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HLA-G5 Induces IL-4 Secretion Critical for Successful Pregnancy through Differential Expression of ILT2 Receptor on Decidual CD4$^+$ T Cells and Macrophages

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Successful pregnancy in humans has been associated with production of IL-4 by T cells at the feto–maternal interface. Soluble HLA-G5 produced by trophoblasts potentially controls the decidual T cell cytokine profile. We studied the effect of HLA-G5 on the cytokine profile of purified human macrophages and Ag-specific T cells in vitro. We demonstrated that HLA-G5 increased production of IL-12 by purified peripheral blood macrophages. Although IL-12 production by macrophages is known to induce IFN-γ production by CD4$^+$ T cells, HLA-G5 increased production of IL-4 but not IFN-γ by CD4$^+$ T cells after Ag presentation by macrophages. We found that this apparent paradox was due to the differential expression of the ILT2 HLA-G5 receptor on activated T cells and macrophages. This receptor was upregulated in the former and downregulated in the latter after Ag presentation and activation of both cell types. This observation was confirmed in situ, where decidual macrophages and T cells are continuously exposed to HLA-G5 produced locally and activated by trophoblast alloantigens. Freshly isolated decidua basalis macrophages expressed lower levels of ILT2 than peripheral blood macrophages from the same pregnant women. They did not spontaneously produce IL-12, whereas freshly isolated decidual CD4$^+$ T cells expressed high levels of activation markers (CD25, HLA-DR, and CD69) as well as ILT2 and spontaneously produced IL-4 but not IFN-γ. Therefore, HLA-G5 could be responsible, at least in part, via its interaction with ILT2, for decidual T cell IL-4 production, known to be crucial for successful pregnancy. ***The Journal of Immunology*****, 2013, 191: 3651–3662.**

The conceptus has been considered to be a semiallograft because of the presence of paternal class I HLA-C molecules on the fetal-derived trophoblast cells that invade the maternal decidua basalis (1). The maternal CD4$^+$ and CD8$^+$ T cells could recognize the paternal alloantigens, after processing and presentation by maternal APCs (2). Uterine macrophages and dendritic cells express HLA class II and costimulatory molecules and comprise APCs necessary for alloantigen presentation to maternal T lymphocytes. The T lymphocytes activated by the paternal MHC Ags could proliferate and secrete cytokines. As the cytokine profile expression of the T cells could be responsible for the rejection or tolerance of allograft by the host, it has been suggested that Th1-type cytokines, including IFN-γ, that promote allograft rejection may compromise pregnancy, whereas the Th2-type cytokines (IL-4) together with IL-10, inhibiting the Th1 responses, promote allograft tolerance and therefore may improve fetal survival (3–9). Accordingly, several years ago, we showed a defect in IL-4 production by both decidual CD4$^+$ and CD8$^+$ T cells and a defect in IL-10 production (6) by decidual CD4$^+$ T cells in women suffering from unexplained recurrent abortion undergoing a spontaneous abortion (with normal chromosomal content), in comparison with the decidual T cells of women with a normal pregnancy undergoing an elective abortion (6, 7). Therefore, in humans, the success of pregnancy seemed to be associated with the production of IL-4 and IL-10 by T cells. Accordingly, in successful pregnancies, the expression of CRTH2 (marker of IL-4–producing cells) by CD4$^+$ and CD8$^+$ T cells at the site of implantation has been reported (10).

CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells, known to control immune responses against self-Ags, have also been defined recently as responsible for alloantigen tolerance (11), although the mechanisms of action of these cells in pregnancy remained unclear.

We did not find any reduction of IL-4 and IL-10 in the peripheral blood (PB) of women suffering from unexplained recurrent abortion in comparison with the PB T cells of women with a normal pregnancy undergoing a voluntary abortion (6), suggesting that this was not an inherent feature of T cells, but rather a microenvironmental alteration. We wondered which factors present in the microenvironment of the T cells present in the decidua basalis could be responsible for the unique cytokine profile shown by the de-

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Abbreviations used in this article: KIR2DL4, killer Ig-like receptor 2DL4; LILR, leukocyte Ig-like receptor; mHLA-G1, membrane-bound HLA-G1; MNC, mononuclear cell; PB, peripheral blood; sHLA-B7, soluble HLA-B7; SK, streptokinase; TT, tetanus toxoid.

cidual T cells. Candidates could include hormones for which concentrations are increased during pregnancy, in particular progesterone, which, at concentrations comparable to those present at the feto–maternal interface during pregnancy, is a potent inducer of production of Th2-type cytokines (i.e., IL-4 and IL-5) (6, 7, 12). Other candidates responsible for the cytokine profile of the decidual T cells could be molecules expressed/produced by trophoblasts. Soluble HLA-G5, secreted by the villous and extravillous cytotrophoblast that are, respectively, in direct contact with maternal blood of the intervillous space and maternal T cells in the decidua basalis (13), could be one of these.

HLA-G is a nonclassical class I molecule characterized by a minimal polymorphism, a restricted constitutive tissue distribution (mostly trophoblast), and some unique structural features, including alternative splicing resulting in several membrane-bound and soluble isoforms (13–15). Among the soluble forms, HLA-G5 is structurally similar to the full-length membrane-bound HLA-G1, except it has neither transmembrane nor intracytoplasmic domains but a translated intron 4 (14). HLA-G5 is able to bind β_2-microglobulin and form dimers (16). Soluble HLA-G5, designated in this study as HLA-G5, has been reported by several groups to be produced by extravillous and villous trophoblast (15, 17–20). One major HLA-G receptor described to date is ILT2, also designed leukocyte Ig-like receptor (LILR)B1 or CD85j. It is expressed in decidual macrophages, dendritic cells, T cells, and NK cells as well as in PB monocytes and some T cells (20–22). ILT2 binds most HLA class I ligands, but the highest affinity is for HLA-G (16, 23) and preferentially the dimeric form of HLA-G (21, 24). It has been shown that HLA-G homodimer engagement of ILT2 on human decidual macrophages triggers the secretion of IL-6, IL-8, and, to a lesser extent, TNF-α (22). As HLA-G was originally discovered on extravillous cytotrophoblast cells invading the decidua basalis (25, 26), HLA-G was considered to be a specific mediator of induction of feto–maternal tolerance during pregnancy (27). This concept was supported by lack of expression of polymorphic class I HLA-A and HLA-B and class II Ags on trophoblast (28) and the limited polymorphism of HLA-G (29). These findings suggested an inability of maternal immune cells to identify fetal cells as foreign, thereby protecting the fetus from allograft rejection by the maternal immune system. However, expression of the classical class I HLA-C Ags on extravillous cytotrophoblast (30), which could activate the decidual T cells as explained above, suggests that the maternal T cells present in the decidua can recognize and interact with trophoblast Ags and consequently secrete cytokines to regulate the growth and differentiation of trophoblast. Soluble HLA-G5 produced by extravillous cytotrophoblast (31) could modulate the cytokine profile of decidual T cells activated by paternal alloantigens exhibited by the conceptus.

