The human fetoembryonic defense system hypothesis: twenty years on

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1. Background

Immunological studies in the 19th and early 20th centuries were focused primarily on the development of vaccines directed against specific pathogens that caused substantial morbidity and mortality (1). This emphasis on therapeutic applications later shifted to the development of antibiotics and other therapies primarily for the victims of war (2). Prior to World War II, the British biologist Peter Medawar studied nerve regeneration and the conditions for optimizing cell culture. Soon after war began, he was enlisted by the Medical Research Council to determine why skin grafts between different individuals were rapidly rejected (3, 4).

These studies led Medawar to refocus his research effort in the areas of immune tolerance and organ transplantation where he made seminal experimental discoveries that supported the theory of acquired immunological tolerance that had been proposed by the Australian virologist Frank Macfarlane Burnet (5). Medawar's subsequent experiments confirmed that early engraftment of donor splenocytes into a mouse induced tolerance to future grafts derived from the donor strain but not from other histoincompatible strains (6). During Medawar's studies, he soon realized that the developing eutherian *in utero* was similar to a foreign organ transplant due to the contribution of paternal antigens. In 1953, he presented this paradox of immune accommodation in the form of a specific question: "How does the pregnant mother nourish within itself for many weeks or months a fetus that is antigenically a foreign body?" (7). This enigma provided a powerful impetus for the development of the area of investigation known as reproductive immunology.

Medawar suggested three different hypotheses to explain this maternal tolerance of her histoincompatible offspring: (i) anatomical separation; (ii) the immaturity of fetal antigens; and (iii) the inertness of the maternal immune response during pregnancy (7). Extravillous cytotrophoblasts (EVT) of fetal origin work in collaboration with maternal decidual leukocytes to remodel uterine spiral arteries into flaccid conduits for blood flow that support fetal development, ruling out anatomical separation (8, 9). Studies in animal models confirm that immunity is triggered by injections of fetal tissue or cells into adult animals, indicating that fetal antigens are mature (10). The immune system of pregnant women responds robustly to

different pathogens, confirming that maternal indolence is not applicable (11). In summary, all of Medawar's hypotheses were unsupported by subsequent experimentation.

2. Development of Models for the Induction of Tolerance to the Human Fetus

The human fetus primarily exists as a hemiallograft during a natural pregnancy. However, the fetus is an allograft if the surrogate mother is unrelated to the genetic parents. This type of surrogate pregnancy was impossible before the advent of human IVF techniques, but has become relatively common today (12). The ability to accommodate the histoincompatible fetus to normal term indicates that a very powerful system of immune deviation must exist in the gravid human uterus and placenta to block histocompatibility-based reactions. Several different models have been suggested to play a role in the uterine accommodation of the histoincompatible human fetus during pregnancy. These models (with one exception) do not rely on carbohydrate recognition. Briefly, these models rely on: (i) the broad immunomodulatory effects of HLA-G; (ii) the expansion of regulatory T (Treg) cells that induce tolerance; (iii) increased expression of TGF- β and PD-L1 which prime Treg cells; (iv) downregulation of NKG2D, a crucial NK cell receptor; (v) stimulation of the pathway for the TNF-related apoptosis-inducing ligand (TRAIL); and (vi) factors that control the migration of maternal T cells at the implantation site (13-20).

The possibility that carbohydrate recognition could play a major role in the induction of tolerance during pregnancy was initially suggested in 1996 (21). Specific carbohydrate sequences linked to glycoproteins and mucins were proposed to act as functional groups that would interact with lymphocytes and inflammatory cells to inhibit potential immune responses that could be directed against both the fetus and the gametes. Coordination of different carbohydrate dependent recognition pathways were proposed to exist constituting a system. The evidence indicated that a human fetoembryonic defense system (Hu-FEDS) was integral for this protective effect (21). There was evidence that human sperm-egg binding was dependent on the recognition of the sialyl-Lewis^x carbohydrate sequence at the time that the Hu-FEDS model was initially proposed (22, 23). This proposal suggested that immune recognition of the human egg was also mediated by recognition of sialyl-Lewis^x sequences presented on the glycans attached to zona pellucida (ZP) glycoproteins. This hypothesis and its potential linkages to pathogenesis were powerfully endorsed by Nobelist Robert Edwards, who was renowned for his contributions to the development of human IVF (24, 25). Nonetheless, the Hu-FEDS hypothesis was not considered in a recent review of the different pathways for the induction of tolerance to the histoincompatible fetus (18).

3. Immunological Methods Employed to Define Medawar's Paradox

When Medawar initially proposed this paradox in 1953, the tools for assessing immune function were highly limited, as was knowledge about how the immune system worked. This lack of understanding made the identification of the mechanism for promoting fetal accommodation in the uterus extremely challenging. Fortunately, there was considerable interest in defining the central molecular mechanisms that operated in the human immune system. The methods and the logic that were developed in other subdisciplines of immunology were rapidly integrated into strategies that were employed by reproductive immunologists to investigate Medawar's paradox.

Leukocytes were induced to proliferate in the presence of phytohemagglutinin (PHA) in 1959 (26, 27). Subsequent glycobiological analyses confirmed that the PHA preparations employed in these studies consisted of a mixture of two different carbohydrate binding proteins, lectins, erythroagglutinating phytohemagglutinin (E-PHA) and leucoagglutinating phytohemagglutinin (L-PHA) (28). L-PHA was shown to be solely responsible for the lymphoproliferative effects of these PHA preparations (28). A similar mitogenic effect was also observed in the presence of two other lectins, Concanavalin A (Con A) and pokeweed mitogen (PWM) (29-31). Modern glycobiological analyses have identified the specific carbohydrate sequences that are bound by L-PHA, ConA and PWM (32-35). The ability of different uterine and placental factors to block the proliferative effect induced by these mitogenic lectins was employed in early studies to assess specific immune suppressive activity of glycoproteins and mucins isolated from the gravid uterus.

