**Human Reproduction Vol.23, No.1 pp. 144–152, 2008** doi:10.1093/humrep/dem326
Advance Access publication on October 18, 2007

# Human decidual stromal cells express HLA-G Effects of cytokines and decidualization

**Osmany Blanco[1], Irene Tirado[1], Raquel Muñoz-Fernández[1], Ana Clara Abadía-Molina[1], Jose Marcos García-Pacheco[2], Jose Peña[3] and Enrique G. Olivares[1,4,5]**

*[1]Departamento de Bioquímica, Biología Molecular 3 e Inmunología, Facultad de Medicina, Unidad de Inmunología, Instituto de Biopatología y Medicina Regenerativa, Universidad de Granada, 18012-Granada,Spain; [2]Servicio de Inmunología, Hospital Virgen de la Arreixaca, Murcia, Spain; [3]Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Spain; [4]Servicio de Análisis Clínicos, Hospital Universitario 'San Cecilio', Servicio Andaluz de Salud, Granada, Spain*

[5]Correspondence address. Tel: +34-958-243107; Fax: +34-958-249015; E-mail: engarcia@ugr.es

**BACKGROUND: Decidual stromal cells (DSC) are the main cellular component of the decidua, the maternal tissue in close contact with fetal trophoblast. Although of mesenchymal origin, DSC exert numerous immune functions that seem to be relevant for the immunological relationship between the mother and fetus. HLA-G, an antigen preferentially expressed by trophoblast, appears to participate in the immune tolerance by the mother of the semiallogeneic fetus. METHODS AND RESULTS: We show by flow cytometry, fluorescence microscopy, western blotting and RT–PCR that DSC isolated and maintained in culture express HLA-G weakly but consistently. We also detected this antigen by flow cytometry in fresh DSC. Interleukin (IL)-10, a cytokine associated with normal pregnancy, increased the expression of HLA-G by DSC ($P < 0.00001$), whereas IL-2, a cytokine involved in spontaneous abortion, showed no effect. Decidualization by progesterone and cAMP also up-regulated the expression of HLA-G by DSC ($P < 0.001$). Interferon γ, a cytokine implicated in the vascular remodelling of the decidua necessary for embryo implantation, also increased the expression of HLA-G by DSC ($P < 0.05$). CONCLUSIONS: Our results suggest the existence of a network in which hormones together with cytokines regulate the expression of HLA-G by DSC, and that may be of relevance in the maintenance of maternal–fetal tolerance.**

*Keywords:* decidual stromal cell; HLA-G; interferon γ; interleukin-10; progesterone

## Introduction

Successful pregnancy has been considered an example of semiallogeneic graft acceptance in which the semiallogeneic fetus is protected from the mother's immune system. Immunological interrelations between the mother and fetus during pregnancy are believed to take place in the decidua, the maternal tissue in closest contact with the fetal trophoblast. Decidual stromal cells (DSC) are the main cellular component of the decidua. These cells comprise a distinctive class whose origin and lineage remained unknown until recently. DSC exert different immune activities that appear to be of relevance in the immunological cross-talk between the mother and the fetus, and that may lead to either a normal pregnancy or abortion (Kimatrai *et al*., 2003, 2005). We have been able to isolate and maintain highly purified cultures of DSC, which allowed us to demonstrate that human DSC are related to bone marrow stromal precursors (Garcia-Pacheco *et al*., 2001), and that their morphology, phenotype and functions are similar to those of myofibroblasts, fibroblastic cells with contractile activity which are involved in wound retraction (Oliver *et al*., 1999; Kimatrai *et al*., 2003, 2005), and also to follicular dendritic cell (Muñoz-Fernandez *et al*., 2006).

Among the multiple immune mechanisms that control maternal–fetal tolerance during normal pregnancy, human leukocyte antigen G (HLA-G) seems to play a key role. This antigen, which was discovered in trophoblast, is preferentially expressed at high intensity by this tissue (Kovats *et al*., 1990). The low number of HLA-G alleles reduce the possibility of allogeneic reaction by the maternal cytotoxic T lymphocytes against the trophoblast (Hunt *et al*., 2005). Furthermore, the potential cytotoxicity of decidual natural killer (NK) cells against trophoblast is probably blocked by inhibitory receptors that bind HLA-G, and that are expressed by these NK cells (Rouas-Freiss *et al*., 1997; Khalil-Daher *et al*., 1999). Here, we show that human DSC express low but significant levels of HLA-G, and that this expression can be modulated by cytokines and decidualization.

## Materials and Methods

### *Tissues*

Twenty-three samples from elective vaginal terminations of first trimester pregnancy (6–11 weeks) from healthy patients aged 20–30 years were used. We excluded women receiving any medication or with infectious, autoimmune or other systemic or local diseases. None of the abortions was pharmacologically induced. The specimens were obtained by vaginal curettage at the Clínica El Sur in Málaga or the Clínica Ginegranada in Granada. Informed consent was obtained from each patient. This study was approved by the research and ethics committee of the Hospital Universitario de San Cecilio in Granada.

### *Isolation and culture of DSC lines*

To establish DSC lines, decidual tissues were examined histologically to exclude the presence of infection or inflammatory infiltration. Samples of decidua from different patients were not pooled so as to avoid inducing cytokine secretion as a result of the allogeneic reaction of leukocytes that initially contaminate DSC cultures. Tissues were thoroughly washed in phosphate-buffered saline (PBS) solution, and the decidua was carefully freed from the trophoblast. Decidual fragments were finely minced between two scalpels in a small volume of RPMI 1640 medium with 100 U/ml penicillin and 50 μg/ml gentamicin, and put in a solution of 0.5% trypsin and 0.2% EDTA (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 37°C. The proteolytic reaction of trypsin was stopped by adding cold RPMI with 20% fetal calf serum (FCS) (Life Technologies, Inc., Paisley, UK); the suspension was then filtered through gauze and centrifuged at 425*g* for 10 min. The supernatant was discarded and the cell pellet was suspended in RPMI and centrifuged on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) for 20 min at 600 *g*. Cells were collected from the interface, suspended in PBS, and washed. This suspension was incubated in culture flasks for 1 h in complete RPMI with 10% FCS to allow macrophages and granulocytes to adhere to the flask. The supernatant was washed and incubated in Opti-Minimum Essential Medium (Opti-MEM, Invitrogen, Paisley, UK) with 100 U/ml penicillin and 50 μg/ml gentamicin, and 2% FCS. After overnight incubation to allow DSC to adhere to the flask, non-adherent cells in the supernatant were discarded. The remaining adherent cells were mainly DSC. Opti-MEM was then replaced and changed twice a week, and after 2–4 weeks, adherent cells covered the whole surface of the 25-$cm^2$ culture flask. Proliferating DSC overgrew other possible contaminant cells such as trophoblast cells or leukocytes, thus further guaranteeing the purity of the cultures. Purity was further confirmed using flow cytometry to detect the co-expression of CD10 and CD29, and the lack of CD45 (which identifies leukocytes) and cytokeratin (which detects epithelial cells and trophoblast) by 95–100% of DSC (Montes *et al*., 1996; Oliver *et al*., 1999; Garcia-Pacheco *et al*., 2001; Kimatrai *et al*., 2003, 2005). With this procedure we obtained 18 finite DSC lines which were assigned individual names. In Opti-MEM, cell lines proliferated for 8–12 weeks before extinction; during this period their antigen phenotype was stable.

