EVT support the findings by Fisher and coworkers (54).
who demonstrated that HLA-G produced by EVT and secreted into amniotic fluid migrates as a polydisperse band between 35-50 kDa that is collapsed into a single 35-36 kDa band after digestion with endo-β-galactosidase, an enzyme that specifically depolymerizes polylactosamine sequences. Together, these data suggests that a substantial proportion of native HLA-G molecules in EVT are decorated with polylactosamine sequences.

The expression of BBSCT N-glycans, though lower than in STB or CTB, could nonetheless provide EVT with some measure of protection from dNK cell responses. However, the exact level of protection remains a matter of conjecture with the currently available data. Results from one study suggest that dNK cells are incapable of forming mature activating synapses with EVT and thus are not cytotoxic (55). Other groups have reported that EVT are susceptible to lysis by dNK cells (14-16). Fisher and coworkers have provided evidence that decidual macrophages induce tolerance to EVT via their secretion of transforming growth factor-β1 (14). However, glycolelin-A and CA125 are produced in abundance in the endometrium and decidua from the initiation of implantation up until the 20th week of gestation, and they also suppress NK cell cytotoxicity in vitro (18, 25, 27, 56, 57). NK cell cytotoxicity is also directly suppressed by galectin-3 that is also expressed at elevated levels in the uterus during pregnancy (58, 59). These findings suggest that there are likely redundant pathways for the induction of NK cell tolerance in the endometrium and decidua during the first two trimesters of human pregnancy.

**Expression of Galectins (LGALS) and Galectin Ligands in the Pregnant Uterus**

Galectins are a family of β-galactoside binding proteins that express one or two conserved carbohydrate recognition domains (59-61). Elevated expression of GALS-1, -3, -8, -9, -13, -14, and -16 has been documented in the placenta and the endometrium/decidua during human pregnancy (59). Galectins act as both extracellular and intracellular modulators of diverse
biological activities including trophoblast migration and invasion, syncytium formation, and immune modulation (60-64).

Several groups have tested the carbohydrate binding specificities of galectins via a variety of different methods (65-68). Considerable data have been obtained by analyzing the binding of galectins to different versions of the glycan array available on the public Consortium for Functional Glycomics (CFG) website (http://www.functionalglycomics.org). Galectins are characterized by a near universal affinity for lacNAc (Galβ1-4GlcNAc). Many can accommodate substitution of the terminal β-linked Gal at the 3 but not the 6 position (65-68). This specificity means that galectins can bind to terminal NeuAcα2-3Galβ1-4GlcNAc sequences, but not to those capped with NeuAcα2-6Galβ1-4GlcNAc sequences (65). Nearly all of the sialic acid associated with STB and CTB was determined to be α2-3 linked in the current analyses. No α2-6 linked sialic acid was identified in the N-glycans derived from EVT. Galectins usually display higher affinity for triantennary and tetraantennary N-glycans terminated with LacNAc sequences than for biantennary N-glycans of the same type (65-68). Galectin-3, -8, and -9 preferentially exhibit higher affinity for polylactosamine sequences (65). Glycomic analyses performed in this study indicate that EVT express a much higher level of polylactosamine sequences that bind to these galectins. Galectin-1 binds to the BBSCT N-glycan based on glycan array data available at the CFG website. This specificity is consistent with the binding of galectin-1 to the surface of CTB reported in a previous study (69).

As noted earlier, polylactosamine sequences are linked to HLA-G associated with human EVT (54). These findings suggest that the HLA-G molecules on the surface of EVT in the decidua and myometrium are likely bound to galectins. Soluble HLA-G could also bind to galectins. Whether glycoforms of HLA-G synthesized in other cell types manifest the same
immune deviating effects as the placental glycoforms bearing polylactosamine sequences is a topic for further investigation (37, 70, 71).