Scientists and physicians who were interested in organ transplantation also pioneered methods for gauging potential immune responses that a transplant recipient would direct against the donated organ. The mixed lymphocyte reaction (MLR) assay was originally developed to be essentially an *in vitro* histocompatibility test (36). During the classical allogeneic "one way MLR", an equal number of stimulator and responder lymphocytes from histoincompatible donors are mixed together. The stimulator cells had been irradiated or treated with mitomycin C to inhibit their proliferation before mixing. After several days in culture, the responder lymphocytes would usually undergo blast formation and proliferation in response to the allogeneic human leukocyte antigen (HLA) class I and class II antigens presented on the stimulator cells. They incorporate either [³H] thymidine or 5-bromo-2'-deoxyuridine (BrdU) into the DNA of cells resulting from this proliferative response. The degree of histocompatibility in this assay is inversely proportional to the level of incorporation of these indicators in lymphocyte culture. The inhibition of the human MLR assay was another method that was employed to measure the immune suppressive activity of different factors in the pregnant uterus during these early studies (36).

Another method that was employed in early studies to measure immune suppressive activity is the antigen-specific lymphocyte transformation assay (37). In this assay, human lymphocytes are usually stimulated with either tetanus toxoid or diphtheria toxoid. These antigens induce blast formation and lymphocyte proliferation, which could also be measured by the

incorporation of either [³H] thymidine or BrdU. The ability of different pregnancy-associated factors to inhibit antigen-induced lymphocyte proliferation was also employed as a measure of immune deviating activity.

Today, these early methods have been augmented by the far more powerful and specific tools of modern immunology. These methods have certainly provided more information about the consequences of the diversity of different types of immune deviation that occur during pregnancy. Nonetheless, even at this point, there is agreement that Medawar's enigma has not been solved (18). **Solution of this enigma is essential for improving outcomes in obstetrics. Pregnant women suffer from many different pathological disorders including preeclampsia, intrauterine growth restriction, preterm labor, preterm premature rupture of membranes, late spontaneous abortion, and abruptio placentae. The molecular basis underlying these disorders, known collectively as the Great Obstetrical Syndromes, have not been defined (38). Whether aberrant glycosylation is the underlying cause of these syndromes needs to be determined.**

4. The Role of Different Immune Cell Types in the Uterus

The delineation of immune effector cell populations has led to the realization that uterine immune cells are functionally distinct from those found outside the uterus. The major immune cell type in the uterus is the natural killer (uNK) cell. uNK cells clearly display a phenotype that is quite different from NK cells found elsewhere in the circulation and peripheral tissues (pNK cells) (39, 40). One of the crucial functions of uNK cells is to collaborate with placental trophoblasts to remodel spiral arteries into flaccid conduits that supply the placenta with blood (41-43). uNK cells also differentiate into large granular lymphocytes during early pregnancy that support trophic functions that promote fetal development (44). Nearly complete elimination of uNK cells as a result of different gene knockouts in mice results in abnormal decidualization and vascularization of the placental bed (45). A consequence of these effects is infertility.

Macrophages constitute another major immune cell type in the pregnant uterus. They play a major role in the deviation of the innate and adaptive immune responses in the pregnant uterus, primarily by secreting a major immune suppressive cytokine (IL-10) and producing prostaglandin E₂ (PGE₂) and indoleamine dioxygenase (46-51). Like uNK cells, uterine macrophages have been proposed to display a specific phenotype (M2) that differentiates them from macrophages present in other organs and tissues (52-54). The M2 phenotype skews the responses toward tolerance rather than the classical immunological activities associated with this effector cell type.

The pregnant uterus is also a haven for regulatory T cells (Treg) that promote fetal accommodation as opposed to classical T cell populations that initiate responses directed against antigens associated with pathogens and tumor cells (55, 56). Treg were initially referred to as suppressor T cells (57). Early evidence supporting Treg activity during pregnancy was obtained by investigating the immune response of female C57BL/6 mice that had been

subjected to multiple syngeneic pregnancies. These multiparous mice displayed delayed rejection of syngeneic male skin grafts expressing the male-associated H-Y antigen as compared to virgin recipients (58). More importantly, this tolerance to the H-Y antigen could be adoptively transferred to syngeneic virgin mice recipients via thymus-dependent cells derived from multiparous females. Excellent reviews focused on Treg expression in the gravid human and mouse uterus are available (14, 17, 56, 59)

5. Glycoconjugates with Immune Deviating Activities Associated with Human Reproduction

Glycoproteins and glycolipids that are specifically expressed in the pregnant uterus have been shown to express carbohydrate sequences that react with specific lectin-like receptors on the surface of immune and inflammatory cells. In some cases, the possibility that carbohydrate sequences are acting as functional groups has been inferred by the differential immune suppressive activities of isoforms obtained from specific organs, tissues or fluids.

5. 1 Human α -Fetoprotein (AFP)

AFP is a member of the albumin family that was initially detected in human serum in 1956 (60). It is a 67 kDa sialylated glycoprotein that is N-glycosylated at Asn-233 (61). AFP is synthesized by embryonic yolk sac cells and fetal liver during early development (62). It is readily detected at elevated levels in the amniotic fluid of fetuses suffering from central nervous tissue disorders (63). AFP is also a major protein in the fetal circulation during the first trimester that is gradually replaced by human serum albumin as development proceeds, becoming a very minor component in the serum six months after parturition (64).

AFP mediates diverse immunomodulatory effects *in vitro* (65-74). Early studies indicated that this glycoprotein inhibited human lymphocyte transformation induced by lectins, anti-human thymocyte serum, and mixed lymphocyte culture. Difference in the immune suppressive activities of different isoforms of AFP isolated from different sources suggested that its glycan could be acting as a key functional group. Glycomic analysis of serum AFP glycoform derived from hepatocellular carcinoma and non-seminomatous germ cell tumors has been performed (75). The investigators in this study defined seven biantennary N-glycans on the tumorigenic AFP, which differed in their degrees of sialylation, galactosylation and core fucosylation. Four simple O-glycan structures were also detected. Butterfield and coworkers recently reported that AFP derived from hepatocellular carcinoma cells diminished the differentiation and T cell stimulatory activity of human dendritic cells (76).

