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HLA-G intracellular expression in decidua trophoblasts in a normal term placenta: a confocal and transmission electron microscopy study.

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ABSTRACT

The placenta acts as an immunological barrier between the mother and the fetal "graft", allowing two antigenically different organisms to tolerate one another. Human leukocyte antigen-G (HLA-G) may be involved in the mechanisms modulating the maternal immune system. Indeed, several authors have found that HLA-G is primarily expressed on the placental trophoblast plasmatic membrane.
However, pregnancy still remains a major immunological enygma and current studies are far from clarifying the mystery. To further investigate this matter, we undertook the current study, using laser scanning confocal (LSCM) and transmission electron microscopy (TEM), in order to deepen knowledge on HLA-G expression in placentas from normal pregnancies, following a spontaneous term delivery. Unexpectedly, we observed that HLA-G expression includes both vesicles and clusters, localized in the cytosol or close to the nucleus.
Keywords: placenta, HLA-G, confocal microscopy, transmission electron microscopy.

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SOMMARIO

La placenta agisce come una barriera immunologica tra la madre ed il " trapianto" fetale, permettendo la tolleranza reciproca di due organismi antigenicamente differenti . Il sistema HLA-G, Human Leukocyte Antigens, può essere coinvolto nei meccanismi che modulano il sistema immunitario materno nei processi di tolleranza. In effetti, diversi autori hanno scoperto che il sistema HLA-G è espresso principalmente sulla membrana plasmatica del trofoblasto placentare.
Tuttavia, la gravidanza rimane ancora uno dei principali enigmi immunologici e gli studi attuali sono ben lungi dalla soluzione del mistero. Per approfondire questo tema, abbiamo intrapreso questo studio, utilizzando la microscopia confocale a scansione laser (LSCM) e la microscopia elettronica a trasmissione (TEM), al fine di approfondire le conoscenze sull'espressione HLA-G in placente da gravidanze fisiologiche, a seguito di un parto spontaneo a termine. Inaspettatamente, abbiamo osservato che l'espressione HLA-G include sia vescicole che aggregati, localizzati nel citosol o in stretta prossimità del nucleo.

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INTRODUCTION

The human placenta is a feto-maternal organ that connects the fetus to the uterine wall. At term, the human placenta is discoid in shape and composed of fetal and maternal sides[1]. The fetal surface of the placenta consists of the wall of the chorion, called the chorionic plate, and the chorionic villi, complex tree-like projections that arise from that region. On the fetal surface, the umbilical cord is inserted in a slightly off-center position into the chorionic plate, with the chorionic vessels ramifying over the surface. These vessels can be seen through the transparent amnion.

The maternal portion of the placenta is formed by the decidua basalis or basal plate, formed from the uterine endometrium. The decidua contains a mixture of fetal extravillous trophoblasts and several kinds of maternal cells of the uterine decidua, such as decidua stroma cells, macrophages and other immune cells.

The fetal and maternal components of the placenta enclose a separate space, the intervillous space, which is occupied by freely circulating maternal blood that directly bathes the surface of the placental villi[2].

Considerable attention has been paid to how the placenta receives maternal nutrients and disposes of the products of metabolism through maternal circulation[3]. This focus rightly recognizes physiological exchange as the primary function of the placenta, but it fails to emphasize the placenta's central role in immunoregulation at the maternal-fetal interface in order to support the pregnancy[4, 5]. One of the major mysteries of pregnancy is why the fetus and placenta, which are immunologically distinct from the mother, are not recognized as foreign tissue and rejected by the mother's immune system[6, 7, 8, 9]. To establish and maintain an immune privileged site where the embryo can survive and grow despite its genetic dissimilarities, the fetal and the maternal immune systems have to cooperate jointly[10,11]. The mother does this through the presence of local regulatory immune cells, such as decidua natural killer (dNK) cells and macrophages[12, 13], and the fetus possibly utilizes several mechanisms. Among all mechanisms contributing to a fetal-maternal tolerance, the unique HLA status of fetal trophoblasts seems to be the most important[14,15]. Fetal extravillous trophoblast cells that infiltrate the decidua, thus making direct contact with maternal immune cells, do not express the polymorphic HLA class I (HLA-A and -B) and class II (HLA-DR, DQ, and DP) molecules, but they do preferentially express HLA-G molecules as well as low amounts of HLA-C, HLA-E and HLA-F. HLA-G plays a crucial role in maternal-fetal tolerance, which is the only example of physiological tolerance to semi-allergenic tissues[16,17].