How HLA-C and HLA-E are glycosylated in the placenta is unknown at this time. Nonetheless, their interactions with receptors for HLA molecules could be drastically affected by their N-glycosylation. The addition of a bulky polylactosamine sequence to the N-glycan expressed on these HLA molecules could block access to the heavy chains of these molecules. Galectins could bind to their carbohydrate ligands expressed on HLA-C and HLA-E to further inhibit access to their heavy chains. This type of steric hindrance could inhibit allore cognition of paternal HLA-C. HLA-E is a class Ib molecule that induces NK cell tolerance (72-74). Why HLA-E does not protect EVT from NK cell response in this case is unknown, but it could be due to the addition of polylactosamine sequences. Though certainly speculative at this time, this model of differential glycosylation and galectin binding would be consistent with the observed lack of allore cognition of HLA-C and the ability of exogenously stimulated dNK cells to lyse EVT in spite of their expression of HLA-E (14-16). Sequencing of the N-glycans linked to HLA-C and HLA-E combined with immunological studies will be required to determine the effect of glycosylation on the recognition of these HLA class I molecules.

Galectins also mediate many immune deviating activities following their interaction with a variety of immune cell types. A pivotal role for galectins in the establishment of fetomaternal tolerance was initially supported by studies performed in a mouse model of stress-induced immunological abortion (75). Galectin-1 mediated its immune deviating effects in this model via the induction of tolerogenic dendritic cells and regulatory T cells (iT\textsubscript{reg}) (75). Fetal loss rates were substantially reduced in these mice by dosing them with galectin-1. Galectin-3 has recently been shown to directly inhibit human NK cell cytotoxicity by binding to the human cytotoxicity
triggering receptor-3 (NCR3), also known as NKp30 expressed on the surface of NK cells (58). Galectin-3 has been shown to form lattices on cell surfaces by cross-linking glycoproteins on T cells that bear polylactosamine sequences on their tri-and tetraantennary N-glycans, thereby blocking adaptive immune responses in mice (76). Galectins could avidly bind to their ligands on EVT, and erect a “galectin shield” around these trophoblasts that would protect them during their invasion of the decidua and myometrium. Galectins also manifest many other immune deviating effects that could block different types of immune responses in the pregnant uterus (60, 61, 77).

**Linkages to the Human Fetoembryonic Defense System (Hu-FEDS) Hypothesis**

This hypothetical model for the induction of tolerance to the gametes and developing human *in utero* was first proposed nearly two decades ago (26, 38). This specific paradigm is based on the concept that immunomodulatory glycoproteins and their complementary lectin-like signaling proteins play a major role in the induction of tolerance to the developing human and the gametes. Glycodelin-A, uromodulin and CA125 are three major immune deviating glycoproteins that have been identified as components of this model in the urogenital tract of pregnant human females. Data obtained in this study clearly define the carbohydrate sequences linked to STB, CTB and EVT as those shown to promote tolerance in the limited number of healthy tissues and cells with similar membrane glycosylation patterns. The present work supports the concept that the glycosylation patterns in human trophoblast cells could play a role in the suppression of both adaptive and innate immune responses in the pregnant uterus, a testable hypothesis that we are presently pursuing.

The existence of such a system implies that the effects of each component should be additive if not potentially synergistic. Ideally, the components should not interfere with their respective immune deviating activities. It is interesting to note in this context that glycodelin-A,
uromodulin, and CA125 do not express galectin ligands (21, 23, 24, 78). This result indicates that these immune modulators should not interfere with galectins or with the recognition of the glycans expressed on STB, CTB or EVT. If components of the hu-FEDS model are involved in the induction of tolerance to the developing human in utero, then aberrant shifts in glycosylation could contribute to the pathological consequences that are observed during the development of the Great Obstetrical Syndromes (79).

Evidence consistent with this possibility was obtained in a recent study. Zhang et al. isolated integrin β1 from villous samples harvested at 6-9 weeks of gestation from patients that developed an early spontaneous miscarriage and normal controls (80). Lectin binding studies indicated that the level of N-glycans bearing Galβ1-4GlcNAcβ1-6 Man sequences on their antenna was decreased in integrin samples isolated from patients that experienced a miscarriage compared to controls. By contrast, bisecting type N-glycans were substantially elevated in the integrin samples derived from miscarriage patients compared to normal pregnancies. These shifts in glycosylation were correlated with the level of the N-acetylgalactosaminyltransferase enzymes designated Mgat 5 and GnT-III that add β1-6 linked GlcNAc and bisecting GlcNAc to the trimannosyl core of N-glycans, respectively. Future studies will be necessary to determine if specific shifts in glycan expression occur during the development of the other Great Obstetrical Syndromes.