How the various decidual immune cells that reside at the materno–fetal interface during pregnancy contribute to success (or failure) of pregnancy requires deeper understanding. The aim of this study was to investigate further the functional effects of HLA-G5 on both decidual macrophages and CD4+ T cells following their interaction with paternal Ags, including HLA-C, exhibited by trophoblast cells.

In this study, using in vitro and ex vivo experiments, we demonstrate that HLA-G5 downregulates IL-12 production by decidual macrophages and increases IL-4 secretion by decidual CD4+ T cells. This HLA-G5–mediated modulation of cytokine secretion is correlated with the downregulation of ILT2 expression on decidual macrophages and increased levels of the same HLA-G–specific receptor on decidual CD3+CD4+ T cells. These results strongly suggest that HLA-G5, produced locally by trophoblasts, could be responsible, at least in part, for the decidual T cell–IL-4 production that is essential for a successful pregnancy.

Materials and Methods

HLA-G5 and soluble HLA-B7

rHLA-G5 and soluble HLA-B7 (sHLA-B7) proteins were purified from specific transfectant cell-culture supernatants, as previously described (32, 33).

Cytokine production and mRNA expression of Ag-specific T cell lines in the absence or presence of HLA-G5 and sHLA-B7

Tetanus toxoid (TT)–specific T cell lines were generated from 12 donors previously vaccinated against TT, as described elsewhere (12). Briefly, 10^6 PBMC in 2 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 2×10^{-5} M 2-ME, and 2.5% human serum (complete medium) were stimulated in 24-well-flat-bottom plates for 5 d with the TT Ag (1 μg/ml) in the absence or presence of either HLA-G5 or sHLA-B7 (0.2, 1, and 2 μg/ml) (Fig. 1) and in the presence or not of recombinant human IL-4 (R&D Systems; 200 pg/ml) and IL-12 (R&D Systems; 5000 pg/ml) as controls of cytokine modulation. Human IL-2 (generous gift from Eurocetus, Milano, Italy) at 20 U/ml was then added and cultures continued for additional 9 d. Viable T blasts were suspended in complete medium and tested for their Ag specificity as follows: T cell lines, 2×10^4 T blasts were seeded in microplates and cocultured for 48 h with irradiated (9000 rad) autologous PBMC (5×10^4) in the presence of medium alone or TT (1 μg/ml). After a 16-h pulse with 0.5 μCi [^{3}H]thymidine deoxyribose (Amersham Biosciences), cultures were harvested and radioactivity measured by liquid scintillation. The phenotype distribution of TT-specific T cells was assessed by flow cytometry analysis. These TT-specific T cell lines were CD4+ cells. To induce the cytokine production by T cell lines, 10^6 T blasts from each TT-specific T line generated in the absence or in the presence of HLA-G5 or sHLA-B7 and in the presence of IL-4 and IL-12 were cultured in the presence of PMA (Sigma-Aldrich; 20 ng/ml) plus anti-CD3 mAb (BD Biosciences; 100 ng/ml). After 36 h, culture supernatants were collected and stored in aliquots at −80°C until used. IL-4, IL-5, IFN-γ, and IL-10 were quantified by an in-house–made capture ELISA using anti–IL-4, –IL-5, –IFN-γ, and –IL-10 mAb bound to microwell plates and biotinylated anti–IL-4, –IL-5, –IFN-γ, and –IL-10 mAb as revealing Abs, respectively (34). Values of the cytokine content 5 SD over those of control supernatants obtained by stimulation of irradiated feeder cells alone were considered as an effective secretion.

mRNA expression for IL-10, IL-4, IFN-γ, and IL-5 in Ag-specific T cell lines was measured using the QuantiGene 2.0 bead-based multiplex assay (Panomics, Fremont, CA). The blasts from each Ag-specific T cell line were washed three times and centrifuged (1100 rpm, 10 min) to obtain a pellet of cells. T blasts were suspended in lysis buffer solution at a concentration of 250,000 cells/ml containing 50% Lysis Mixture (Panomics) and 1 g/l proteinase K. The mixture was shaken at 65°C for 30 min to lyse the T cell blasts. The lysates were stored at −80°C until use. A panel of oligonucleotide capture probes, each with a unique sequence of 15 bases, was covalently linked to carboxylated fluorescently encoded beads (Luminex). We mixed each lysate diluted at 1:1 and 1:2 and the pooled capture beads in a round-bottom assay well and hybridized for 16 h at 54°C (final volume in each well, 100 μl). The assay mixture was transferred to a MultiScreen filter plate (Millipore, Billerica, MA), and unbound material was washed from the wells three times with wash buffer. The plate was then hybridized at 54°C for 1 h with 100 μl/well bDNA amplifier in Amplifier Diluent (Panomics). After the plate was washed twice, it was incubated at 50°C for 1 h with 100 μl/well 5′-dT(biotin)–conjugated label probe (Panomics) diluted in Label Probe Diluent (Panomics). After two washes, streptavidin-conjugated R-PE diluted in streptavidin-PE diluent (20 mmol/l Tris-HCl, 400 mmol/l lithium chloride, 1 ml/l Tween 20, 1 ml/l BSA, and 5 ml/l Micr-O-protect [Roche Applied Science]) was added, and the plate was put in a shaker and incubated at room temperature for 30 min. We washed the beads to remove unbound streptavidin-PE and then analyzed them with Bio-Plex 200 system (Bio-Rad). The streptavidin-PE fluorescence measured from each bead was proportional to the number of mRNA transcripts captured by the beads. Expression of target-specific RNA molecules was calculated as the mean values from triplicate cultures and normalized against GAPDH.