In contrast to classical HLA class I molecules, HLA-G displays a low degree of polymorphism, and an unusually large variety of molecular structures due to alternative splicing of the primary transcript. Seven isoforms of HLA-G have been identified, all products of alternative splicing of a unique mRNA transcript. Four of these are membrane bound (HLA-G1 to -G4), and three are soluble isoforms (HLA-G5 to -G7). HLA-G1 and HLA-G5 isoforms present the typical structure of the classical HLA class I molecules formed by a 3 globular domain heavy-chain, noncovalently associated to a β-2-microglobulin (B2M) and a nonapeptide[18, 19].

With regard to function, HLA-G is able to protect the fetus by inhibiting immune responses of maternal immunocompetent cells. HLA-G binds to several receptors, including ILT2, ILT4, and KIR2DL4 receptors, to inhibit immune responses of myelomonocytic cells, dendritic cells, T cells, B cells, and NK cells[20].

Despite numerous studies, the intracellular organization of HLA-G remains unclear (i.e. light immunohistochemical studies were not able to determine whether immunoreactive proteins are expressed on the cell membrane or in the cytoplasm)[21]. However, in vitro experiments on trophoblast cell lines or HLA-G transfectants showed HLA-G clustered on the cell surface[22, 23].

The aim of the present study was to analyze the cellular localization and the macromolecular organization up to ultra structural level of the HLA-G protein on decidua trophoblasts from normal pregnancies at 36-40 weeks following a spontaneous delivery, using LSCM and TEM. In all experiments two different monoclonal antibodies specific for HLA-G were used in order to confirm our LSCM and TEM results.

Transmission Electron Microscopy

TEM was carried out on placental specimens taken close to the same zone processed for LSCM. Samples were fixed in 3% glutaraldehyde (0.1 M phosphate buffer pH 7.4), post-fixed in 1% $OsO4$ buffered solution, dehydrated in ethyl alcohol and embedded in epoxidic resin. Ultrathin sections of placentas were cut at ultramicrotome, collected on nickel grids, and processed for post-embedding immunoelectron microscopy.

After deosmication in aqueous saturated solution of sodium metaperiodate, the grids were treated with PBS (10% goat serum and 0.2% saponin) to block nonspecific antigenic sites. The incubation in mAb MEM-G/1 (1:10) in PBS or in mAb 4H84 antibodies (1:10) in PBS was carried out overnight at 4°C. The secondary gold-conjugated antibody (10 nm) was diluted 1:10. After incubation in 1% of glutaraldehyde for 3 min., the grids were contrasted for TEM and examined using a Jeol JEM SX 100 electron microscope (Jeol, Tokyo, Japan). Control sections were incubated only with the secondary antibody.

RESULTS

Immunofluorescence labelling of HLA-G in decidua trophoblast cells

HLA-G protein expression in human placental trophoblast cells was investigated by LSCM using two anti- HLA-G mAbs: MEM-G/1 and 4H84.
In the negative control samples incubated with the substrate solution only, no signals above background fluorescence were observed, thus minimizing false-positive signals (**Figure 1A**).
In all placental tissues, immunofluorescence staining combined with confocal microscopy revealed that HLA-G protein was expressed on the surface of decidua cells. This finding was in accordance with previous studies on placental tissues and cell lines[16, 22, 23].