Relationship to Other Mammals That Employ Hemochorial Placentation.

Eutherians undergo endotheliochorial, epitheliochorial, or hemochorial placentation during pregnancy (81, 82). Humans employ hemochorial placentation, as do many other mammals. This form of placentation requires direct contact between the maternal blood and trophoblasts at the
villous interface. Specialized adaptations are required to block maternal immune responses directed against trophoblasts in all species that employ hemochorial placentation.

Jones and coworkers investigated lectin binding to the villous interface in hemochorial placentas from five widely separated species of mammals: lesser hedgehog tenrec (*Echinops telfairi*), spotted hyena (*Crocuta crocuta*), nine-banded armadillo (*Dasypus novemcinctus*), human (*Homo sapiens*) and guinea pig (*Cavia porcellus*) (83). They analyzed the binding of 23 different lectins, including E-PHA and *Canavalia ensiformis* agglutinin (ConA). These investigators observed intense binding of E-PHA and ConA to the microvillous surface of STB from all five species, consistent with an elevated expression of bisecting type and high mannose type N-glycans. The profile of human STB glycans is dominated by high mannose type N-glycans and BBSCT N-glycans that bind to ConA and E-PHA, respectively (Fig. 2). Some minor differences in lectin binding were observed between these species, but overall the profiles were quite similar. Jones and coworkers suggested that the similarities in the glycosylation of the villous interface in these five species may have arisen by convergent evolution (83).

In summary, in this study we have performed glycomic analysis of CTB, STB and EVT. We observed complex N-glycosylation on all of these cell types. Based on the knowledge that immunoreactivity can be strongly modulated by the types of glycans present on trophoblasts, we propose that maternal-fetal tolerance is influenced by trophoblast glycosylation. Furthermore, we propose that alterations in glycosylation may, at least in part, contribute to inappropriate maternal-fetal immune response and poor pregnancy outcomes. Testing of these hypotheses will involve future work analyzing glycosylation in trophoblasts in normal and complicated pregnancies, characterization of glycosylation changes in trophoblasts during development, and
immunological analysis of any observed differences to determine their effects on maternal-fetal tolerance.

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Oligosaccharide specificity of galectins: a search by frontal affinity chromatography.

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Figure Legends

Fig. 1. Annotated MALDI-TOF MS spectra of permethylated N-glycans from CTB. The top panel shows the glycans in the mass range from 1499 to 3250 and the bottom panel shows the glycans in the mass range from 3249 to 4700. In the top panel, minor peaks are only labeled with their m/z values; their putative structures can be found in supplementary Table 2. Compared to the top panel, the bottom panel has been magnified approximately 53 times. Due to the fact that the peak signal in the mass range from 3900 to 4700 in the bottom panel is very weak, this part has been further magnified 5 times (upper trace). Profiles were obtained from the 50% acetonitrile fraction from a C18 Sep-Pak column. All ions are [M+Na]+. Putative structures are based on the molecular weight, N-glycan biosynthetic pathway, MS/MS data and enzymatic digestion results. Glycans at m/z 2966, 3777 and 4587 are clearly annotated, which is due to the fact that their structures are unequivocal because each antenna is capped with a sialic acid and thus they are homogeneous bi-, tri- and tetraantennary glycans. However, the glycan structure is not always as unequivocal as the glycan at m/z 2966 as biosynthetically non-fully sialylated glycan molecular ion species could be made up of mixtures of structural isoforms. Annotations are simplified to biantennary structures, with additional LacNAc units and capping sugars listed outside the bracket, for mixtures of isobaric multiantennary glycans, some of which have antennae containing extended LacNAc repeats.