Cytokine production by TT-stimulated PB macrophages

A total of 10^6 macrophages purified by adherence from PBMC of seven donors previously vaccinated against TT (adherent fractions contain 97–99% of CD14+ cells) were suspended in 1 ml complete medium in 96-well U-bottom plates and stimulated for 5 d with the TT Ag (Istituto Sclavo, Siena, Italy; 1 μg/ml) in the absence or presence of HLA-G5 (0.2, 1, and

2 μg/ml). Supernatants were collected and stored in aliquots at −80°C until the quantification of IL-1β, IL-6, TNF-α, IL-12, IL-18, IL-10 (R&D Systems), and IFN-γ (PBL Biomedical Laboratories) by ELISA.

Kinetics of ILT2 expression on macrophages and T cells present in a bulk culture of PBMC stimulated with an Ag

A total of 10^6 PBMC obtained from four TT-vaccinated donors were stimulated with TT (1 μg/ml) in 24-well-flat-bottom plates in 2 ml complete medium for 0, 6, 24, 48, 72, and 96 h. As control, 10^6 PBMC obtained from the same four TT-vaccinated donors were cultured without any stimulation in 2 ml complete medium in 24-well-flat-bottom plates for 0, 6, 24, 48, 72, and 96 h. The ILT2 expression on $CD3^+$ T cells and $CD14^+$ macrophages present in the bulk cultures was assessed by flow cytometry analysis on an FACSCalibur (BD Biosciences) using CD3–PE-Cy7, CD14–FITC, and ILT2–PE mAb (Beckman Coulter).

Purification of macrophages, T cells, and extravillous trophoblast from decidua basalis of early pregnancy

Samples of decidua basalis were obtained from healthy pregnant women undergoing vaginal elective termination of pregnancy (8–12 wk of gestation). This study was approved by the local ethics committee of the Centre Hospitalier de l'Université Toulouse, and all pregnant women signed informed consent. Decidual mononuclear cells (MNC) were isolated from the decidua basalis by collagenase digestion and gradient centrifugation as previously described (35). Decidual $CD4^+$ T cells were purified from nonadherent cells using MACS negative selection (Miltenyi Biotec). Purity was routinely >98%. Decidual $CD14^+$ cells were isolated from adherent cell fraction by using MACS CD14 isolation kit (Miltenyi Biotec; positive selection). Purity was >88%. PBMC from the same pregnant women were obtained as described (36). PB $CD4^+$ T cells and $CD14^+$ cells were isolated by using MACS CD4 isolation kit II (negative selection) or MACS CD14 isolation kit (positive selection), respectively. Extravillous trophoblast was obtained from samples of decidua basalis after collagenase digestion and Percoll gradient, as described (17). Purity of extravillous trophoblast was assessed by cytospin and immunostaining using anti–cytokeratin-7 and anti-vimentin mAbs (DakoCytomation), as described (37). Trophoblast purity was routinely >95% cytokeratin-7 positive and >95% vimentin negative.

Quantification of cytokines produced by purified decidual $CD4^+$ T cells and macrophages

Freshly isolated decidual and PB $CD4^+$ T cells purified by MACS negative selection were cultured in 96-well plates (2×10^5 cells/well) coated with anti-CD3 mAb (BD Pharmingen, Le Pont de Claix, France) or its IgG isotype control or uncoated. After 24 or 48 h of incubation, the concentrations of IFN-γ or IL-4 in the culture supernatants (triplicates) were measured using the CBA kit (BD Pharmingen). Freshly isolated decidual $CD14^+$ cells were cultured in 96-well plates (5×10^4 cells/well) for 24, 48, 72, or 96 h in the absence or presence of LPS (10 or 100 ng/ml; Cayla-InvivoGen Europe) or normal culture medium, and the concentrations of IL-12, IL-6, or TNF-α in the culture supernatants (triplicates) were measured using the CBA kit (BD Biosciences).

Quantification by real-time quantitative RT-PCR of ILT2 and killer Ig-like receptor 2DL4 mRNA levels in decidual MNC and purified decidual $CD4^+$ T cells

Total RNA was extracted from freshly isolated PB and decidual MNC and $CD4^+$ T cells by using TRIzol (Invitrogen) and treated with DNase I (Qiagen). First-strand cDNA was prepared from 1–5 μg each RNA sample using Superscript II Reverse transcriptase according to the manufacturer's instructions (Invitrogen). Total RNA was extracted with the RNAEasy Kit and treated with DNase I (Qiagen), and cDNA was synthetized by using TaqMan Reverse Transcription Reagents (Applied Biosystems). RT-PCR was then performed by using TaqMan methodology, as described (38). Quantitative analysis of ILT2, killer Ig-like receptor 2DL4 (KIR2DL4), and β-actin was performed by using Assay on Demand (Applied Biosystem, Warrington, U.K.). β-actin was used for normalization.

Flow cytometry analysis of CD69, CD25, HLA-DR, and ILT2 cell-surface expression on purified decidual $CD3^+$ T cells and $CD14^+$ macrophages

Freshly isolated decidual $CD4^+$ T or PB $CD4^+$ T cells were stained simultaneously with $CD4^+$Pacific Blue, CD3–PE-Cy7, CD69–allophycocyanin-Cy7, CD25-allophycocyanin, HLA-DR–FITC (BD Biosciences), and ILT2-PE mAb (Beckman Coulter) or their respective IgG1–PE-Cy7, IgG1–Pacific Blue, IgG1–allophycocyanin-Cy7, IgG1-APC, IgG2a-FITC (BD Biosciences), or IgG1-PE (Beckman Coulter) isotype controls. Freshly isolated decidual- or PB-$CD14^+$ macrophages were stained simultaneously with CD14-PE.Cy7 (BD Pharmingen) and ILT2-PE mAb (Beckman Coulter) or their respective IgG1–PE-Cy7 (BD Pharmingen) or IgG1-PE (Beckman Coulter) isotype controls. Results were analyzed by an LSRII flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences). For setting gates and addressing fluorescence spillover or determining positive/negative boundaries, we have used the fluorescence-minus-one approach (39).