AFP is also expressed during the development of some types of cancer.(65) In 1962, Abelev and coworkers isolated a protein from mouse hepatoma (designated hepatoma antigen) that was

subsequently identified in fetal liver and serum (77). This murine form of AFP was the first oncofetal antigen ever identified (78). The current diagnostic test for hepatocellular carcinoma based on AFP expression in the serum was developed by Abelson and coworkers in 1967 (79).__

5.2 Human Chorionic Gonadotropin (hCG)

hCG is a heterodimeric 26 kDa glycoprotein hormone consisting of an α subunit (92 amino acids) and a β subunit (145 amino acids) (80, 81). It is synthesized by human syncytiotrophoblast (STB) and released into the maternal circulation. Modern pregnancy tests are based on the detection of this glycoprotein hormone in maternal urine (82, 83). hCG is also employed to induce the final maturation of human eggs during IVF stimulation protocols (84).

The potential functional significance of its glycan sequences in ~~the~~ mediating ~~of~~ its biological activities has been the subject of intense investigation. The glycosylation of hCG has been suggested to vary depending on the stage of pregnancy, the site of its synthesis, and the pathological state (85). This glycoprotein has been reported to mediate many different immune deviating effects in different *in vitro* assays. These effects are summarized in **Table 1**.

How the glycosylation of hCG affects its biological activities is a topical issue with this glycoprotein. Bahl and coworkers were the first investigators to report that each subunit is decorated with two N-linked glycans (80, 81). The β -subunit also bears four O-linked sequences (81). The total carbohydrate content of hCG was determined to be 30% based upon these early studies.

Cole and coworkers sequenced the glycans associated with hCG by employing classical methods of structural analysis in combination with glycosidase digestion and pulsed amperometric methods to detect oligosaccharides (86). However, these investigators did not perform methylation analysis to determine the positions of substitution of the monosaccharides within the glycans. They also did not employ any of the sensitive biophysical tools available at the time, such as fast atom bombardment-mass spectrometry (FAB-MS) to precisely sequence the N- and O-glycans (86).

A “hyperglycosylated” form of hCG (H-hCG) was initially isolated from the urine of a patient that developed choriocarcinoma (86, 87). An antibody directed against H-hCG was subsequently developed, enabling the detection of H-hCG at high levels in the maternal blood during the early first trimester (87). However, the structural differences between hCG and H-hCG have not been fully defined.

5.3 Human leukocyte antigen G (HLA-G)

HLA-G is a nonclassical major histocompatibility complex (MHC) class I (MHC-1b) antigen that was initially identified in human trophoblasts in 1990. MHC is a set of cell surface proteins necessary for the adaptive arm of the immune system to recognize foreign molecules. HLA-G displays greatly restricted allelic polymorphism compared to the major classical HLA class I antigens (88). An excellent review of the HLA-G gene and its different isoforms and multimeric presentations has been published recently (89). This glycoprotein is specifically expressed on human EVT in the pregnant uterus (90). A soluble form of HLA-G has also been detected (91, 92). Many investigative groups have proposed that soluble and cell surface associated forms of HLA-G represent the major immune modulatory factor in the uterus that suppresses the maternal immune response directed against the fetus (93-96).

All HLA class I molecules including HLA-G bind to immunoglobulin-like transcript 2 (ILT2 also known as LILRB1), an immune receptor that is broadly expressed on NK cells, T cells, B cells, monocytes, macrophages and dendritic cells (DC) (97). ILT4 (LILRB2) is a closely related receptor with the same binding specificity, but its expression is limited to monocytes, macrophages and DCs (98). Both ILT2 and ILT4 possess cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs), suggesting that they transmit a negative signal to the immune cell after binding to a HLA class I molecule. Other immune cell receptors that bind to HLA-G include the killer cell immunoglobulin-like receptor KIR2DL4, CD8, and CD160 (99-101).

HLA-G is a glycoprotein that bears one N-linked glycan at Asn-86 like other classical HLA class Ia antigens (102, 103). However, HLA-G linked to EVT is decorated with polylectosamine sequences, unlike the classical HLA class I molecules that exist as a relatively restricted set of glycoforms (102). The conclusion that HLA-G bears polylectosamine sequences is based on a major decrease in its molecular weight that is observed after digestion of this glycoprotein with endo- β -galactosidase (102).

Polylectosamine sequences bind to several galectins, including gal-1, gal-3, gal-8, and gal-9 that are abundant in the pregnant human uterus (104, 105). There is credible evidence for galectin binding to HLA-G. Blois and coworkers recently reported that the incubation of galectin-1 (gal-1) with the human EVT cell line HIPEC65 increased the surface expression of HLA-G by 2.1 fold (106). It is quite possible that gal-1 stabilizes the expression of HLA-G on the cell surface by binding to its carbohydrate sequences. HLA-G could primarily function as a platform that binds galectins that mediate diverse immune deviating activities *in vivo* (107, 108). This hypothesis focused on this aspect of HLA-G interaction needs to be thoroughly tested.

The immune modulatory activities of recombinant forms of HLA-G have been investigated in many cell types, whereas the native glycoforms of HLA-G synthesized by human EVT cytotrophoblasts have not (89, 109, 110). Recombinant HLA-G was employed because of the inability to easily obtain sufficient amounts of the natural glycoprotein from EVT. Whether recombinant HLA-G synthesized in these non-uterine cell types is decorated with polylectosamine sequences or not is unknown at this time. The addition of bulky

polylactosamine sequences to HLA-G could affect its interaction with its proposed receptors on immune cells (ILT2, ILT4, KIR2DL4, CD8, CD160). The binding of galectins to HLA-G in the pregnant human uterus could also have a substantial effect on its interactions with these immune cell receptors. Future studies focused on the immune deviating activities of HLA-G should test specific glycoforms that bear polylactosamine type sequences. Galectin binding to HLA-G bearing these sequences should also be investigated to determine if the presence of these lectins affects the interaction of HLA-G with immune receptors. Such experiments would more closely approximate the physiological environment in the pregnant uterus.