Unexpectedly, the cytoplasm and, in particular, the perinuclear area of decidua cells, often showed a strong HLA-G immunofluorescence positivity (**Figure 1B**). To exclude the possibility that the detection of the HLA-G protein in the cytoplasm was a result of MEM-G/1 over-sensitivity, the 4H84 antibody was also employed. However, we found that 4H84 mAb had the same staining characteristics as the MEM-G/1 mAb, thus confirming our results using MEM-G/1 (**Figure 1C**). To gain further insight into the staining pattern for HLA-G in decidua cells, we used a higher

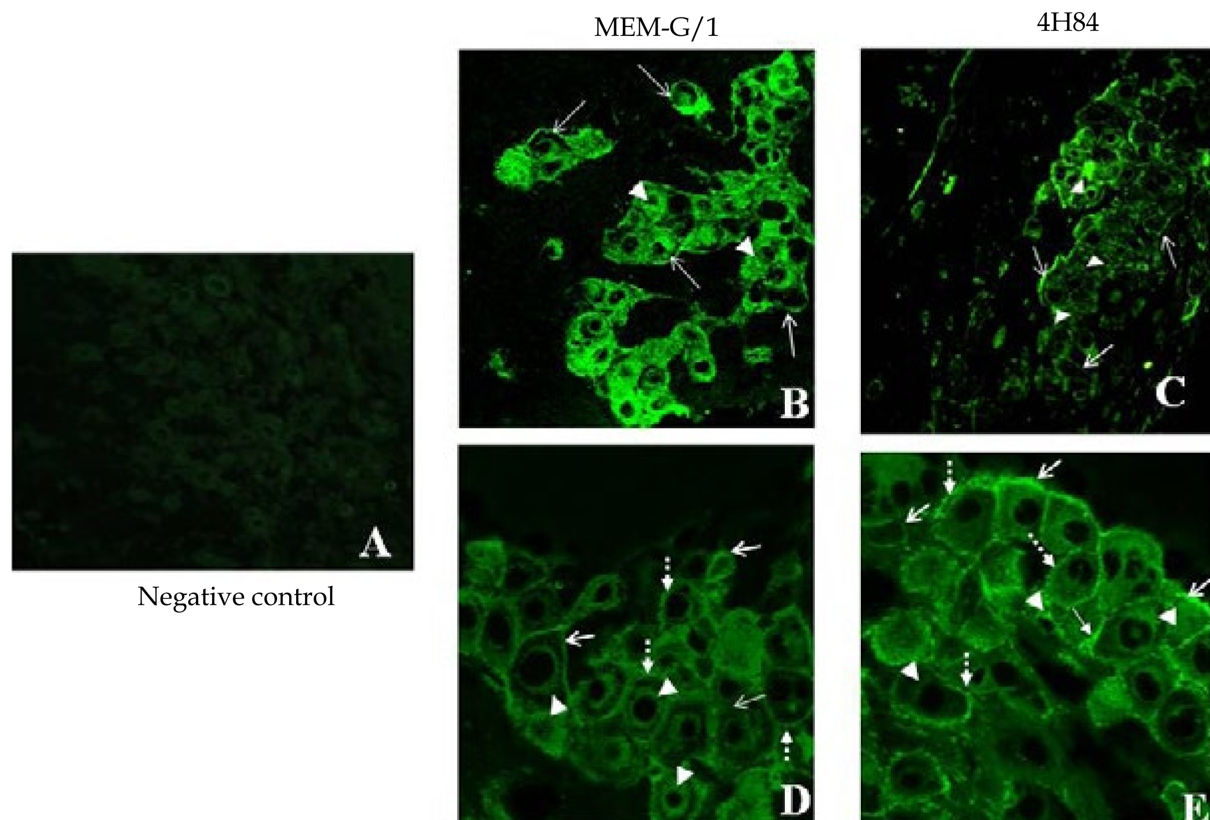

Figure 1.
Immunofluorescence detection of HLA-G in decidua trophoblast cells: HLA-G membrane and cytoplasmatic staining. *Staining with MEM-G/1 and 4H84 mAb showed the presence of HLA-G molecules on the surface of decidua cells (B, C, arrows). The cytoplasm and the perinuclear area of decidua cells, often showed a strong HLA-G immunofluorescence positivity (B, C, arrowheads). Original magnification 40x. Magnified view of tissue sections showed that membrane HLA-G stain was distinct in a linear (D, E arrows) and a dot pattern (D, E, dashed arrows). Cytoplasmic HLA-G signals were prevalently in a dot nature (D, E arrowheads). Original magnification 40x; 3,6x electronic zoom.* ***Negative control, A.*** *The negative control sample: green autofluorescent signals were minimal; Original magnification 40x.*