### *Isolation of fresh DSC*

To obtain a suspension of fresh cells containing DSC, we used a protocol similar to that reported by Montes *et al*. (1996). The decidua was washed in PBS and minced between two scalpels in a small volume of RPMI 1640 with 10% FCS. The cell suspension was filtered through sterile gauze, washed by centrifugation and suspended in culture medium. This suspension was centrifuged at 650*g* for 30 min over a discontinuous gradient of 20 and 30% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). Cells were collected from the 20/30% interphase and washed in PBS. Five decidua samples were studied independently by flow cytometry.

### *Reagents*

Human interleukin (IL)-10, interferon γ (IFNγ), progesterone and 8-Bromo-cAMP were purchased from Sigma-Aldrich. Recombinant human IL-2 was obtained through the acquired immune deficiency syndrome (AIDS) Research and Reference programme (National Institute of Health, Rockville, MD, USA).

### *Treatment of DSC*

DSC isolated and maintained in culture as indicated above were cultured for 48 h with 10 ng/ml IL-10 or 10 ng/ml IFNγ or 50 IU/ml IL-2. To induce decidualization, DSC were treated with 300 nM progesterone and 500 μM cAMP for 15 days. Decidualization was assessed by changes in cell morphology, and secretion of prolactin (PRL).

### *Flow cytometry analysis*

Cultured DSC were detached from the culture flask by treatment with 0.04% EDTA at 37°C. The cells were centrifuged, the supernatant was discarded, and the pellet was suspended in PBS at $10^6$ cells/ml. For direct staining, 100 μl of the cell suspension was incubated with 10 μl of the appropriate monoclonal antibody (mAb) for 30 min at 4°C in the dark. Cells were washed, suspended in 0.5 ml PBS and immediately analysed in a flow cytometer (Ortho-Cytoron, Ortho Diagnostic Systems, Raritan, NJ, USA). To identify dead cells we incubated DSC with propidium iodide (Sigma-Aldrich). The percentage of cells that were antibody-positive was calculated by comparison with the appropriate isotype control. For double labelling, we followed the same procedure except that a second mAb with a fluorescent marker different from that of the first mAb was also added. For intracytoplasmic labelling, DSC were fixed with 4% paraformaldehyde for 20 min at 4°C and permeabilized with cold acetone for 10 min before the mAb was added. DSC were stained with the following mAbs: HLA-G (MEM-G/9)- fluorescein isothiocyanate (FITC) (Abcam, Cambridge, UK), cytokeratin (CAM 5.2), phycoerithrin (PE) (BD Biosciences, San Jose, CA, USA), CD10-PE (DAKO, Glostrup, Denmark), CD14-PE, CD-45-FITC (Sigma-Aldrich), CD23-FITC (The Binding Site, Birmingham, UK) and CD29-FITC (Caltag, San Francisco, CA, USA). Fresh DSC were gated based on side scatter and CD10 antigen expression.

### *Immunofluorescence microscopy*

DSC were plated onto slides in Opti-MEM. After 24 h cells were washed with PBS, and HLA-G (MEM-G/9)-FITC was added. As negative control, an immunoglobulin G1-FITC isotype control was used (Sigma-Aldrich). Preparations were examined with a Leica microscope (Leica Microsystems, Wetzlar, Germany).

### *RT–PCR*

Total RNA from cells was extracted by the Ultraspec RNA isolation method according to the manufacturer's protocol (Biotecx Laboratories, Inc., Houston, TX, USA). A single-strand complementary DNA copy was made from total RNA using random hexamers (Amersham Pharmacia Biotech, Uppsala, Sweden) and Moloney murine leukaemia virus H minus ribonuclease reverse transcriptase (Promega Corp., Madison, WI, USA). After heating to 65°C for 5 min and quickly cooling to 4°C in a thermal cycler (Geneamp PCR System 9600, PerkinElmer/Cetus, Norwalk, CT, USA) for denaturation, reverse transcription was performed for 1 h at 37°C. Starting with the equivalent of 75 ng RNA, amplification was

carried out in a total volume of 12.5 μl of the amplification mix, 10 mM Tris (trishydroxylmethylaminomethane)–Cl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxy-NTPs, 5% glycerol, 0.25 mM of each primer and 0.02 U/ml *Taq* DNA polymerase (Promega Corp.). After incubation for 5 min at 96°C, each cycle consisted of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, for a total of 32 cycles. Oligonucleotide sequences for HLA-G were designed according to sequences available from GenBank (http://www.ncbi.nlm.nih.gov/Genbank) and synthesized by Genset (Paris, France): 5′-CCACTCCATGAGGTATTTCAG-3′ (forward) and 5′-GGTCCTCGTTCAGGGCGAG-3′ (reverse) (389 bp). The PCR products were size-separated on ethidium bromide-stained 2% agarose gels, and a 100-bp DNA ladder was included in each gel.

#### *Western immunoblotting*

Cells were lysed with 1% Nonidet P-40 lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich), diluted 1:1 in 2× concentrated electrophoresis sample buffer [125 mM Tris pH 6.8, 4% sodium dodecyl sulphate (SDS), 10% glycerol, 0.006% bromophenol blue, 1.8% beta-mercaptoethanol], heated at 95°C for 5 min, resolved by SDS– polyacrylamide gel electrophoresis (10% polyacrylamide gels, reducing conditions) and electrotransferred to Hybond-P polyvinylidene difluoride membranes (Amersham). Membranes were blocked with 5% non-fat milk and probed for 1 h at room temperature with an anti-HLA-G mAb (MEM-G/1) (Abcam) followed by incubation for 1 h at room temperature with horse-radish peroxidase-labelled goat anti-mouse antibody (Caltag). The immune reaction was developed by chemiluminescence (ECL, Amersham) and exposed to autoradiographs (Amersham). Loading controls were carried out by rehybridization of stripped membranes with an anti-extracellular regulated kinase (ERK) polyclonal antibody (anti-mitogen-activated protein kinase one-half, Upstate Biotechnology, UK) or with monoclonal anti-α-tubulin (Sigma-Aldrich). Bands were quantified by densitometry.