The discovery of HLA-G greatly decreased interest in investigating other pregnancy associated glycoconjugate factors that clearly manifest immune deviating activities *in vitro*. This focus on HLA-G has been due solely to its connection to the MHC complex, a linkage that is deeply rooted in classical immunology (90). It would indeed be ironic if future experiments reveal that the major immune-deviating activities of HLA-G in the pregnant uterus rely primarily on its N-linked polylactosaminoglycans.

5.4 Glycodelin (Gd)

Gd is a glycoprotein that is also known as placental protein 14 (PP14), pregnancy-associated endometrial alpha-2 globulin (PAEG), and progestagen-associated endometrial protein (PAEP). Gd is the name that is now broadly accepted because this glycoprotein is: (i) synthesized in the endometrium and not by placental cells, though it was originally isolated from this organ (111, 112); (ii) also expressed in the male reproductive system and immune cells of megakaryocytic lineage (113, 114); and (iii) differentially glycosylated depending on its origin (115-119).

The endometrial isoform of Gd exists as a 27 kDa glycoprotein that is expressed from the mid-luteal phase until the end of pregnancy (120, 121). The level of endometrial Gd is at its highest between weeks 7-11 of development, representing 4-16% of the total protein synthesized by decidual cells (122). This glycoprotein is released into the amniotic fluid and taken up by the placenta, where it also becomes a major component known as placental protein 14 (PP14) (111). The synthesis of endometrial Gd declines rapidly after the 20th week of gestation, becoming a minor glycoprotein in the amniotic fluid and decidua (120, 122).

Gd was identified as a major immune suppressive factor in decidual tissue extracts in 1987. These extracts inhibited proliferation in mixed lymphocyte cultures (123). The level of this inhibitory effect was directly proportional to the amount of Gd in the culture. In addition, antibodies directed against Gd blocked this anti-proliferative effect, identifying this glycoprotein as the major decidual factor responsible for inhibiting lymphocyte proliferation in culture (123).

Gd isolated from amniotic fluid (GdA) affects many different immunological activities *in vitro*. GdA inhibits the synthesis of interleukins 1 and 2 (IL-1, IL-2) as well as soluble IL-2 receptors by mononuclear cells isolated from peripheral blood (PBMC) (6-8). This glycoprotein also inhibits

the cytotoxic response of pNK cells directed against K562 erythroleukemia cells via the caspase-independent induction of apoptosis (124, 125). GdA has also been reported to bind to the glycans expressed on CD45 presented on T cells via a lectin-like activity (126). GdA also decreases IgM secretion and HLA class II expression in B lymphocytes (127). This glycoprotein also inhibits the interaction of neutrophils with the vascular endothelium mediated by E-selectin (128). GdA also binds to Siglec-6, a receptor associated with the surface of trophoblasts (129). In summary, the myriad immune suppressive activities of GdA and its prodigious expression during an early stage of remodeling of the maternal spiral arteries suggest that this glycoprotein plays an essential role in the induction of tolerance in the gravid human uterus.

The crystal structure of the protein backbone of Gd synthesized in *E. coli* was recently defined (130). Unlike other lipocalins, the central pocket inside the β -barrel is completely absent, consistent with the inability of Gd to tightly bind hydrophobic ligands. This protein core is more closely packed than other lipocalins because it lacks this pocket. Gd also expresses a homodimeric quaternary structure that is optimized for the presentation of the N-glycans at its two N-glycosylation sites (Asn28, Asn-63). This glycoprotein primarily exists as a dimer with its four N-glycans presented in a very close spatial arrangement (patch) on the same side of the dimer. The investigators in this study suggested that this type of presentation likely optimizes the interaction of Gd with lectin-like receptors on other cells (130).

Only specific suborders of primates express orthologues of Gd during their menstrual cycle, suggesting that a role for this glycoprotein emerged only recently during evolution (130). Therefore a murine orthologue of Gd does not exist, indicating that knockout strategies are not accessible in the mouse model. In summary, all of the available evidence indicates that Gd is a platform for presenting specific carbohydrate functional groups. This concept was initially suggested over twenty years ago (115).

Several isoforms of Gd have been isolated in addition to GdA. They include forms present in seminal plasma (GdS), follicular fluid (GdF), cumulus matrix (GdC) and cells of megakaryocyte lineage (GdM). Ultra-high sensitivity mass spectrometry analysis has been performed on the N-glycans linked to these different forms with the exception of GdM (115-119). The three glycoforms present in the female urogenital tract (GdA, GdF and GdC) express complex type N-glycans terminated with both lacNAc (Gal β 1-4GlcNAc) and lacdiNAc (GalNAc β 1-4GlcNAc) antennae, in most cases decorated with fucose or α 2-6 linked sialic acid (118). The Sda antigen (NeuAc α 2-3(GalNAc β 1-4)Gal) was also detected at the terminal ends of some antenna in many of the multiantennary glycans (114). GdA was more highly sialylated than either GdF or GdC. Sialic acid was present only in the context of the Sda antenna on the N-glycans linked to GdC. GdA and GdF were similarly immunosuppressive, whereas GdC and GdS were not (118).

Interestingly, GdA obtained at term pregnancy from women with gestational diabetes had substantially reduced levels of α 2-6 linked sialic acid plus increased levels of Sda, compared with GdA from normal term pregnancies, and thus was similar to GdC (114). Like GdC, diabetic

GdA showed reduced immunosuppressive activities in *in vitro* assays. Another interesting discovery from the gestational diabetic study was the observation that bisected glycans were strikingly more abundant in normal GdA at term, compared with mid-trimester GdA.