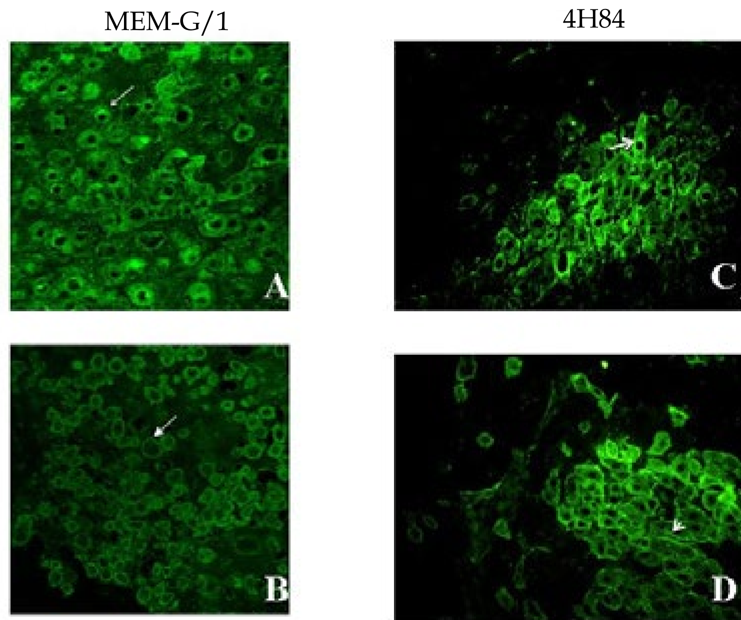

Figure 2.
Immunofluorescence detection of HLA-G in decidua trophoblast cells: prevalent HLA-G membrane or cytoplasmatic staining. *Staining with MEM-G/1 and 4H84 mAb showed that HLA-G expression was not uniform in all placenta tissues analyzed; different area showed higher HLA-G expression in the cytoplasm (A, C, arrows) , compared with the cell surface (B, D, arrows). Original magnification 40x.*

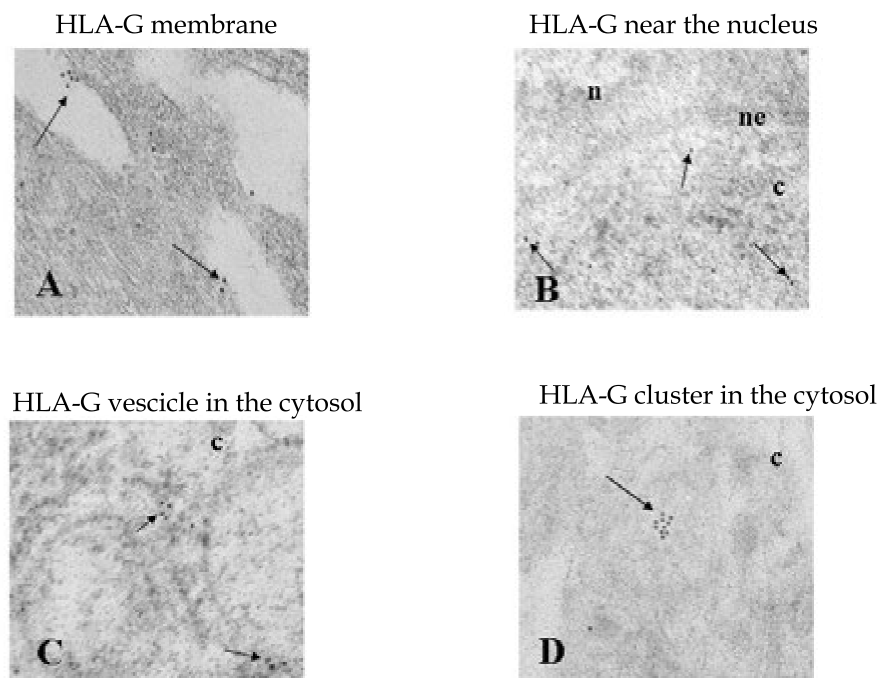

Figure 3.
TEM analysis. *Reactivity with MEM-G/1 and 4H84 mAb showed that HLA-G immunogold positivity was localized on cell membrane (A, arrows) and closed to the nuclear envelop (B, arrows); in the cytoplasm HLA-G immunogold particles were localized in vesicles (C, arrows), otherwise in clusters without limiting membrane (D, arrow). (n: nucleus; ne: nuclear envelop; c: cytoplasm).*

magnification of tissue sections.