Coculture of PB $CD4^+$ T cells and purified trophoblast

Freshly isolated PB $CD4^+$ T cells or decidual $CD4^+$ T cells purified by MACS negative selection were cultured in 12-well plates (10^6 cells in 1 ml) in the presence or not of purified trophoblast cells from the same decidua (2×10^5 cells/well). After 24, 48, or 72 h of coculture, PB or decidual $CD4^+$ T cells were incubated simultaneously with CD4–Pacific Blue, CD3–PE-Cy7, CD69–APC-Cy7, CD25-APC, HLA-DR–FITC (BD Biosciences), and ILT2-PE mAb (Beckman Coulter) or their respective IgG1–PE-Cy7, IgG1-Pacific Blue, IgG1–APC-Cy7, IgG1-APC, IgG2a-FITC (BD Biosciences), or IgG1-PE (Beckman Coulter) isotype controls. Following incubation for 30 min at 4°C, cells were washed and analyzed with LSRII flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences).

Statistics

Statistical analyses were performed using SSPS software (SPSS, Evanston, IL). Due to nonparametric distribution, all comparisons between cytokine concentrations in basal and stimulated conditions were performed by Wilcoxon test. Data are reported as median and ranges unless otherwise stated. A p value < 0.05 was accepted as statistically significant.

Results

HLA-G5 stimulates the production of IL-12 by TT-activated PB macrophages

We first investigated the effect of HLA-G5 on the cytokine production by purified PB $CD14^+$ cells stimulated for 5 d with TT in the presence or absence of HLA-G5 (Fig. 1). A significant increase of IL-12 concentration ($p = 0.01$) was observed in the culture supernatants of macrophages cultured in the presence of HLA-G5 (1 μg/ml) compared with those cultured in the absence of HLA-G5 (Fig. 2). In contrast, HLA-G5 had no effect on the production of IL-1α, IL-6, TNF-α, IL-18, IL-10, and IFN-γ by the PB macrophages (data not shown). As IL-12 is a potent inducer of the IFN-γ production by the $CD4^+$ T cells (40), HLA-G5–induced IL-12 production by activated macrophages present among PBMC could trigger the development of IFN-γ–producing Ag-specific T cells.

HLA-G5 stimulates the IL-4 production of TT-specific T cell lines

To investigate the possible role of HLA-G5 on the cytokine profile of Ag-specific T cells, we generated TT-specific T cell lines from

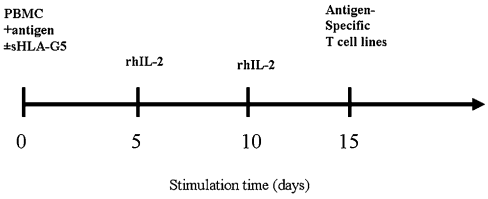

FIGURE 1. Generation of Ag-specific T cell lines in the absence or presence of HLA-G5. TT-specific T cell lines were generated from 12 donors previously vaccinated against TT. PBMC were stimulated for 5 d with TT (1 μg/ml) in the absence or presence HLA-G5 (0.2, 1, and 2 μg/ml). Human IL-2 was then added and cultures continued for additional 10 d. After additional 5 d of culture, the Ag-specific T cell lines are obtained. rhIL-2, Recombinant human IL-2.

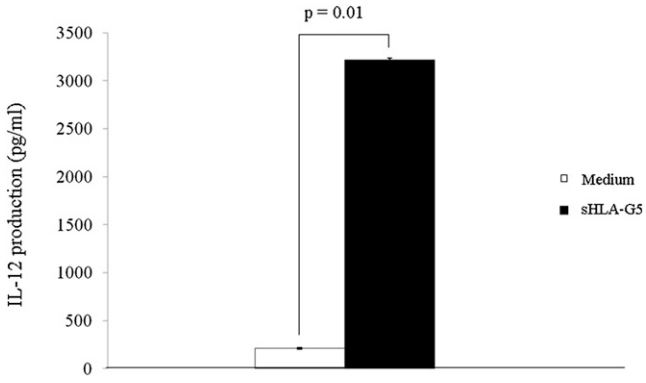

FIGURE 2. HLA-G5 stimulates the production of IL-12 by TT-activated macrophages. PB CD14+ macrophages were purified by adherence from PBMC of five donors previously vaccinated against TT and stimulated for 5 d with TT in the presence or absence of HLA-G5 (1 μg/ml). The IL-12 concentration was measured in culture supernatants by ELISA.

12 donors cultured in the absence or presence of HLA-G5 and analyzed their ability to produce IFN-γ, IL-4, IL-10, and IL-5 (Fig. 3). As control, PBMC from the same donors were stimulated with TT in the presence of IL-4, a powerful inducer of Th2 differentiation (41), or IL-12, a potent inducer of Th1 differentiation (40).

We first measured the cytokines present in the supernatants of TT-specific T cell lines (Fig. 3A). We found a significant increase of IL-4 secretion ($p = 0.02$) by the TT-specific T cell lines in response to IL-4 and a significant increase of IFN-γ ($p = 0.028$) in response to IL-12, suggesting that the culture conditions were satisfactory for the modulation of the T cell line cytokine profile. A statistically significant increase of the IL-4 production ($p = 0.02$) was observed with the TT-specific T cell lines generated in the presence of HLA-G5 (Fig. 3A) with a maximum effect at 1 μg/ml (Supplemental Fig. 1). In contrast to what was expected, the slight increase of IFN-γ production in response to HLA-G5 is not statistically significant, and also, no significant difference was observed in IL-10 and IL-5 production between the T cell lines generated in the presence or in the absence of HLA-G5 (Fig. 3A). In comparison, incubation of the same TT-specific T cell line with sHLA-B7, a classical soluble HLA class I molecule, had no significant effect on the IL-4 and IL-10 production, whereas it induced a significant increase of IFN-γ production ($p = 0.05$) and a significant decrease of IL-5 production ($p = 0.01$). Similar results were obtained by using another Ag-specific (streptokinase [SK])