The N-glycans linked to GdS are completely different from those associated with GdA, GdF and GdC (131). High mannose type N-glycans are linked exclusively at Asn-28. By contrast, Asn-63 is decorated with biantennary complex type N-glycans terminated with Lewis^x (Gal β 1-4[Fuc α 1-3]GlcNAc) and Lewis^y (Fuc α 1-2Gal β 1-4[Fuc α 1-3]GlcNAc). This complete absence of sialic acid is unusual for a secreted glycoprotein. Though GdS does not express the same immune suppressive activities as GdA or GdF, high mannose and oligosaccharides terminated with Lewis^x/Lewis^y sequences bind to DC-SIGN and the mannose receptor (132-134).

5.5 Cancer Antigen 125 (CA125) or Mucin 16 (MUC16)

CA125 was originally detected by screening monoclonal antibodies developed against human ovarian carcinoma cells (135). The tumor antigen that reacted with monoclonal antibody OC125 eventually turned out to be a very large mucinous glycoprotein (24,000 amino acids) of endometrial origin bearing numerous N- and O-glycans (136, 137). Both GdA and CA125 are synthesized in the endometrium during the mid-luteal phase up until the end of the first trimester, if pregnancy ensues (121). Its coincident expression with GdA and its re-expression during the development of cancer indicated that it could also be an immune deviating factor during the first trimester of pregnancy.

An initial issue that arose was which type of immune cell would be the target of CA125 during both pregnancy and tumor development in the ovary. As noted previously, uNK cells are the major immune cell type in the uterus. pNK cells are known to specifically lyse tumorigenic cells. uNK cells migrate toward the implantation site in response to a specific chemoattractant (chemokine MIP-1 α) that is secreted [by](#) trophoblasts. uNK cells actually make direct physical contact with trophoblasts at the implantation site. These trophoblasts do not express any HLA class I molecules that are recognized by NK cells. The possibility was considered that the elevated levels of both CA125 and GdA could block uNK cells from attacking trophoblasts at the site of implantation.

The ovarian carcinoma cell line designated OVCAR-3 was employed as a source of CA125. This mucinous glycoprotein was isolated from the media and subjected to glycomic analysis (137). Both core type 1 and type 2 O-glycans were detected. Unusually, a major portion of the core type 1 glycans were found to be branched (LacNAc β 1-4(LacNAc β 1-6)Gal). The content of N-glycans linked to CA125 was quite substantial, unlike other mucins. The N-glycan fraction contained primarily high mannose type N-glycans (Man₅-Man₉GlcNAc₂) and complex type bi/tri/tetra-antennary bisecting type N-glycans capped with either sialic acid or fucose (137).

Incubation of CA125 for three days at the concentrations present in the pregnant uterus with pNK cells inhibited their lytic activity by 50-70% (138). This loss of lytic activity was accompanied by a 40-70% decrease in the expression of a specific NK cell marker (CD16) compared to native pNK cells. A substantial proportion (90%) of pNK cells bind well to monoclonal antibodies directed against CD16 (CD16^{pos}), whereas uNK cells show substantially diminished binding of CD16 (CD16^{dim}) (139-141). This finding indicated that the incubation of CA125 with pNK cells shifted them toward the uNK phenotype.

Desialylation of CA125 led to a complete loss of its immune-deviating activities *in vitro* (142). This loss was correlated with the inability of desialylated CA125 to bind to pNK cells. The immune receptor on pNK cells that must be engaged to mediate these immune modulating activities was identified as Siglec-9 (142). The data obtained in these studies clearly indicate that CA125 could be yet another glycoconjugate that promotes tolerance of the histoincompatible human fetus during pregnancy.

5.6 Tamm-Horsfall Protein (THP) and Uromodulin

Tamm-Horsfall protein (THP) was the first glycoprotein to be purified to nearly complete homogeneity from human urine (143, 144). THP was initially shown to react with influenza, mumps and Newcastle disease virus (144). In 1986, Muchmore and coworkers isolated an 85 kDa glycoprotein designated uromodulin from the urine of pregnant women that blocked human T cell and monocyte assays *in vitro* at 10^{-9} - 10^{-11} M (145). Structural analysis of this immune deviating glycoprotein confirmed that uromodulin was the pregnancy associated form of THP (146). THP derived from males and nonpregnant females was 13 fold less active as an inhibitor in the antigen-induced T cell proliferation assay compared to uromodulin (147). This difference suggested that uromodulin was a differentially glycosylated form of THP produced by pregnant women (148). However, definitive biophysical analyses of the N-glycans linked to THP and uromodulin did not reveal any differences in their glycosylation (149, 150).

In contrast, application of FAB-MS analysis to the O-glycans linked to uromodulin confirmed the expression of unusual core 2 O-glycans terminated with up to three terminal sialyl-Lewis^x sequences (150). Like in CA125, many of these glycans exhibited branching of their core 1 antenna. By contrast, simple mono- and di-sialylated core 1 O-glycans, lacking antenna extension or branching, were observed in THP isolated from the urine of males and nonpregnant females. Analysis of THP derived from women two months after delivery indicated a complete reversion back to the same types of O-glycans observed in non-pregnant women (150). This latter observation indicates that the expression of these unusual O-glycans linked to uromodulin are under hormonal regulation (progesterone and/or estrogen) or some other pregnancy-associated factor(s). The role of uromodulin in the maintenance of tolerance during pregnancy is currently unknown, but remains a viable topic for future investigation.

5.7 Placental Gangliosides

Gangliosides are cell surface glycolipids that are composed of a glycosphingolipid (ceramide and oligosaccharide) with one or more sialic acid linked to the sugar chain. The major gangliosides in human placenta based on the percentage of lipid bound sialic acid include GM3 (~40%), GD1a (~10%), GD3 (8-10%) and GM1 (3%), (151). The remainder consist of neolacto- and branched polylactosamine type gangliosides that are exclusively capped with α 2-3 linked sialic acid (151, 152). This predominance of α 2-3 linked sialic acid is consistent with the elevated level of this enzyme in the placenta (153).