A magnified view of the tissue sections revealed that membrane HLA-G staining was clearly distinctive, with a pattern of lines and dots, while the intracellular HLA-G signals were prevalently in a dotted pattern. (**Figure 1D** and **Figure 1E**).

Interestingly, HLA-G immunofluorescence dots were very heterogeneous in size and distribution (Figure 1), suggesting a variable aggregation of HLA-G molecules in the cell membrane and the cytoplasm.

Moreover, confocal microscopy analysis indicated that HLA-G expression was not uniform among different areas of all placental tissues; some populations of analyzed cells showed higher HLA-G expression in the cytoplasm compared with the cell surface (**Figure 2**).

Transmission Electron microscopy

TEM is one of the most sensitive methods for detecting ultrastructural localization and conformation of cell structure/macromolecules. At the ultrastructural level, we observed that HLA-G immunogold positivity was localized on the cell membrane, close to the nuclear envelope and in the cytoplasm (**Figure 3A** and **Figure 3D**). In particular, TEM analysis revealed that the cytoplasmic HLA-G immunogold particles were localized in vesicles or otherwise in clusters without a limiting membrane (**Figure 3C** and **Figure 3D**). These TEM analyses are in line with the LSCM data.

In all samples analyzed, the pattern of reactivity of the mAb MEM-G/1 was similar to that of the mAb 4H84.

DISCUSSION

One of the most characteristic features of human embryonic development is the intimate relationship between the embryo and the mother. To survive and grow during intrauterine life, the fetus must maintain an essentially parasitic relationship with the body of the mother to acquire oxygen and nutrients and eliminate waste. The fetus displaying antigens inherited from the father on its cell surfaces must avoid being rejected as a foreign body by the immune system of its maternal host. Reprogramming of immune cell functions into suppressive modes is believed to be a central feature of maternal tolerance to the semiallogenic fetus. A key role in maternal tolerance to the fetus has been attributed to the expression of nonclassical class I MHC molecules HLA-G on trophoblast cells.

This molecule has garnered a lot of interest due to its ability to confer protection to the semiallogenic fetus from the maternal immune system, creating a general state of tolerance via interaction with inhibitory receptors presented in maternal killer (NK) cells, T cells and antigen-presenting cells (APC)[27, 28].

Previous studies clearly demonstrate an additional unique capability of the HLA-G protein: its ability to form molecular complexes on the surface of trophoblast cell lines[23, 29, 30, 31, 32]. Moreover, emerging studies have revealed that multimeric complexes of HLA-G enhance the inhibitory signal mediated by immune inhibitory receptors compared with HLA-G monomers, thereby favoring inhibition of NK cell-mediated cytotoxicity[33, 34, 35].

To date, no study has investigated whether HLA-G multimeric complexes are present in placental tissue cells. Here, we document for the first time the existence of HLA-G multimeric complexes not only in the membrane, but also in the cytoplasm of cells in term placental tissue.

In all samples analysed, we showed by LSCM that HLA-G was expressed on the plasma membrane of term placental decidua cells. The HLA-G specific stain was clearly distinct in a linear and dot pattern (Figure1 and Figure2) , suggesting a variable aggregation of the HLA-G protein on the cell surface. These results are consistent with previous studies[16, 22, 23].

Unexpectedly, in all placental tissue analyzed, the cytoplasm and perinuclear region of cells also showed a strong HLA-G immunofluorescence punctate staining. These intracellular dots were strongly heterogeneous in size and distribution, suggesting an irregular aggregation of HLA-G proteins in the cell cytoplasm.