#### *Determination of PRL and IL-10 in the culture supernatants*

To determine the concentration of PRL, supernatants from confluent DSC cultures were collected. The presence of PRL was studied with an electrochemiluminescence immunoassay (Roche, Indianapolis, IN, USA). Concentrations of IL-10 in the supernatants of the DSC cultures were determined by a commercially available enzyme immunoassay (R&D, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instructions, and all samples were determined in duplicate.

#### *Statistical analysis*

Constitutive or induced expression of HLA-G by different DSC lines was compared with Student's *t*-test. A *P*-value of 0.05 was considered statistically significant. Quantitative results of the electroluminescence assay were carried out in triplicate or quadruplicate and expressed as mean ± SD.

## Results

### *Expression of HLA-G by cultured DSC*

In culture, DSC proliferate and overgrow any non-proliferating cells, leading to a practically pure population of spindle-shaped DSC (Fig. 1), with no contaminating trophoblast, epithelial cells or leukocytes. This procedure has been used to study DSC lines in previous reports in which the phenotypical and functional characteristics of these cells were determined (Montes *et al*., 1996; Oliver *et al*., 1999; Garcia-Pacheco *et al*., 2001; Kimatrai *et al*., 2003, 2005). With Opti-MEM, we obtained DSC lines, which with weekly passages, proliferated for 8–12 weeks. By flow cytometry, almost all DSC co-expressed CD29 and CD10 and lacked CD45 and cytokeratin. These cells also expressed HLA-G weakly, although consistently (Fig. 1). The intensity of expression of HLA-G varied among the different DSC lines (range: 2–20% positive cells). This variation was expected, as DSC lines are polyclonal normal cells which, as previously reported, can vary in their functional properties (Kimatrai *et al*., 2003, 2005). By immunofluorescesce, we observed HLA-G-positive cells with the typical fibroblastic morphology. These findings were supported by the detection of mRNA for HLA-G by RT–PCR (Fig. 1).

### *Expression of HLA-G by fresh DSC*

Fresh DSC were isolated on Percoll gradients and analysed by flow cytometry according to Montes *et al*. (1996). Under these experimental conditions, fresh DSC with an antigen phenotype similar to that of cultured DSC have been observed (Montes *et al*., 1996; Garcia-Pacheco *et al*., 2001) (Fig. 2). Fresh DSC preparations, however, are not so pure as cultured DSC, as they may contain a small proportion of leukocytes (CD45+ cells) and extravillous trophoblast cells (EVT). Although we observed that in the fresh DSC preparations, CD45+ leukocytes did not express HLA-G, contaminant EVT expressed this antigen (not shown). To distinguish the potentially HLA-G expressing DSC from EVT in fresh preparations, we used a mAb against CD10. This antigen is considered a marker of endometrial stromal cells or DSC (Sumathi and McCluggage, 2002; Iwase *et al*., 2006) and not expressed by EVT (Toki *et al*., 2002). Cells were then gated in flow cytometry by the expression of CD10. We found that a proportion of CD10+ DSC co-expressed HLA-G (range: 12–23% positive cells) (Fig. 2).

### *Modulation by cytokines and decidualization of the expression of HLA-G on cultured DSC*

IL-10 and IFNγ increased the expression of HLA-G by DSC, as shown by flow cytometry and western blotting. IL-2, however, did not have any effect (Fig. 3). Progesterone and cAMP, which induced decidualization as determined by the secretion of PRL by DSC, also up-regulated their expression of HLA-G (Fig. 4). The effects of the cytokines and progesterone and cAMP were quantified and represented in Fig. 5. Progesterone may have a direct effect on the expression of HLA-G by DSC, as it has on trophoblast (Yie *et al*., 2006a). Furthermore, an indirect effect through the IL-10 may also contribute to the up-regulation of HLA-G, as progesterone and cAMP increase the secretion of IL-10 by DSC (Fig. 6). IFNγ, however, up-regulated the expression of HLA-G, but decreased the secretion of IL-10 by DSC (Fig. 6).

## Discussion

HLA-G, a class Ib major histocompatibility complex (MHC) antigen with a low degree of polymorphism, is highly