The immunomodulatory effects of placental gangliosides have also been investigated. GM3, GD1a, GD3 and GM1 specifically inhibit pNK cell activity by 55-70% at 50 μ M (154). How GM3, GD1A and GM1 mediate this specific immune deviating effect is unknown. By contrast, the oligosaccharide associated with GD3 has been reported to bind to Siglecs-5, -7 and -8 during glycan array analysis (155, 156). Crocker and coworkers demonstrated that GD3 on target cell surfaces decreases cytotoxic responses by NK cells via pathways that are both dependent and independent of Siglec-7 interactions (157). Sialylated nLc₆ (NeuAc α 2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc-Cer) derived from human placenta inhibits pNK cell cytotoxicity by 79% at 50 μ M. How this effect is mediated is unknown, but this ganglioside should be able to bind to many different galectins based on its carbohydrate sequence (104, 158-160).

6. Roles of Galectins in Pregnancy Related Immune Deviation

Galectins are proteins that specifically bind to β -galactoside sugars, such as N-acetyllactosamine (lacNAc) (161). Galectins have been associated with many different immune deviating activities (107, 108). A role for galectins in human pregnancy was suggested by the observation that a specific placental protein (PP13) is also a galectin (162). Subsequent analyses indicated that all human galectins are expressed in the human uterus during pregnancy (163). The major galectins in the pregnant uterus include gal-1, gal-3, gal-8, and gal-9 (105).

Galectins represent a group of thirteen lower molecular weight soluble lectins that express 1-2 binding sites with affinity for either lactose and/or N-acetyllactosamine (lacNAc) (161). However, some galectins can also bind to lacNAc sequences that have been modified with a terminal sequence (e.g. α 2-3 linked sialic acid) (159, 164). Many galectins also display enhanced affinity for N-glycans bearing multiple lacNAc terminals, while others bind more robustly to polylactosamine chains (104, 158, 159). Dimers and oligomers of galectins promote the formation of lattices within a single cell or between cells (158, 165). This capability enables galectins to participate in many different biological activities (158).

As noted previously, HLA-G expresses poly lactosamine sequences, which could enable this HLA class Ib molecule to bind with high affinity to gal-3, gal-8, and gal-9 in the human uterus (104, 105). Human placental membranes express exceptionally high levels of α 2-3 sialyltransferase compared to α 2-6 sialyltransferase activity (153). This result suggests that the majority of the sialylated N- and O-glycans in the placenta are terminated with α 2-3 linked sialic acid. Galectins readily accommodate terminal NeuAc α 2-3Gal β 1-4GlcNAc but not NeuAc α 2-6Gal β 1-4GlcNAc. This result suggests that many other placental glycoproteins could be ligands for galectins (104, 158, 159). However, this result has yet to be demonstrated. Galectins are also neoexpressed in metastatic tumor cells, further supporting the overlap between the environment in the pregnant uterus and aggressive cancers (166, 167).

7. Glycans and the Reproductive Imperative: Linkage to Cancer

Cancer is a pathological state that involves the aberrant division of cells in organs, tissues or the circulatory system (168). Malignant cells in solid tumors or tissues have the ability to invade other parts of the body (metastasize) to form tumors at distal sites. Metastatic tumors wreak havoc by negatively impacting normal physiological processes and crowding out essential cells, tissues and organs. The outcome of metastatic cancer is usually organ failure resulting in a fatal outcome. Cancer has been suggested to be the 'Emperor of all Maladies' due to its wide range of clinical presentations and numerous pathological effects (169).

There are eight distinct hallmarks of cancer that have been universally recognized (170, 171). One of the most recent hallmarks to be formally accepted is immune evasion (170). Due to genomic constraints, neoplastic cells likely employ existing pathways to inhibit antitumor responses. There are specific organs and tissues in the body where the immune response is highly regulated to prevent bystander injury that could compromise either reproduction or survival (172). These organs and tissues are known as immune privileged sites. Classical immune privileged sites include the brain, the testes, the fetus, the placenta, the anterior chamber of the eye and the inner ear (11, 173-175).

Evidence that tumor development induces shifts in the glycosylation of glycoproteins was obtained as early as 1956 (176, 177). The neoexpression of many carbohydrate sequences on tumor cells was more readily detected by employing monoclonal antibodies (MAbs) (178, 179). Sequences that were defined by employing MAb technology are often referred to as tumor associated carbohydrate antigens (TACAs) (180). The major human TACAs and tumor associated carbohydrate sequences are outlined in Table 2. There is compelling evidence that aggressive tumor cells (but not their progenitor cells) express TACAs that also act as carbohydrate functional groups for immunomodulatory glycoconjugates in the human reproductive system (placenta, uterus, gametes, and testes).

Some of the human tumor associated carbohydrate sequences presented in Table 2 are expressed at immune privileged sites unrelated to reproduction. For example, gangliosides GM2, GD2, and GD3 are found in the human brain (181-183). These glycolipids are overexpressed in many neoplastic cell types and have been the targets of many immunotherapeutic approaches (179, 184-196). This interaction could evoke tolerance in the immune cell types that express these glycolipids. GM2 and GD2 could also be expressed in the reproductive organs, but at this time there is no confirmation. Only those tumor associated carbohydrate sequences that have been shown to be specifically expressed in the male and female reproductive system will be considered here.

7.1 Lewis^y Antigen (*Fuc*α1-2*Gal*β1-4[*Fuc*α1-3]*GlcNAc*-)

The Lewis^y antigen was the first oligosaccharide epitope to be defined as a TACA based on its binding to the monoclonal antibody (MAb) designated AH6 (197). However, the AH6 MAb was not sufficiently specific to detect Lewis^y due to its cross-reactivity with the H2 antigen (*Fuc*α1-2*Gal*β1-4*GlcNAc*). To address this issue, Hellstrom and colleagues developed two other MAb (BR64, BR96) that displayed specific binding to Lewis^y (198). Immunostaining with these MAb indicated that 70% of all adenocarcinomas but not their precursor cells expressed Lewis^y (198).