Supporting immunofluorescence results, TEM experiments clearly indicated a cluster distribution along with single HLA-G protein expression on the cell surface. TEM analysis also revealed that the immunofluorescence of the intracellular HLA-G dots correspond at the ultrastructural level to HLA-G protein aggregates either enclosed in vesicles or without a limiting membrane. Our LSCM and TEM findings in human term placental tissue clearly revealed for the first time, the unusual presence of cytoplasmic multimeric HLA-G protein aggregates localized in clusters without a limiting membrane or otherwise in vesicles.

Moreover, immunoexpression of the HLA-G

protein showed a varying degree of cellular distribution among different areas of all placental tissue analyzed, but at the moment the significance of these findings is unclear.

In recent years, up-regulation of HLA-G has been found in many types of primary tumors such as trophoblastic tumors, breast carcinoma and various gastrointestinal cancers, and metastases[36, 37, 38], suggesting that HLA-G antigen expression is one strategy used by tumor cells to escape immune surveillance.

HLA-G has been detected in tumors mainly by immunohistochemistry and usually with the 4H84 antibody[20, 39]. In these studies, an over-expression of HLA-G was observed either in the membrane and/or in the cytoplasm of tumor cells. In addition, a study on hepatocellular carcinoma[40] showed a mainly cytoplasmic staining of HLA-G. Membrane and/or cytoplasmic expression of HLA-G, suggested that not only membranous, but even cytoplasmic HLA-G might participate in the regulation of tumor progression.

Recently, an in-vitro study[41] indicated a new role of HLA-G in trophoblast cells. This work supports the hypothesis that HLA-G may affect the invasiveness of trophoblast-derived cell lines JEG-3 without interacting with the immune cells.

Considerable attention has been paid to the HLA-G protein's capability to form disulfide-linked homodimers on the cell surface that bind to inhibitory receptors with increased avidity compared to HLA-G monomers. This has been proven only on the surface of trophoblasts and HLA-G transfectants. Recently the existence of HLA-G dimers was also documented on the surface of tumor cells[42]. These data emphasize the physiological relevance of HLA-G complexes on the surface of cells, highlighting the relationship between HLA-G conformation and its efficiency at inhibiting the immune response.

Very interestingly, our study revealing cytoplasmic HLA-G expression in placental tissue seems to be in agreement with HLA-G expression observed in tumors. Moreover, our LSCM and TEM results provide evidence for the first time that HLA-G is present as protein aggregates not only on the cell surface but also in the cytoplasm in human term placental tissue.

In light of our findings and literature data we hypothesize that membranous and cytoplasmic HLA-G aggregates represent a biologically active form of this molecule. In particular, cytoplasmic complexes of the HLA-G molecule could be active structures that play important roles in modulating immune responses in the context of pregnancy. In addition, cytoplasmic HLA-G complexes may be part of autocrine networks that regulate non-immunological functions, such as promoting cell growth and control of trophoblast invasion.

In summary, based on our LSCM and TEM staining results, we showed for the first time a marked HLA-G sub-cellular variability in expression and localization. Still, the topic of HLA-G is far from being fully exhausted. Further investigations are necessary to determine whether and how the level of expression and the subcellular distribution of the HLA-G protein complexes are affected in a pathological pregnancy.