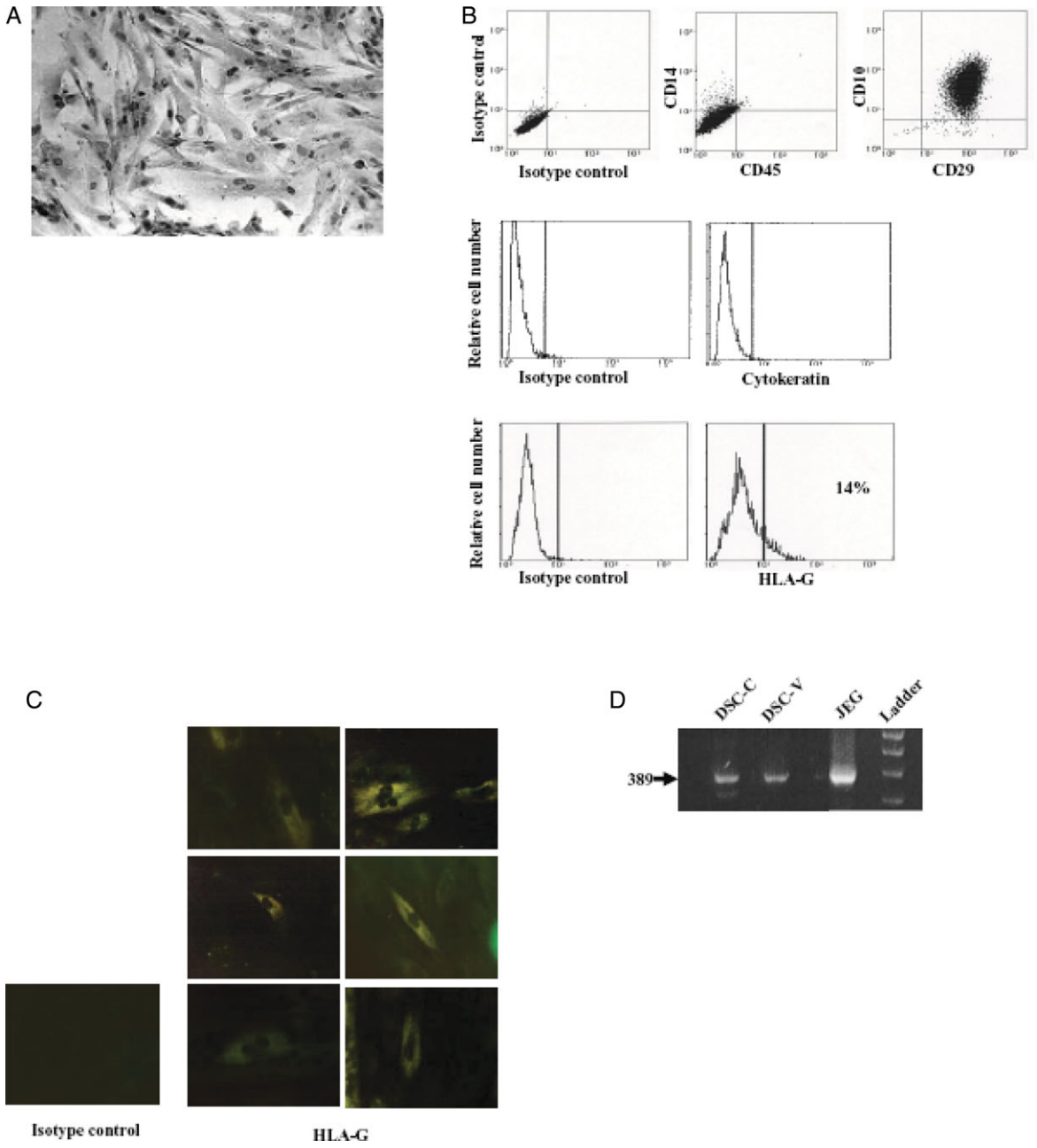


**Figure 1:** Expression of HLA-G by cultured DSC
DSC of the DSC-C line cultured in Opti-MEM showing fibroblast-like morphological features (**A**). Flow cytometric analysis of the antigens expressed by cells of the DSC-C line (**B**). Immunofluorescence detection of HLA-G in cells of the DSC-C line (**C**). Expression of HLA-G mRNA by the DSC-C and DSC-V lines, detected by RT–PCR. The extravillous trophoblast cells tumour line JEG-3 was used as a positive control (**D**). Results are shown from one representative experiment out of ten.

expressed only by EVT (Kovats *et al*., 1990). However, lower levels of expression of this antigen or HLA-G mRNA has been detected in many different tissues, in which expression was associated with immunotolerance (Le Discorde *et al*., 2003; Wiendl *et al*., 2003). In our cultured DSC, HLA-G protein was detected by flow cytometry, immunofluorescence microscopy and western blotting, and HLA-G mRNA was found by RT–PCR (Figs 1–4). This expression, although weak, was upmodulated by IL-10, INFγ and progesterone plus cAMP, but not by IL-2 (Figs 3 and 4). We also found HLA-G in a proportion of fresh DSC (Fig. 2).

The possibility that our results were due to contamination with EVT can be ruled out, since it is highly improbable that these cells survived for weeks in culture as did DSC. Moreover, our DSC lines were negative for cytokeratin (Fig. 1). Furthermore, immunofluorescence microscopic observations disclosed cells with fibroblastic morphology typical of cultured DSC, and which expressed HLA-G (Fig. 1). Although HLA-G expression by cells of the human maternal–fetal interface has been extensively studied by immunohistological methods, the detection of this antigen on DSC has not previously been reported. Immunohistological methods may not have been as sensitive as

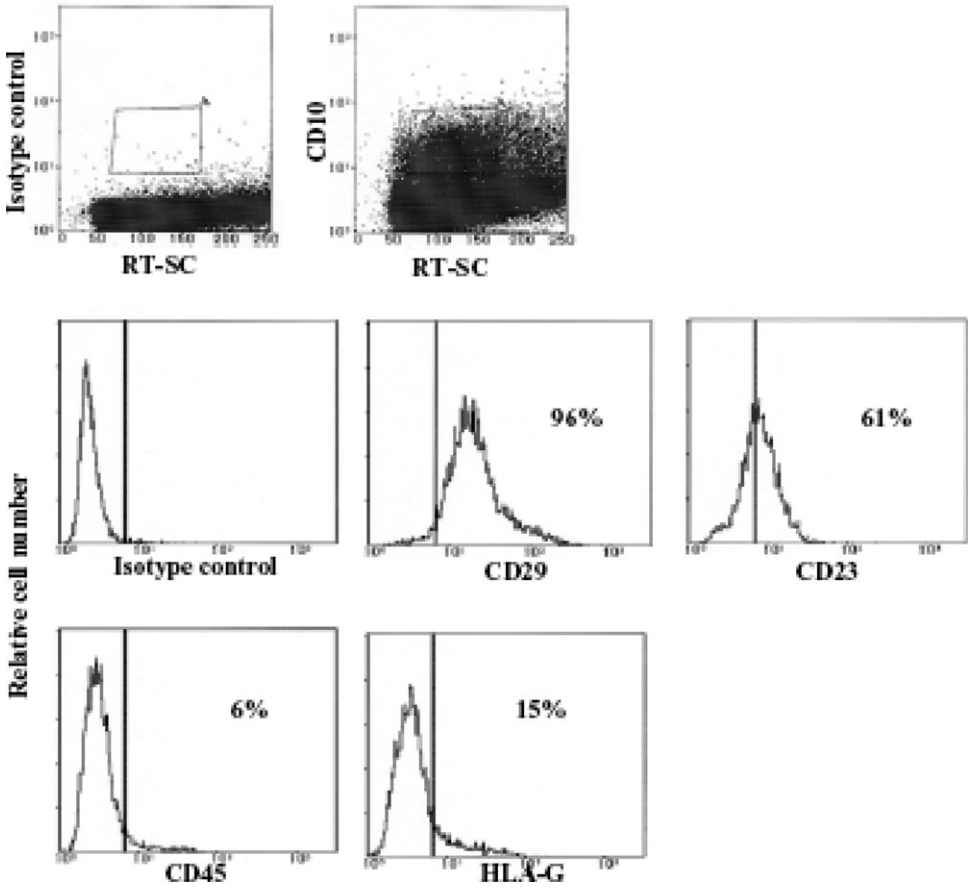


**Figure 2:** Expression of HLA-G by fresh DSC
Cells were isolated by Percoll gradients and analysed by flow cytometry. Cells were gated based on CD10 expression versus side scatter (RT-SC). CD29 and CD23, antigens previously detected in cultured DSC, were found on fresh DSC. A small proportion (6%) of contaminant leukocytes, probably CD10+ neutrophils, were observed in this preparation. Results are shown from one representative experiment out of five.

flow cytometry in disclosing the weak expression of HLA-G by some, but not all, DSC. HLA-G expressed by EVT is considered to play a key role in maternal–fetal tolerance. This antigen appears to bind to the inhibitory receptor of decidual NK and T cells, leading to inhibition of the potential cytotoxicity of these cells against fetal tissues (Rouas-Freiss *et al*., 1997; Khalil-Daher *et al*., 1999). The expression of HLA-G by DSC may constitute an additional mechanism of inhibition of cytotoxic cells.

IL-10 is a Th2 cytokine which is associated in mice and humans to mechanisms of maternal tolerance of normal pregnancy (Chaouat *et al*., 1995; Marzi *et al*., 1996). In humans, a deficiency of placental IL-10 is seen in preeclampsia (Hennessy *et al*., 1999), and a decrease in decidual IL-10 has been observed in missed abortion (Plevyak *et al*., 2002). We recently demonstrated that IL-10 reduced the contractility of DSC, and proposed that this relaxing effect would favour the normal progression of pregnancy (Kimatrai *et al*., 2005). The present results show that IL-10, in addition, increases the expression of HLA-G by DSC (Fig. 3). This effect may potentiate the inhibitory effect of DSC on NK cytotoxicity. The effect of IL-10 on HLA-G expression is not limited to DSC, since this cytokine also increased the expression of this antigen by trophoblast (Moreau *et al*., 1999).