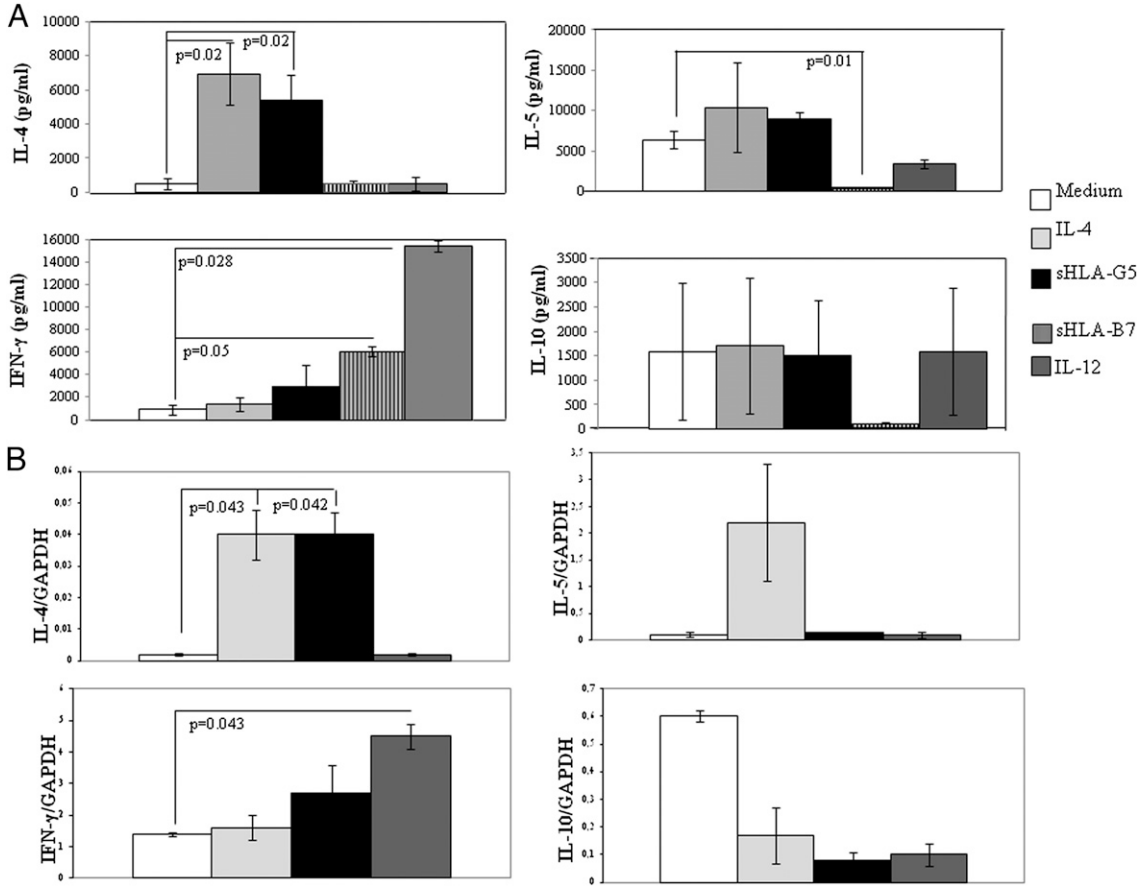

FIGURE 3. HLA-G5 stimulates the IL-4 production of TT-specific T cell lines. (**A**) TT-specific T cell lines from 12 donors were generated by a 5-d culture in the absence or presence of sHLA-G5 and their production of IFN-γ, IL-4, IL-10, and IL-5 measured by ELISAs. To control the specific effect of HLA-G5 on the cytokine profile of the TT-specific T cell lines, TT-specific T cell lines from the same donors cultured in the absence or presence of sHLA-B7 were also generated and their IL-4, IFN-γ, IL-5, and IL-10 production was measured by ELISA. PBMC from the same donors were stimulated with TT in the presence of IL-4, a powerful inducer of Th2 differentiation, and IL-12, a powerful inducer of Th1 differentiation. These indicate that the culture conditions were satisfactory for the modulation of the T cell lines cytokine profiles. p values not indicated are >0.05. (**B**) TT-specific T cell lines from five donors cultured in the absence or presence of HLA-G5 were generated and their IL-4, IFN-γ, IL-5, and IL-10 mRNA expression was measured by Quantigene 2.0. As controls, PBMC from the same donors were stimulated with TT in the presence of IL-4, an inducer of Th2 differentiation, and in the presence of IL-12, a potent Th1 inducer, which both indicate that the culture conditions were satisfactory for the modulation of the T cell lines cytokine profile. The p values not indicated are >0.05 (NS).

T line (Supplemental Fig. 2): HLA-G5 (1 μg/ml) significantly stimulated IL-4 production from SK-specific T cell lines derived from the PBMC of 23 additional donors.

We then analyzed the cytokine mRNA levels of TT-specific T cell lines by Quantigene 2.0 (Panomics) (Fig. 3B). We found a significant increase of IL-4 mRNA expression ($p = 0.043$) by the TT-specific T cell lines in response to IL-4 and a significant increase of IFN-γ mRNA expression ($p = 0.043$) by the TT-specific T cell lines in response to IL-12, suggesting that the culture conditions were satisfactory for the modulation of the T cell lines cytokine mRNA expression. A statistically significant increase ($p = 0.042$) of IL-4 mRNA expression was observed with the TT-specific T cell lines generated in the presence of HLA-G5 at 1 μg/ml compared with the TT-specific T cell lines generated in the absence of HLA-G5 (Fig. 3B). In contrast, no significant differences were observed for IFN-γ, IL-10, and IL-5 mRNA expression between the T cell lines generated in the presence or in the absence of HLA-G5 (Fig. 3B). These results confirmed the results obtained at the protein level.