REFERENCES

1) Huppertz B. **The anatomy of the normal placenta.** J Clin Pathos 2008;61:1296-302.
2) Burton GJ, Fowden AL. **The placenta: a multifaceted, transient organ.** Phil. Trans. R. Soc. 2015;5:370.
3) Gudea NM, Roberts CT, Kalionisa B, King RG. **Growth and function of the normal human placenta.** Thrombosis Research 2004; 114: 397—407.
4) Lynge NL, Djurisic S, Hviid TV. **Controlling the Immunological Crosstalk during Conception and Pregnancy: HLA-G in Reproduction.** Front Immunol. 2014; 5:198.
5) Hunt JS, Petroff MG, McIntire RH, Ober C. **HLA-G and immune tolerance in pregnancy.** FASEB J. 2005;19: 681–693.
6) Guleria I, Sayegh MH. **Maternal acceptance of the fetus: true human tolerance.** J Immunol. 2007;178:3345-3351.
7) Gregori S, Amodio G, Quattrone F, Panina-Bordignon P. **HLA-G orchestrates the early interaction of human trophoblasts with the maternal niche.** Front. Immunol. 2015; 6: 128. doi: 10.3389/fimmu.2015.00128.
8) Hunt JS. **Stranger in a strange land.** Immunol Rev. 2006; 213:36-47.
9) Huppertz B. **The feto-maternal interface: setting the stage for potential immune interactions.** Semin Immunopathol. 2007;29:83-94.
10) Hunt JS, Petroff MG. **IFPA Senior Award Lecture: Reproductive immunology in perspective--reprogramming at the maternal-fetal interface.** Placenta 2013; 34 Suppl:S52-5.
11) Alba Munoz-Suano Alexander B. Hamilton Alexander G. **Betz Gimme shelter: the immune system during pregnancy** Immunological Reviews 2011; 241: 20–38.
12) Le Bouteiller P. **HLA-G in Human Early Pregnancy: Control of Uterine Immune Cell Activation and Likely Vascular Remodeling.** Biomed J 2015;38: 32-38.
13) Dahl M, Djurisic S, Hviid TVF. **The Many Faces of Human Leukocyte Antigen-G: Relevance to the Fate of Pregnancy.** Journal of Immunology Research 2014.
14) Djurisic S, Hviid TV. **HLA Class Ib Molecules and Immune Cells in Pregnancy and Preeclampsia.** Front Immunol. 2014; 5:652.
15) Hunt J, Pace JL, Gill RM. **Immunoregulatory molecules in human placentas: potential for diverse roles in pregnancy.** Int. J. Dev. Biol. 2010;54: 457-467.
16) McMaster MT, Librach C, Zhou Y, Lim K-H, Janatpour MJ, DeMars R et al. **Human Placental HLA-G Expression Is Restricted to Differentiated Cytotrophoblasts.** The journal of Immunology 1995; 154: 3771-3778.
17) Rebmann V, da Silva Nardi F, Wagner B, Horn PA. **HLA-G as a Tolerogenic Molecule in Transplantation and Pregnancy.** Journal of Immunology Research 2014; Article ID 297073.
18) Curigliano G, Criscitiello C, Gelao L, Goldhirsch A. **Molecular Pathways: Human Leukocyte AntigenG(HLA-G).** Clin Cancer Res 2013;19:5564-71.
19) Carosella ED, LeMaoult Jl. **HLA-G: a look back, a look forward.** Cell. Mol. Life Sci. 2010.
20) González A, Rebmann V, LeMaoult J, Horn PA., Carosella ED, Alegre E. **The immunosuppressive molecule HLA-G and its clinical implications.** Critical Reviews in Clinical Laboratory Sciences 2012; 49: 63–84.
21) Bhalla A, Stone PR, Liddell HS, Zanderigo A, Chamley LW. **Comparison of the expression of human leukocyte antigen (HLA)-G and HLA-E in women with normal pregnancy and those with recurrent miscarriage.** Reproduction 2006; 131 : 583-589.
22) Boyson JE, Erskine R, Whitman MC, Chiu M, Lau JM, Koopman LA et al. **Disulfide bond-mediated dimerization of HLA-G on the cell surface.** Proc Natl Acad Sci U SA 2002; 99: 16180-16185.
23) Gonen-Gross T, Mandelboim O. **HLA-G complexes are observed on the cell surface.** Hum Immunol. 2007;68: 227-232.
24) Paul P, Rouas-Freiss N, Moreau P, Cabestre FA, Menier C, Khalil-Daher I et al. **HLA-G, -E, -F preworkshop: tools and protocols for analysis of non-classical class I genes transcription and protein expression.** Hum Immunol. 2000;61:1177-95.
25) Blaschitz A, Hutter H, Leitner V, Pilz S, Wintersteiger R, Dohr G et al. **Reaction patterns of monoclonal antibodies to HLA-G in human tissues and on cell lines: a comparative study.** Hum Immunol. 2000;61:1074-85.
26) Menier C, Saez B, Horejsi V, Martinozzi S, Krawice-Radanne I, Bruel S et al. **Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules.** Hum Immunol. 2003;64: 315-326.
27) Menier C, Rouas-Freiss N, Favier B, LeMaoult J, Moreau P, Carosella ED. **Recent advances on the non-classical major histocompatibility complex class I HLA-G molecule.** Tissue Antigens 2010;75:201-6.
28) LeMaoult J, Le Discorde M, Rouas-Freiss N, Moreau P, Menier C, McCluskey J et al. **Biology and functions of human leukocyte antigen-G in health and sickness.** Tissue Antigens 2003 ;62: 273-284.
29) Gonen-Gross T, Achdout H, Arnon TI, Gazit R, Stern N, Horejsí V et al. **The CD85J/leukocyte inhibitory receptor-1 distinguishes between conformed and beta 2-microglobulin-free HLA-G molecules.** J Immunol. 2005;175:4866-74.
30) Apps R, Gardner L, Sharkey AM, Holmes N, Moffett A. **A homodimeric complex of HLA-G on normal trophoblast cells modulates antigen-presenting cells via LILRB1.** Immunol. Eur. J. 2007; 37: 1924–1937.
31) Morales PJ, Pace JL, Platt JS, Phillips TA, Morgan K, Fazleabas AT, et al. **Placental Cell Expression of HLA-G2 Isoforms Is Limited to the Invasive Trophoblast Phenotype.** The Journal of Immunology 2003; 171: 6215–6224.
32) Morales PJ, Pace JL, Platt JS, Langat DK, Hunt JS. **Synthesis of beta(2)-microglobulin-free, disulphide-linked HLA-G5 homodimers in human placental villous cytotrophoblast cells.** Immunology 2007; 122:179-88.
33) Howangyin KY, Loustau M, Wu J et al. **Multimeric structures of HLA-G isoforms function through differential binding to LILRB receptors.** Cellular and Molecular Life Sciences 2012; 69: 4041–4049.
34) Kuroki K, Maenaka K. **Immune modulation of HLA-G dimer in maternal-fetal interface.** Eur J Immunol. 2007;37:1727-9.
35) Howangyin KY, Loustau M, Wu J, Alegre E, Daouya M, Caumartin J et al. **Multimeric structures of HLA-G isoforms function through differential binding to LILRB receptors.** Cell Mol Life Sci. 2012.
36) Rouas-Freiss N, Moreau P, LeMaoult J, Carosella ED. **The Dual Role of HLA-G in Cancer.** Journal of Immunology Research 2014; Article ID 359748, 10 pages.