Jokhi *et al*. (1994) found no or scant amounts of IL-2 mRNA in normal decidua and suggested that this cytokine played no role in pregnancy. Other authors, however, detected significant amounts of IL-2 mRNA in normal pregnancy (von Rango *et al*., 2003), and found this cytokine to be associated with abortion (Marzi *et al*., 1996; Lim *et al*., 2000), which suggested that IL-2 may be induced in this pathological situation. In this connection, IL-2 blocks decidualization (Kanda *et al*., 1999) and increases the contractility of DSC, effects which may contribute to expulsion of the trophoblast (Kimatrai *et al*., 2003). In the present study, this cytokine had no effect on the expression of HLA-G by DSC (Fig. 3).

High concentrations of Th1 cytokines have been proved to be deleterious for murine and human pregnancy, including recurrent miscarriages, preeclampsia and fetal growth retardation (Marzi *et al*., 1996; Lim *et al*., 2000). In mice, however, IFNγ is involved in vascular remodelling necessary for embryo implantation and subsequent placenta formation (Ashkar *et al*., 2000). Furthermore, IFNγ, usually considered a proinflammatory cytokine, can drive cells into immunosuppressive profiles when linked with other modulators, and could therefore paradoxically serve as an anti-inflammatory cytokine (Wood and Sawitzki, 2006). This situation may occur during normal pregnancy. In line with the regulatory

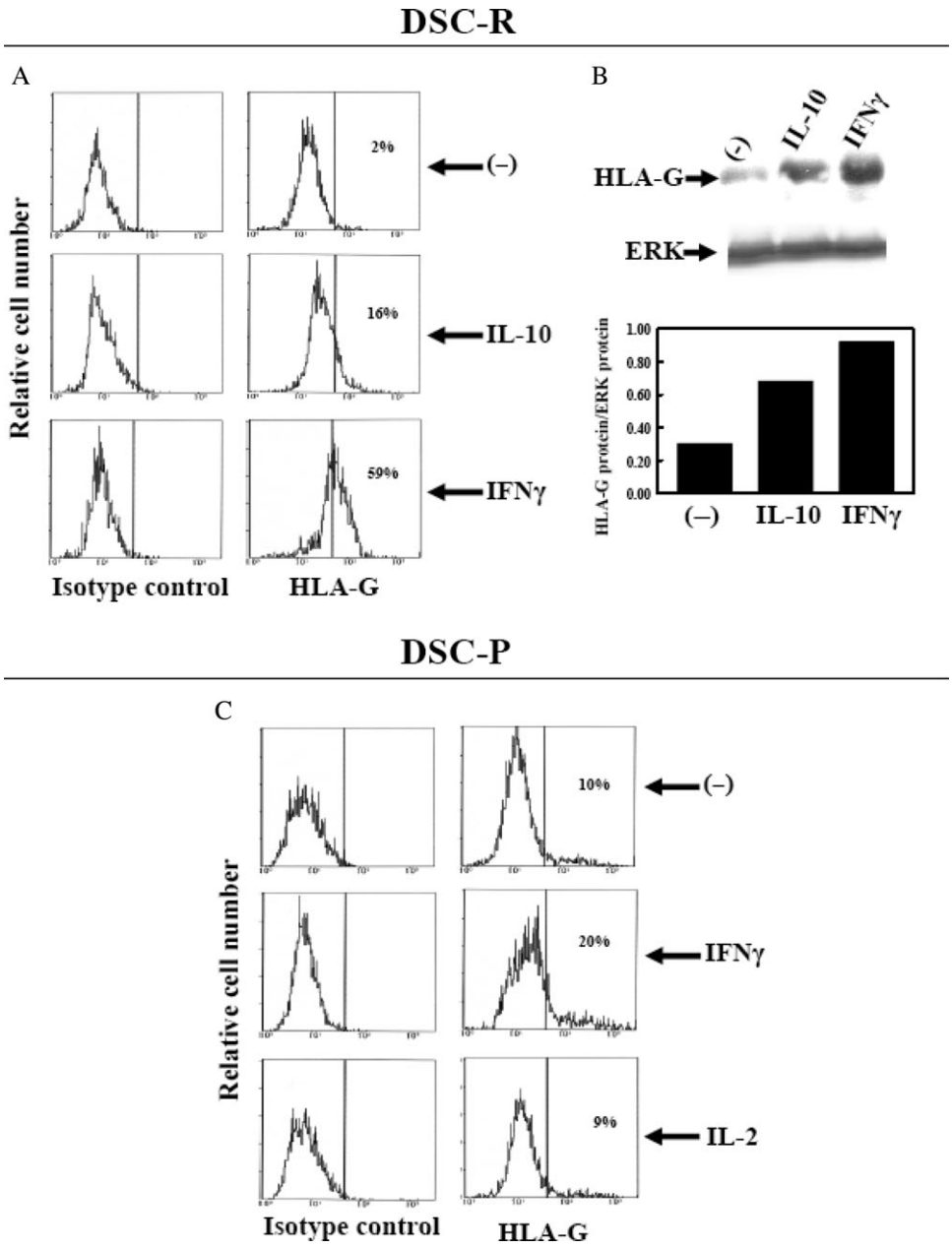


**Figure 3:** Effects of cytokines on the expression of HLA-G by DSC
The DSC line DSC-R was cultured in Opti-MEM independently with 10 ng/ml IL-10 or IFNγ for 48 h, and the expression of HLA-G was studied by flow cytometry (**A**) and western blotting (**B**). Mitogen-activated protein kinase extracellular regulated kinase (ERK) was used as the loading control in western blotting. The bar graph shows the densitometric quantification of western blot results. The DSC line DSC-P was treated independently with 50 IU/ml IL-2 or 10 ng/ml IFNγ for 48 h, and the expression of HLA-G was studied by flow cytometry (**C**). Results are shown from one representative experiment out of five.