The above findings indicate that HLA-G5 increases the production of IL-12 by purified PB macrophages, and, although IL-12 secretion by macrophages is known to induce IFN-γ production by activated T cells, such IFN-γ production by Ag-specific T cells in response to HLA-G5 is not significant. Surprisingly, the production of IL-4 by these T cells in response to HLA-G5 increased significantly. This apparent paradox could be due to the fact that when Ag-specific T cell lines are generated (Fig. 1), the bulk cultures comprised T cells and some APCs (monocytes/macrophages), which both express receptors for HLA-G. ILT2, the receptor for HLA-G also known as LILRB1, or CD85j, is mainly expressed by macrophages and T cells (20).

ILT2 HLA-G receptor expression is downregulated on activated macrophages and upregulated on Ag-specific T cells present in a bulk culture of Ag-stimulated PBMC

The apparent paradox mentioned above could be due to differential ILT2 expression on $CD4^+$ T cells and macrophages. To investigate this possibility, we activated PBMC of four donors with TT for 6, 24, 48, 72, and 96 h and examined the ILT2 expression on $CD3^+$ T cells and $CD14^+$ macrophages present in the bulk cultures using flow cytometry analysis. The ILT2 expression of one representative experiment is shown in Fig. 4. At time 0, when the PBMC are not yet activated by TT, $CD3^+$ T cells did not express ILT2 (Fig. 4, *top panel*), whereas 100% of $CD14^+$ macrophages expressed this receptor (Fig. 4, *bottom panel*). In the first 24 h of culture, the expression of ILT2 is upregulated on the activated TT-specific $CD3^+$T cells (Fig. 4, *top panel*; 10–26% increase in the four experiments performed) and downregulated on the $CD14^+$ cells (Fig. 4, *bottom panel*). After 48–96 h of stimulation, the percentage of $ILT2^+$ macrophages further increased, whereas the percentage of T cells expressing ILT2 decreased, suggesting that these T cells were less and less activated by the Ag and reverted to a homeostatic state.

During the first 24 h of stimulation, there is a downregulation of the ILT2 receptor expression on macrophages, which thus cannot efficiently bind its specific ligand HLA-G5 added in the culture medium. Such macrophages cannot produce enough IL-12 to induce T cells to differentiate into IFN-γ–producing T cells. In contrast, the Ag-stimulated T cells express higher levels of ILT2, allowing more efficient binding of HLA-G5 and subsequent differentiation into IL-4–producing T cells (Fig. 3A, 3B).

To investigate the influence of HLA-G5 produced locally at the feto–maternal interface, we next evaluated the expression of ILT2 on both decidual macrophage and T cells from early pregnancy.

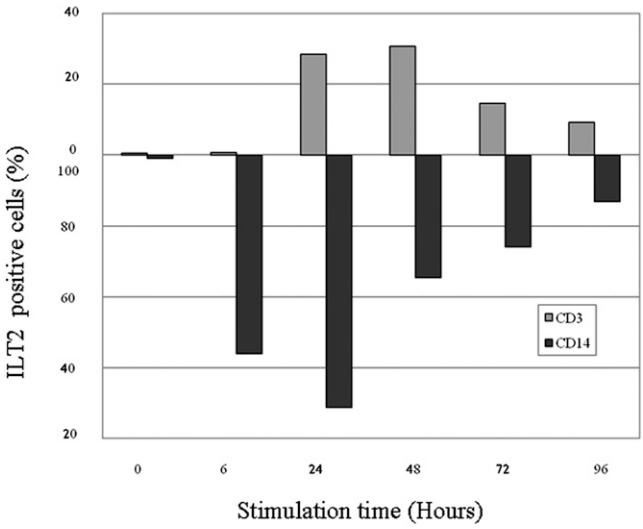

FIGURE 4. ILT2 expression is downregulated on activated macrophages and upregulated on Ag-specific T cells present in a bulk culture of Ag-stimulated PBMC. PBMC were stimulated with TT for 0, 6, 24, 48, 72, and 96 h and examined for the ILT2 expression on $CD3^+$ T cells and $CD14^+$ monocytes present in the bulk cultures using flow cytometry analysis. Representative experiment out of four.

Lower expression of ILT2 on decidual than PB macrophages in pregnant women

$CD14^+$ macrophages were purified from the decidua basalis and from the PB of the same five pregnant women who underwent an elective termination (first-trimester pregnancy). Expression of ILT2 on freshly isolated, unstimulated in vitro decidual and PB macrophages was analyzed by flow cytometry (Fig. 5). The level of ILT2 expression was significantly lower in decidual macrophages (mean 30 ± 6% of positive cells) compared with PB monocytes (mean 76 ± 10% of positive cells) ($p = 0.043$) (Fig. 5, *left panel*). Representative flow cytometric profiles of PB (82.6%) and decidual (20.5%) macrophages from the same decidua and expressing both CD14 and ILT2 are shown in Fig. 5, *right panel*. These results suggest that the local activation of decidual macrophages at the feto–maternal interface may contribute to the downmodulation of their ILT2 cell-surface expression.

Decidual $CD3^+CD4^+$ T cells are activated in situ by trophoblast cells and express ILT2 receptor

To evaluate their activation status, freshly isolated decidual $CD3^+$ $CD4^+$ T cells (90–98% positivity) were analyzed for the presence of different activation markers by flow cytometry. A significant number of decidual T cells expresses CD69 (43 ± 6%), HLA-DR (58 ± 9%), and CD25 (18 ± 4%) (Supplemental Fig. 3). These observations indicate that decidual $CD4^+$ T cells are activated locally at the feto–maternal interface. Trophoblast cells expressing paternal alloantigens are likely to be responsible for such activation. To verify this hypothesis, a comparison was made between $CD3^+CD4^+$ decidual and PB T cells of the same pregnant women using a multicolor flow cytometry analysis. As shown in Fig. 6A (*top panel*), a high number of $CD3^+CD4^+$ decidual T cells express both CD69 and $HLA\text{-}DR^+$ (81.3%) or CD69 and CD25 (45.4%). By comparison, almost no $CD3^+CD4^+$ T cells from PB are $CD69^+$ $CD25^+$ (0.043%) or $CD69^+$ $HLA\text{-}DR^+$ (0.22%) (Fig. 6A, *middle panel*). When $CD3^+CD4^+$ PB T cells were cocultured with irradiated trophoblast cells purified from the same pregnant woman, they acquire the expression of CD69/HLA-DR (32.2%) as well as CD69/CD25 (16.2%) (Fig. 6A, *bottom panel*), thus resembling to the decidual $CD4^+$ T cell phenotype. These latter results suggest