Lewis^y binds with high affinity to the dendritic cell lectin known as DC-SIGN (133, 199). This TACA also binds to the mannose receptor with lower affinity (132-134). Lipopolysaccharides bearing this sequence induce the dissociation of the KSR1-CNK-Raf-1 complex from the signalosome (200). This dissociation triggers the increased release of IL-10 from dendritic cells coincident with the decreased expression of IL-12 and IL-6. Overall, this effect results in a Th1→ Th2 shift that favors tolerance.

Lewis^y is abundantly expressed on human sperm and seminal plasma glycoproteins in the male urogenital tract (201, 202). However, this sequence is almost undetectable in human females. Free oligosaccharides terminated with Lewis^y are also present in human seminal plasma (203). Another significant aspect of Lewis^y is its expression in some pathological states including breast and pancreatic cancer. Lewis^y is also a major sequence linked to the lipopolysaccharides associated with strains of *H. pylori* that induce tolerance, and it is expressed on CD4⁺ T cells in HIV infected patients (204-207). Cercarial forms of schistosomes express a close structural analogue known as pseudo-Lewis^y (*Fuc*α1-3*Gal*β1-4[*Fuc*α1-3]*GlcNAc*) that binds with higher affinity to DC-SIGN than Lewis^y (208, 209).

7.2 Sialyl-Lewis^x Antigen (*NeuAc*α2-3*Gal*β1-4[*Fuc*α1-3]*GlcNAc*-)

Sialyl-Lewis^x was initially isolated from a ganglioside fraction of human kidney in 1976 (210). In 1984 sialyl-Lewis^x was discovered on the polylactosamine antennae of N-linked carbohydrates in

glycoproteins isolated from healthy human granulocytes and was subsequently shown to be more highly expressed in chronic myelogenous leukemia cells (211, 212). Around the same time, this sequence was confirmed as a TACA, based on its binding to the MAb designated CSLEX (213). CSLEX reacted with tumor cells associated with lung, esophageal, ovarian, breast, pancreatic, bladder stomach and colorectal cancers, but did not bind to progenitor cells or tissues (Table 2) (213-221). Sialyl-Lewis^x is also substantially elevated on serum glycoproteins in patients with lung, ovarian, stomach and breast cancer (222-226). This sequence was also identified as the universal ligand for E-, P- and L-selectin binding (227, 228). Sialyl-Lewis^x would later be identified as the major carbohydrate sequence that mediates the binding of human sperm to the outer covering of the human egg, known as the zona pellucida (229).

The expression of sialyl-Lewis^x on the surface of tumor cells has been associated with their interactions with selectins on the endothelium thereby promoting metastasis (230). Sialyl-Lewis^x is also a ligand for Siglec-9 on specific immune cell populations, enabling tumor cells to evoke a tolerizing response (231, 232). The secretion of tumor cell glycoproteins bearing this sequence could also inhibit lymphocyte recirculation and antigen presentation. As indicated in studies with uromodulin, glycoproteins bearing sialyl-Lewis^x on their glycans could also block the antigen-induced proliferation of T cells, a prerequisite for mounting an adaptive immune response (145, 150). Clearly, tumor cells that decorate their glycoproteins with sialyl-Lewis^x could inactivate the immune system in cancer patients.

7.3 *Gangliosides GD2 (NeuAc α 2-8NeuAc α 2-3[GalNAc β 1-4]Gal β 1-4Glc-Cer) and GD3 (NeuAc α 2-8NeuAc α 2-3Gal β 1-4Glc-Cer)*

The shedding of antigens and membranes by tumor cells was initially reported in 1975 (233). Elevation in the level of circulating gangliosides was subsequently reported in female breast and colorectal cancer patients (234). Ganglioside GD3 was first reported in human malignant melanoma in 1979 (235). Ganglioside GD3 is the precursor for ganglioside GD2, so they are often found in similar amounts on tumor cell surfaces. These gangliosides are often elevated on melanoma, neuroblastoma, small cell lung cancer, glioma, fibrosarcoma, retinoblastoma, liposarcoma, Ewing's sarcomas, leiomyosarcoma, rhabdomyosarcoma, malignant fibrous histiosarcoma, spindle cell sarcoma and osteosarcoma (236-242). They have been utilized as targets for immunotherapy directed against these tumor types (189, 243). The specific immune deviating pathway evoked by ganglioside GD3 has been discussed previously (154).

7.4 *Polylactosaminoglycans (Gal β 1-4GlcNAc β 1-3[Gal β 1-4GlcNAc]_n)*

Cold agglutinin disease is a rare blood disorder that is also known as autoimmune hemolytic anemia. This disease was first described in 1957 (244). This disorder is caused primarily by a humoral response directed against Ii blood group antigens (245). The basic unit of the i

antigenic determinant was subsequently determined to be a linear polylectosamine sequence (Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc) (246). The basic unit of I antigenic determinant was subsequently reported to be a branched polylectosamine sequence (Gal β 1-4GlcNAc β 1-3[Gal β 1-4GlcNAc β 1-6]Gal β 1-4GlcNAc) (247, 248). Linear polylectosamine sequences are often readily detected during glycan analysis due to their sensitivity to digestion with endo- β -galactosidase (249-251).

Polylectosamine sequences are overexpressed on many different types of organ-specific tumors and leukemias (Table 2) (212, 252-273). The broad distribution of polylectosaminoglycans on human neoplastic cells is consistent with specific functional roles. Many different carbohydrate sequences are expressed on the terminal ends of polylectosaminoglycans, so they could be useful for projecting terminal sequences that can interact with immune lectins. Several galectins (gal-3, gal-8, and gal-9) that bind with high affinity to polylectosamine sequences are expressed at elevated levels in the pregnant uterus (104, 105). As noted previously, HLA-G expresses polylectosamine chains on its N-glycans, unlike the other classical HLA molecules. Human fetal fibronectin and trophoblast integrins are also decorated with polylectosamine sequences (274, 275). It is likely that many different glycoproteins in the pregnant uterus will be decorated with polylectosamine sequences.