Fig. 2. Annotated MALDI-TOF MS spectra of permethylated sialidase S treated N-glycans from CTB (upper panel), STB (middle panel) and EVT (lower panel). Profiles were obtained from the 50% acetonitrile fraction from a C18 Sep-Pak column. All ions are [M+Na]+. Putative structures are based on the molecular weight, N-glycan biosynthetic pathway, MS/MS data and enzymatic digestion results. Annotations are simplified to biantennary structures, with additional LacNAc units and capping sugars listed outside the bracket, for mixtures of isobaric multiantennary glycans, some of which have antennae containing extended LacNAc repeats. Minor peaks are only labeled with their m/z values; their putative structures can be found in supplementary Table 2.
Fig. 3. Annotated MALDI-TOF MS spectra of permethylated N-glycans from STB86. The top panel shows the glycans in the mass range from 1499 to 3250 and the bottom panel shows the glycans in the mass range from 3249 to 4700. In the top panel, minor peaks are only labeled with their m/z values; their putative structures can be found in supplementary Table 2. Compared to the top panel, the bottom panel has been magnified approximately 37 times. Due to the fact that the peak signal in the mass range from 3900 to 4700 in the bottom panel is weaker, this part has been further magnified 5 times (upper trace). Profiles were obtained from the 50% acetonitrile fraction from a C18 Sep-Pak column. All ions are [M+Na]+. Putative structures are based on the molecular weight, N-glycan biosynthetic pathway, MS/MS data and enzymatic digestion results.

Glycans at m/z 2966, 3777 and 4587 are clearly annotated, which is due to the fact that their structures are unequivocal because each antenna is capped with a sialic acid and thus they are homogeneous bi-, tri- and tetraantennary glycans. However, the glycan structure is not always as unequivocal as the glycan at m/z 2966 as biosynthetically non-fully sialylated glycan molecular ion species could be made up of mixtures of structural isoforms. Annotations are simplified to biantennary structures, with additional LacNAc units and capping sugars listed outside the bracket, for mixtures of isobaric multiantennary glycans, some of which have antennae containing extended LacNAc repeats.

Fig. 4. Annotated MALDI-TOF MS spectra of permethylated N-glycans from EVT. The top panel shows the glycans in the mass range from 1499 to 3250 and the bottom panel shows the glycans in the mass range from 3249 to 4700. Minor peaks are only labeled with their m/z values; their putative structures can be found in supplementary Table 2. Compared to the top panel, the bottom panel has been magnified approximately 12 times. Profiles were obtained from the 50% acetonitrile fraction from a C18 Sep-Pak column. All ions are [M+Na]+. Putative
structures are based on the molecular weight, N-glycan biosynthetic pathway, MS/MS data and enzymatic digestion results. Glycans at m/z 2966, 3777 and 4587 are clearly annotated, which is due to the fact that their structures are unequivocal because each antenna is capped with a sialic acid and thus they are homogeneous bi-, tri- and tetraantennary glycans. However, the glycan structure is not always as unequivocal as the glycan at m/z 2966 as biosynthetically non-fully sialylated glycan molecular ion species could be made up of mixtures of structural isoforms. Annotations are simplified to biantennary structures, with additional LacNAc units and capping sugars listed outside the bracket, for mixtures of isobaric multiantennary glycans, some of which have antennae containing extended LacNAc repeats.

Fig. 5. Differential glycosylation pattern expression on human trophoblast. This figure summarizes the types of glycans that are characteristic of the cytotrophoblast (CTB), syncytiotrophoblast (STB) and extravillous cytотrophoblast (EVT). All three cell types express abundant high mannose glycans in addition to complex glycans. BBSCT N-glycans are found in all cell types, but are more abundant in CTB and STB than in EVT. EVT cells displayed higher levels of multiantennary and polylactosamine type N-glycans than CTB and STB. Abbreviations: fetal vessel (fv); maternal blood; uterine artery (ua). □ GlcNAc ○ Man ○ Gal ▲ Fuc ◊ NeuAc.
Figure 2
Figure 4
Figure 5

[Diagram of fetal anchoring villus with labels STB, CTB, fv, mb, EVT, and ua.]

[Diagram showing molecular structures similar to the fetal anchoring villus.]