functions of IFNγ, this cytokine increased the expression of HLA-G by DSC (Fig. 3). Progesterone, a hormone that supports pregnancy, up-regulates the secretion of Th2 cytokines by lymphocytes (Piccinni *et al*., 1995), inhibits the production of Th1 cytokines (Choi *et al*., 2000), differentiates DSC to progestational status, and blocks their immune activities (Montes *et al*., 1995; Ruiz *et al*., 1997). In our results, decidualization also increased the expression of HLA-G by DSC (Fig. 4). This is consistent with the results reported by Yie *et al*., (2006b), who showed that progesterone enhanced HLA-G gene expression in JEG-3 choriocarcinoma cells and human cytotrophoblasts *in vitro*. Although progesterone appears to exert a direct effect on the induction of HLA-G expression (Yie *et al*., 2006a), an indirect effect through IL-10 on DSC is likely, as decidualization increased the secretion of IL-10 by DSC (Fig. 6). This indirect effect, however, could not be demonstrated *in vitro*, since the constitutive or induced secretion of IL-10 by DSC ($<$70 pg/ml) (Fig. 6) (Vigano *et al*., 2002; Kimatrai *et al*., 2005) was below the concentration necessary to induce an increase in HLA-G expression by DSC ($\sim$10 ng/ml) (Fig. 3). Nevertheless, this cytokine, locally secreted by DSC, together with that secreted by the

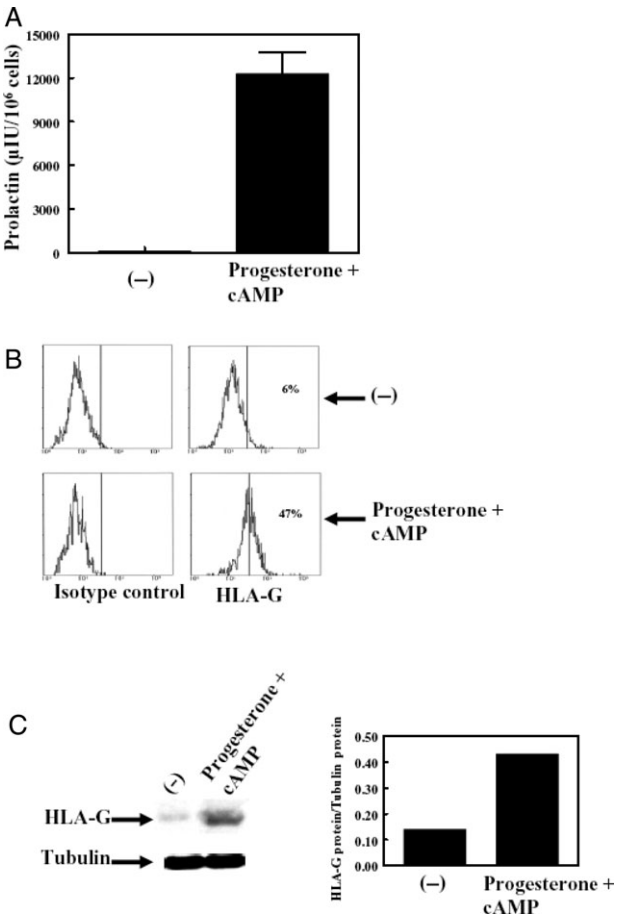


**Figure 4:** Effect of decidualization on the expression of HLA-G by DSC

DSC line DSC-N cells were cultured in Opti-MEM with 300 nM progesterone and 500 μM cAMP for 15 days to induce decidualization. The secretion of PRL was determined to confirm decidualization (**A**). The expression of HLA-G was analysed by flow cytometry (**B**) and western blotting (**C**). α-tubulin was used as the loading control in western blotting. The bar graph shows the densitometric quantification of western blot results. Results are shown from one representative experiment out of three.

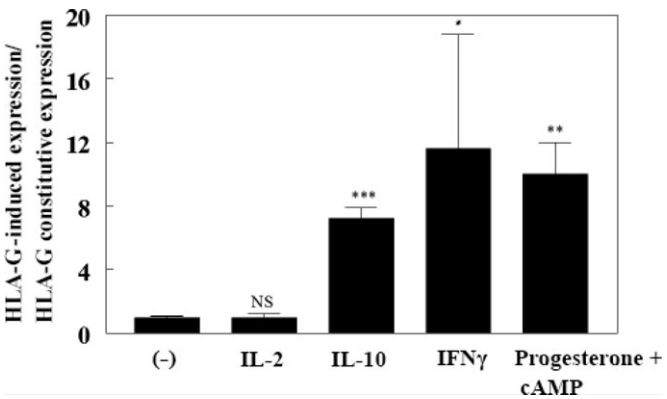


**Figure 5:** Expression of HLA-G induced in DSC by different cytokines and progesterone plus cAMP

Results from flow cytometry are shown as the ratio between the proportion of HLA-G-positive treated DSC and the proportion of HLA-G-positive untreated DSC. Columns represent the mean ± SD of all experiments from Figs 3 and 4. Results are compared with control (−) DSC. NS, not significant. $*P < 0.05$; $**P < 0.001$; $***P < 0.00001$ versus control.

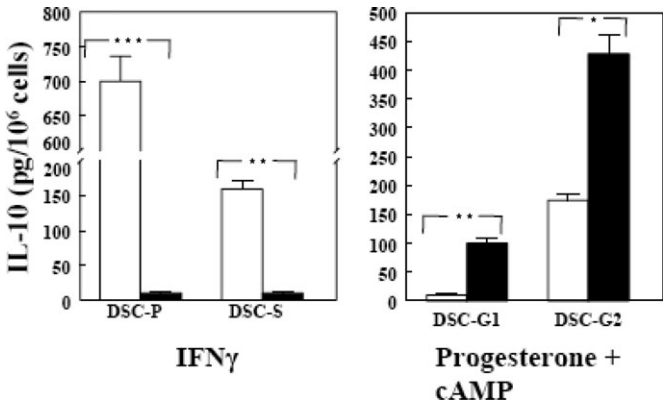


**Figure 6:** Effects of IFNγ (10 ng/ml for 48 h) and treatment with progesterone (300 nM) and cAMP (500 μM) for 15 days on the secretion of IL-10 by different DSC lines cultured in Opti-MEM. Results are shown from one representative experiment out of three. Open bars represent the amount of IL-10 secreted by untreated cultures. Black bars represent the amount of IL-10 secreted by cytokine-treated cultures. $*P < 0.001$; $**P < 0.0001$; $***P < 0.00001$.

trophoblast—which is the main source of IL-10 at the maternal–fetal interface (Roth *et al*., 1996)—may make the concentration of the cytokine high enough to induce the expression of HLA-G *in vivo*.

IFN γ, as well as progesterone plus cAMP are able to induce the expression of HLA-G by DSC (Figs 3–5). However, they have opposite effects on the secretion of IL-10 by these cells: progesterone and cAMP increases it, whereas IFNγ decreases it (Fig. 6). Although both IL-10 and progesterone favour gestation, the effect of IFNγ can be viewed not so much as a factor that favours abortion, as a regulatory mechanism. In fact, progesterone induces both IL-10 and IL-15 secretion by DSC (Okada *et al*., 2000), and this latter cytokine activates decidual NK cells (Verma *et al*., 2000), which are the main source of IFNγ (Ashkar and Croy, 1999). IFN γ, on the other hand, may control the local secretion of IL-10 by DSC.