7.5 Fucosylated LacdiNAc Sequence (GalNAc β 1-4[Fuc α 1-3]GlcNAc)

As noted earlier, the fucosylated lacdiNAc sequence is expressed on the glycodelin isoforms designated GdA, GdF, and GdC (115, 118). This sequence is also present at elevated levels on the tegmental surface of schistosomes (276, 277). Like many other fucosylated sequences, this sequence is also a ligand for DC-SIGN (278). However, not much is currently known about the specific immune-deviating activity of terminal fucosylated lacdiNAc. This sequence has thus far only been detected on two human colorectal cancer cell lines (279). LIM1899 cells were isolated from a moderately differentiated primary cancer, whereas LIM2405 was derived from a poorly differentiated primary cancer.

8. Summary

The Hu-FEDS hypothesis suggesting that functional glycosylation could play a major role in protecting the gametes and the developing human in utero from both maternal and paternal immune responses was initially presented in 1996 (21, 25). The aggregate findings outlined in this review provide ample support for this model for immune evasion. However, it is apparent that this hypothesis has not been generally accepted by other scientists working in the area of reproductive immunology (13-20).

This issue is not without major consequences. **As noted previously, pregnant women suffer from several pathological states known collectively as the Great Obstetrical Syndromes (38).** These syndromes have a profoundly negative impact on maternal and/or fetal health. They are of great concern for both public and private funding agencies across the world. The potential role of aberrant glycosylation in these disease states has not been investigated at this time.

Additional support for the Hu-FEDS hypothesis comes from data confirming that the same carbohydrate functional groups that are associated with immune deviating glycoproteins in the human reproductive system are also neoexpressed in aggressive tumor cells. This expression is only logical since immune responses must be carefully regulated within the reproductive system to enable procreation between histoincompatible individuals. When this evidence is considered in aggregate, it is unlikely that this recapitulation is only a chance event. The possibility exists that such shifts in glycosylation could be absolutely required for the survival of aggressive tumor cells. Whether this extrapolation is correct or not is certainly a matter for future experimentation.

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Table 1: Major Immune Deviating Activities Associated with Human Chorionic Gonadotropin (hCG)

<u>Immune Deviating Activity</u>	<u>Reference</u>
<u>Inhibition of lymphocyte proliferation induced by PHA</u>	<u>Adcock et al., 1973</u>
<u>Inhibition of MLR assay with maternal and paternal lymphocytes</u>	<u>Teasdale et al., 1973</u>
<u>Inhibition of NK cell cytotoxicity</u>	<u>Sulke et al., 1985</u>
<u>Blockage of the development of Th1 autoimmune diabetes in the nonobese diabetes (NOD) mouse model</u>	<u>Khan et al., 2001</u>
<u>Attraction of regulatory T cells (Treg) to the fetal-maternal interface</u>	<u>Schumacher et al., 2009</u>
<u>Promotion of an immune tolerant microenvironment at the fetal-maternal interface</u>	<u>Zenclussen et al., 2006</u>
<u>Regulation of uterine NK cell proliferation via interaction with the mannose receptor</u>	<u>Kane et al., 2009</u>
<u>Upregulation of Fas Ligand (FasL) expression in the human endometrium</u>	<u>Kayisli et al., 2003</u>
<u>Upregulates MHC class II levels and increases IL-10 and Indole dioxygenase (IDO) expression in activated dendritic cells</u>	<u>Wan et al., 2008</u>
<u>Upregulates IDO in the placenta</u>	<u>Ueno et al., 2007</u>
<u>Induces IL-8 production by monocytes</u>	<u>Kosaka et al., 2002</u>

Table 2: Tumor Associated Carbohydrate Sequences

<u>Tumor Glycan</u>	<u>Carbohydrate Sequence</u>	<u>Organ/Tissue/Cell*</u>
<u>Sialyl-Tn</u>	<u>NeuAcα2-6GalNAc</u>	<u>Pan, Brst, Col, Ova, Pro, Lu</u>
<u>Sialyl-Lewis^x</u>	<u>NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc</u>	<u>Pan, Brst, Col, Lu, Eso</u>
<u>Sialyl-Lewis^a</u>	<u>NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAc</u>	<u>Pan, Brst, Col, Lu, Eso</u>
<u>GD2</u>	<u>GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc-Cer</u>	<u>Mel, Lym, Brn, Sar</u>
<u>GD3</u>	<u>Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc-Cer</u>	<u>Brn, Mel, Sar</u>
<u>Fucosyl-GM1</u>	<u>Fucα1-2Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc</u>	<u>Lu, Liv</u>
<u>Lewis^y</u>	<u>Fucα1-2Galβ1-4[Fucα1-3]GlcNAc</u>	<u>Ova, Pan, Brst, Col, Pro, Lu</u>
<u>Globo-H</u>	<u>Fucα1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc</u>	<u>End, Ova, Pan, Brst, Col, Pro, Lu</u>
<u>Polylactosamine</u>	<u>Galβ1-4GlcNAcβ1-3[Galβ1-4GlcNAcβ1-4]₁₋₁₅</u>	<u>Pan, Mel, Col, Cerv, Brn, Lym, Kid, Gas, Lu, Leu, Liv, EC, Thy</u>

TF (T antigen)	Galβ1-3GalNAc	Ova, Brst, Col, Pro
Polysialic acid	NeuAcα2-8 polymer	Brst, Ova Col, Pro
GM2	GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc	Ova, Pan, Brst, Col, Br, Mel, Lu
Fucosylated LacdiNAc	GalNAcβ1-4[Fucα1-3]GlcNAc	Col

[Key to symbols:](#)

[Key to abbreviations:](#) **Brn**, brain; **Brst**, female breast; **Cerv**, cervical; **EC**, embryonal carcinoma; **Endo**, endometrial; **Eso**, esophageal; **Gas**, gastric; **Kid**, kidney; **Leu**, leukemia; **Liv**, liver; **Lu**, lung; **Lym**, lymphoma; **Mel**, melanoma; **Ova**, ovary; **Pan**, pancreatic; **Pro**, prostate; **Sar**, sarcoma; **Cer**, ceramide