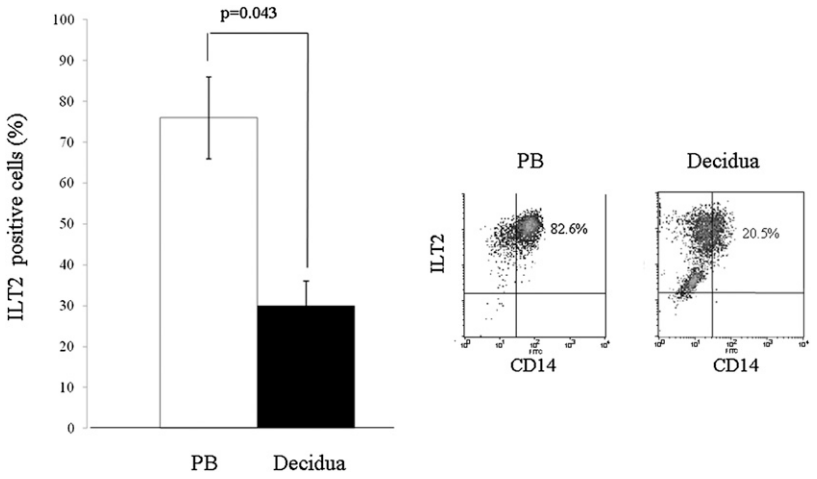

FIGURE 5. Lower expression of ILT2 on decidual macrophages in situ than on PB macrophages of pregnant women. CD14+ macrophages were purified from the decidua and from the PB of the same pregnant women ($n = 5$), who underwent an elective termination of first-trimester pregnancy. Freshly isolated, unstimulated in vitro, purified decidual and PB macrophages were analyzed by flow cytometry for their cell-surface expression of ILT2.

that in these coculture studies the trophoblast cell alloantigens presented by the APCs enriched from decidual MNC fraction could be responsible for the activated status of the CD4+ PB T cells incubated with trophoblast. By choosing the semiallogeneic combination, we wanted to be in a situation that could be linked to what could be found in normal pregnancy, in which the paternal HLA-C Ags would be able to stimulate the specific decidual CD4+ T cells after processing and presentation by maternal APCs. The decidual CD4+ cells activated in response to these alloantigens would be able to express the receptor for HLA-G5, ILT2, thus linking HLA-G5 and producing IL-4. The IL-4 secretion by CD4+ T cells in response to HLA-G5 is completely related to the expression of the receptor for HLA-G5 (ILT2) after the stimulation of CD4+ T cells. Experiments of proliferation with allogeneic combinations of 46 decidual CD4+ T cell clones obtained from the same pregnant woman stimulated with 15 different samples of purified irradiated trophoblast cells obtained from other women undergoing elective termination of pregnancy show that the proliferation of the CD4+ T cell clones in response to the 15 different allogeneic trophoblast samples is significantly increased ($p < 0.00001$) (cpm; mean ± SE, 1350 ± 189 T cells alone and 3920 ± 717 T cells plus trophoblast cells) (data not shown). In contrast, we found that the irradiated maternal PB MNC are unable to stimulate the same CD4+ T cell clones and thus cannot significantly increase the proliferation of the decidual CD4+ T cells, indicating that the maternal decidual CD4+ T cells respond solely to paternal alloantigens. These findings suggest that in allogeneic, as well as in semi-allogeneic combinations, the decidual CD4+ T cells are activated by the paternal HLA-C Ags expressed on the trophoblast cells. The result of the CD4+ T cell activation is the expression of ILT2 by the CD4+ T cells that could link HLA-G5 and produce IL-4. Accordingly, a time course of the expression of ILT2 on CD4+ cells after the stimulation of PB MNC with PMA plus ionomycin show an increase expression of ILT2 by flow cytometry analysis on the CD3+/CD4+ cells after the stimulation (time 0: 0% ILT2; 6 h: 1% ILT2; 24 h: 7% ILT2; 48 h: 17,5% ILT2; and 96 h: 28.7% ILT2; data not shown). These findings clearly indicate that the type of stimulation has no influence on ILT2 expression. The IL-4 production by CD4+ T cells in response to HLA-G5 is completely linked to the expression of the receptor for HLA-G5 (ILT2), which depends of the state of activation of CD4+ T, but is independent of the type of stimulation used (i.e., TT Ag in Fig. 4, semialloantigens, alloantigens, and PMA/ionomycin).

We confirm in a representative experiment (Fig. 6B) that CD4+ CD3+ T cells purified from the decidua of elective abortion, without any in vitro stimulation, express spontaneously high levels of activation markers HLA-DR (99.4%) or CD69 (99.2%) and CD25 (41.7%) and have been activated in vivo (Fig. 6B, *left panel*). Among these activated CD4+ T cells, many of them spontaneously coexpress ILT2 (CD4+HLA-DR+ILT2+, 13.4%; CD4+CD69+ ILT2+, 5.4%; and CD4+CD25+ILT2+, 11.9%), indicating that the total percentage of activated CD4+ T cells expressing ILT2 is 30.7% in the case of this decidua (Fig. 6B, *right panel*) and a mean of 22.33% when using six different decidua (data not shown). Although the fresh CD4+ T cells are always activated after their purification from the decidua and express activation markers (CD25, CD69, and HLA-DR), suggesting strongly that the decidual CD4+ cells have been activated in vivo, the percentage of these three markers and the percentages of the different combinations of these three markers expression vary from one decidua to another. However, not all decidual CD4+ T cells coexpress CD69 and HLA-DR (Supplemental Fig. 4). We have performed a further analysis of the six-color flow cytometry of five different decidual samples to investigate the ILT2 expression on the CD4+ CD3+ decidual T cells expressing both CD69 and HLA-DR (Supplemental Fig. 4). In the sample shown in Supplemental Fig. 4, 58.1% of decidual T cells express both CD69 and HLA-DR activation markers. Among this decidual T cell subset, 6% are ILT2+. In the other analyzed samples of CD69+HLA-DR+ decidual T cells, between 10.3 and 3.8% were ILT2+ (data not shown).