37) de Kruijf EM, Sajet A, van Nes JGH, Natanov R, Putter H, Smit VTHBM et al. **HLA-E and HLA-G Expression in Classical HLA Class I-Negative Tumors Is of Prognostic Value for Clinical Outcome of Early Breast Cancer Patients.** The Journal of Immunology 2010; 185: 7452–7459.
38) Ye S-R, Yang H, Li K, Dong Da-D, Lin X-M, Yie S-M. **Human leukocyte antigen G expression: as a significant prognostic indicator for patients with colorectal cancer.** Modern Pathology 2007; 20: 375–383.
39) Kalhor N, Pedro TR, MD, Deavers MT, Malpica A, Silva EG. **Immunohistochemical Studies of Trophoblastic Tumors.** Am J Surg Pathol 2009;33:633–638.
40) Cai M-Y, Xu Y-F, Qiu S-J, Ju M-J, Gao Q, Y-W Li et al. **Human LeukocyteAntigen-GProtein Expression Is an Unfavorable Prognostic Predictor of Hepatocellular Carcinoma following Curative Resection.** Clin Cancer Res 2009;15: 4686-4693.
41) Liu X, Gua W, Li X. **HLA-G regulates the invasive properties of JEG-3 choriocarcinoma cells by controlling STAT3 activation.** Placenta 2013; 34: 1044-1052.
42) Zilberman S, Schenowitz C, Agaugu´e S, Favier B, Riteau B, Rouzier R et al. **HLA-G1 and HLA-G5 active dimers are present in malignant cells and effusions: The influence of the tumor microenvironment.** Eur. J. Immunol 2012; 42: 1599–1608.