Although several studies suggest that HLA-G controls the cytotoxicity of decidual NK cells against trophoblast (Rouas-Freiss *et al*., 1997; Khalil-Daher *et al*., 1999), this cytotoxic activity is controversial, since trophoblast is resistant to NK cell-mediated lysis, regardless of HLA class I expression (Avril *et al*., 1999; Zdravkovic *et al*., 1999). Some authors have proposed that decidual NK cells, rather than harming the trophoblast through their cytotoxic activity, actually favour trophoblast functioning by enhancing its ability to invade the decidua (Le Bouteiller and Tabiasco, 2006). Nevertheless, most studies of cytotoxicity have analysed only necrosis, and not apoptosis (Rouas-Freiss *et al*., 1997; Avril *et al*., 1999; Khalil-Daher *et al*., 1999; Zdravkovic *et al*., 1999). We have also confirmed that decidual lymphocytes were unable to kill trophoblast by necrosis; however, we found that they induced apoptosis in EVT cells (Olivares *et al*., 2002). We also observed that apoptotic cytotoxicity, which in normal pregnancy probably controls the physiological invasion of the decidua by EVT, is exacerbated during spontaneous abortion (Olivares *et al*., 2002). In this connection,

experimental evidence suggests that decidual NK cells also induce apoptosis in DSC under normal and pathological situations (Ashkar and Croy, 1999). Mice lacking IFNγ receptor α exhibited a high incidence of dead DSC. This was attributed to an IFNγ signalling deficit which would lead to a drop in MHC expression (HLA-G according to our data) to levels below those necessary to engage the killer-inhibitory receptors of NK cells. Thus decidual NK cells become lytically active and destroy the DSC (Ashkar and Croy, 1999). Decidual NK cells proliferate and are activated through their interaction with DSC (King, 2000; Verma *et al*., 2000). In addition, IFNγ secretion by decidual NK cells may be induced by interaction with the HLA-G of DSC (van der Meer *et al*., 2004). According to our results, this cytokine increases the expression of HLA-G (Fig. 3), and this may play a role in a mechanism by which DSC defend themselves from the putative cytotoxic attack of NK cells they have previously activated. In fact, DSC apoptosis is a normal event in pregnancy, although its mechanism is not fully understood (Gu *et al*., 2004). HLA-G and NK cells may be involved in this mechanism. Experiments are in progress to study the capacity of decidual NK cells to induce apoptosis in DSC.

In conclusion, our results show that DSC express HLA-G. This expression is weak but is up-regulated by IL-10, IFNγ and progesterone and cAMP, which are molecules that promote normal pregnancy. The expression of HLA-G by DSC may control the cytotoxic activity of NK cells against trophoblast and the physiological decay (by apoptosis) of DSC.

## Acknowledgements

We are grateful to Dr S. Jordán from the Clínica el Sur (Málaga) and Dr F. Garcia Gallego from the Clínica Ginegranada (Granada) for providing us with decidual specimens. We thank K. Shashok for improving use of English in the manuscript.

## Funding

This work was funded by the following Grants: SAF2003-06799; SAS PFI 3/02, TCMR 0010/2006.

## References

Ashkar AA, Croy BA. Interferon-γ contributes to the normalcy of murine pregnancy. *Biol Reprod* 1999;**61**:493–502.

Ashkar AA, Di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J Exp Med* 2000;**192**: 259–270.

Avril T, Jarousseau AC, Watier H, Boucraut J, Le Bouteiller P, Bardos P, Thibault G. Trophoblast cell line resistance to NK lysis mainly involves an HLA class I-independent mechanism. *J Immunol* 1999;**162**: 5902–5909.

Chaouat G, Assal Meliani A, Martal J, Raghupathy R, Elliot J, Mosmann T, Wegmann TG. IL-10 prevents naturally occurring fetal loss in the CBAx DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. *J Immunol* 1995;**154**:4261–4268.

Choi BC, Polgar K, Xiao L, Hill JA. Progesterone inhibits in-vitro embryotoxic Th1 cytokine production to trophoblast in women with recurrent pregnancy loss. *Hum Reprod* 2000;**15**:46–59.

García-Pacheco JM, Oliver, Kimatrai M, Blanco FJ, Olivares EG. Human decidual stromal cells express CD34 and STRO-1 and are related with bone marrow stromal precursors. *Mol Hum Reprod* 2001;**7**:151–157.

Gu Y, Jow GM, Moulton BC, Lee C, Sensibar JA, Park-Sarge OK, Chen TJ, Gibori G. Apoptosis in decidual tissue regression and reorganization. *Endocrinology* 1994;**135**:1272–1279.

Hennessy A, Pilmore HL, Simmons LA, Painter DM. A deficiency of placental IL-10 in preeclampsia. *J Immunol* 1999;**163**:3491–3495.

Hunt JS, Petroff MG, McIntire RH, Ober C. HLA-G and immune tolerance in pregnancy. *FASEB J* 2005;**19**:681–693.

Iwase A, Ando H, Nagasaka T, Shibata D, Harata T, Shimomura Y, Goto M, Kikkawa F. Neutral endopeptidase expressed by decidualized stromal cells suppresses Akt phosphorylation and deoxyribonucleic acid synthesis induced by endothelin-1 in human endometrium. *Endocrinology* 2006;**147**:5153–5159.

Jokhi PP, King A, Sharkey AM, Smith SK, Loke YW. Screening for cytokine messenger ribonucleic acids in purified human decidual lymphocyte populations by the reverse-transcriptase polymerase chain reaction. *J Immunol* 1994;**153**:4427–4435.

Kanda Y, Jikihara H, Markoff E, Handwerger S. Interleukin-2 inhibits the synthesis and release of prolactin from human decidual cells. *J Clin Endocrinol Metab* 1999;**84**:677–681.

Khalil-Daher I, Riteau B, Menier C, Sedlik C, Paul P, Dausset J, Carosella ED, Rouas-Freiss N. Role of HLA-G versus HLA-E on NK function: HLA-G is able to inhibit NK cytolysis by itself. *J Reprod Immunol* 1999;**43**:175–182.

Kimatrai M, Oliver C, Abadía-Molina AC, García-Pacheco JM, Olivares EG. Contractile activity of human decidual stromal cells. *J Clin Endocrinol Metab* 2003;**88**:844–849.

Kimatrai M, Blanco O, Muñoz-Fernández R, Tirado I, Martin F, Abadia-Molina AC, Olivares EG. Contractile activity of human decidual stromal cells. II. Effect of interleukin-10. *J Clin Endocrinol Metab* 2005;**90**:6126–6130.

King A. Uterine leukocytes and decidualization. *Hum Reprod Update* 2000;**6**:28–36.

Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. A class I antigen, HLA-G, expressed in human trophoblasts. *Science* 1990;**248**: 220–223.

Le Bouteiller P, Tabiasco J. Killers become builders during pregnancy. *Nat Med* 2006;**12**:1065–1074.

Le Discorde M, Moreau P, Sabatier P, Legeais JM, Carosella ED. Expression of HLA-G in human cornea, an immune-privileged tissue. *Hum Immunol* 2003;**64**:1039–1044.

Lim KJ, Odukoya OA, Ajjan RA, Li TC, Weetman AP, Cooke ID. The role of T-helper cytokines in human reproduction. *Fertil Steril* 2000;**73**:136–142.

Marzi M, Vigano A, Trabattoni D, Villa ML, Salvaggio A, Clerici E, Clerici M. Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clin Exp Immunol* 1996;**106**:127–133.

Montes MJ, Alemán P, Tortosa CG, Borja C, Ruiz C, Olivares EG. Cultured human decidual stromal cells express antigens associated with hematopoietic cells. *J Reprod Immunol* 1996;**30**:53–66.

Montes MJ, Tortosa CG, Borja C, Abadía AC, González-Gomez F, Ruiz C, Olivares EG. Constitutive secretion of interleukin-6 by human decidual stromal cells in culture. Regulatory effect of progesterone. *Am J Reprod Immunol* 1995;**34**:188–194.

Moreau P, Adrian-Cabestre F, Menier C, Guiard V, Gourand L, Dausset J, Carosella ED, Paul P. IL-10 selectively induces HLA-G expression in human trophoblasts and monocytes. *Int Immunol* 1999;**11**:803–811.

Muñoz-Fernandez R, Blanco FJ, Frecha C, Martin F, Kimatrai M, Abadia-Molina AC, Garcia-Pacheco JM, Olivares EG. Follicular dendritic cells are related to bone marrow stromal cell progenitors and to myofibroblasts. *J Immunol* 2006;**177**:280–289.

Okada H, Nakajima T, Sanezumi M, Ikuta A, Yasuda K, Kanzaki H. Progesterone enhances interleukin-15 production in human endometrial stromal cells in vitro. *J Clin Endocrinol Metab* 2000;**85**:4765–4770.

Olivares EG, Muñoz R, Tejerizo G, Montes MJ, Gómez-Molina F, Abadía-Molina AC. Decidual lymphocytes of human spontaneous abortions induce apoptosis but not necrosis in JEG-3 extravillous trophoblast cells. *Biol Reprod* 2002;**67**:1211–1217.

Oliver C, Montes MJ, Galindo JA, Ruiz C, Olivares EG. Human decidual stromal cells express α-smooth muscle actin and show ultrastructural similarities with myofibroblasts. *Hum Reprod* 1999;**14**:1599–1605.

Piccinni MP, Giudizi MG, Biagiotti R, Beloni L, Giannarini L, Sampognaro S, Parronchi P, Manetti R, Annunziato F, Livi C *et al.* Progesterone favours the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. *J Immunol* 1995;**155**:128–133.

Plevyak M, Hanna N, Mayer S, Murphy S, Pinar H, Fast L, Ekerfelt C, Ernerudh J, Berg G, Matthiesen L *et al.* Deciency of decidual IL-10 in first trimester missed abortion: A lack of correlation with the decidual immune cell profile. *Am J Reprod Immunol* 2002;**47**:242–250.

Roth I, Corry DB, Locksley RM, Abrams JS, Litton MJ, Fisher SJ. Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. *J Exp Med* 1996;**184**:539–548.

Rouas-Freiss N, Goncalves RM, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. *Proc Natl Acad Sci USA* 1997;**94**:11520–11525.

Ruiz C, Montes MJ, Abadía-Molina AC, Olivares EG. Phagocytosis by fresh and cultured human decidual stromal cells. Opposite effects of interleukin-1α and progesterone. *J Reprod Immunol* 1997;**33**:15–26.

Sumathi VP, McCluggage WG. CD10 is useful in demonstrating endometrial stroma at ectopic sites and in confirming diagnosis of endometriosis. *J Clin Pathol* 2002;**55**:391–392.

Toki T, Shimizu M, Takagi Y, Ashida T, Konishi I. CD10 is a marker for normal and neoplastic endometrial stromal cells. *Int J Gynecol Pathol* 2002;**21**:41–47.

van der Meer A, Lukassen HG, van Lierop MJ, Wijnands F, Mosselman S, Braat DD, Joosten I. Membrane-bound HLA-G activates proliferation and interferongamma production by uterine natural killer cells. *Mol Hum Reprod* 2004;**10**:189–195.

Verma S, Hiby SE, Loke YW, King A. Human decidua natural killer cells express the receptor for and respond to the cytokine interleukin 15. *Biol Reprod* 2000;**62**:959–968.

Vigano P, Omigliana E, Angioni S, Vignali S, Vignali M, Di Blasio AM. Expression of interleukin-10 and its receptor is up-regulated in early pregnant *versus* cycling human endometrium. *J Clin Endocrinol Metab* 2002;**87**:5730–5736.

von Rango U, Classen-Linke I, Raven G, Bocken F, Beier HM. Cytokine microenvironments in human first trimester decidua are dependent on trophoblast cells. *Fertil Steril* 2003;**79**:1176–1186.

Wiendl H, Mitsdoerffer M, Weller M. Hide-and-seek in the brain: a role for HLA-G mediating immune privilege for glioma cells. *Semin Cancer Biol* 2003;**13**:343–351.

Wood KJ, Sawitzki B. Interferon γ: a crucial role in the function of induced regulatory T cells in vivo. *Trends Immunol* 2006;**27**:183–187.

Yie SM, Li LH, Li GM, Xiao R, Librach CL. Progesterone enhances HLA-G gene expression in JEG-3 choriocarcinoma cells and human cytotrophoblasts in vitro. *Hum Reprod* 2006b;**21**:46–51.

Yie SM, Xiao R, Librach CL. Progesterone enhances HLA-G gene expression through a novel progesterone response element. *Hum Reprod* 2006a;**21**: 2538–2544.

Zdravkovic M, Aboagye-Mathiesen G, Guimond MJ, Hager H, Ebbesen P, Lala PK. Susceptibility of MHC class I expressing extravillous trophoblast cell lines to killing by natural killer cells. *Placenta* 1999;**20**:431–440.

*Submitted on April 24, 2007; resubmitted on September 12, 2007; accepted on September 20, 2007*


# QUALITY, INNOVATION, AND SERVICE—IT'S ALL AT THE CENTER OF EVERYTHING WE DO.

From developing assisted reproductive technologies that maximize performance, like the first ART media and cultures, to expertise that streamlines productivity, FUJIFILM Irvine Scientific brings together decades of industry expertise with a powerhouse of innovation, turning opportunities into realities. Together, we're working to support healthy futures—from retrieval to realization.

***ALL IN*** *FOR LIFE.*
irvinesci.com/*ALLIN*

FUJIFILM
Value from Innovation

IrvineScientific