Thus, decidual CD4+ T cells could be activated at the feto–maternal interface and then express the receptor for HLA-G5, ILT2.

The expression of ILT2 by purified decidual CD4+ T cells was also confirmed at the mRNA level. RT-PCR analysis of purified decidual CD4+ T cells demonstrates that they express high levels of ILT2 transcripts, whereas decidual MNC, from which the CD4+ T cells have been purified, express very low levels of ILT2 mRNA (Fig. 6C), but high levels of KIR2DL4 mRNA. Knowing that ~70% of the decidual MNC populations consists of NK cells (42), KIR2DL4 mRNA detected in the decidual MNC fraction is likely to derive from these leukocytes. The levels of ILT2 transcripts expressed by PB CD4+ T cells obtained from the same pregnant women are much lower than the levels of transcripts for ILT2 expressed by decidual CD4+ T cells (Fig. 6C), suggesting that at the feto–maternal, interface CD4+ T cells are extensively exposed to and stimulated by paternal alloantigens compared with PB CD4+ T cells. Altogether, these results strongly suggest that decidual CD4+ T cells are highly activated in situ by trophoblast and expressed ILT2 receptor, whereas PB CD4+ T cells of the same

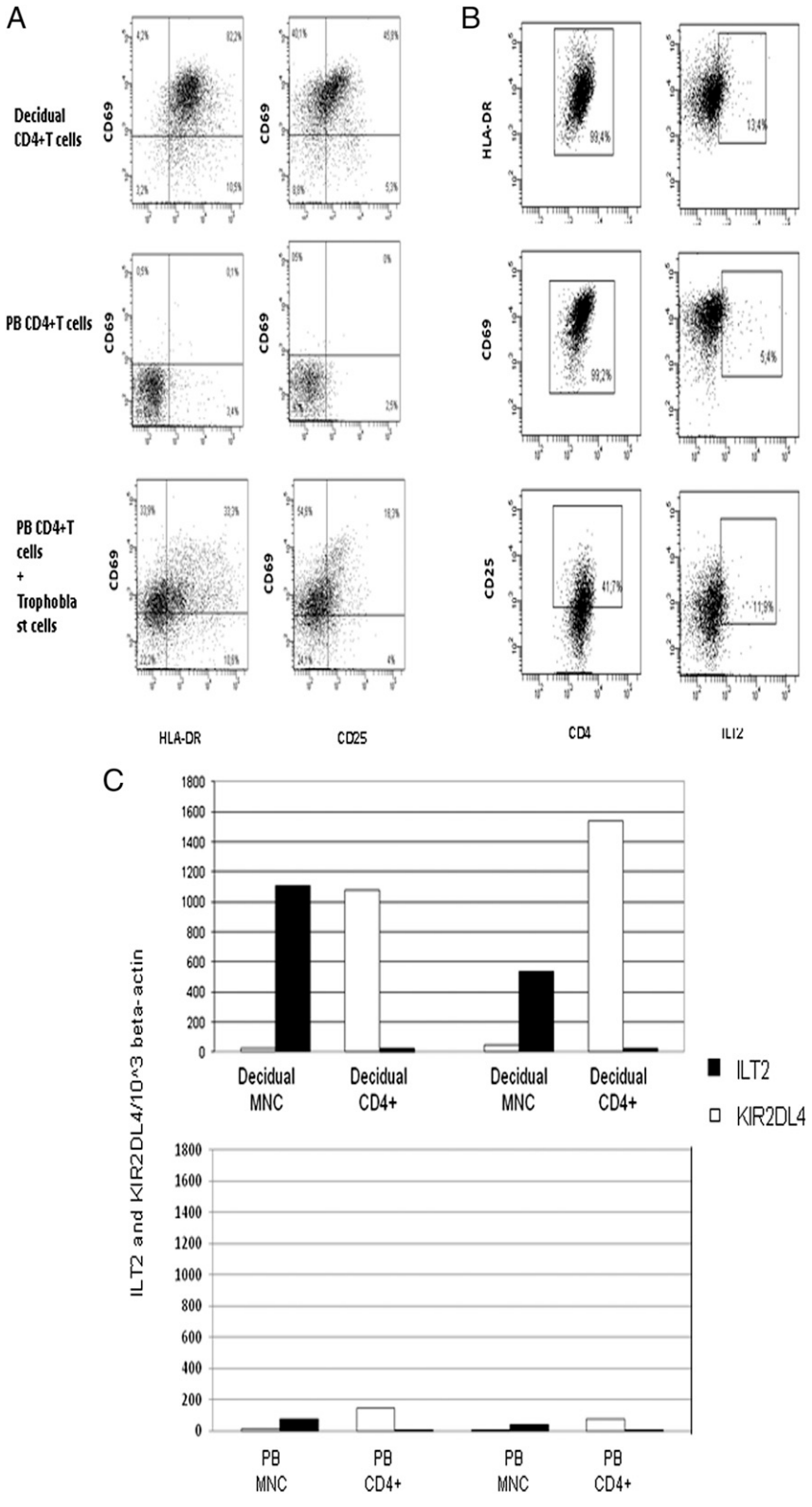

FIGURE 6. Decidual $CD3^+CD4^+$ T cells are activated in situ by trophoblast cells and express ILT2 receptor. (**A**) Multicolor flow cytometry analysis of the expression of CD69 and HLA-DR or CD69 and CD25 at the cell surface of purified, unstimulated $CD3^+CD4^+$ decidual T cells (*top panel*), PB T cells cultured alone (PB $CD4^+$ T cells, *middle panel*) from the same pregnant women, or PB T cells cocultured for 24 h with irradiated trophoblast cells enriched from the decidual MNC fraction obtained from the same pregnant woman (PB $CD4^+$T cells + Trophoblast cells, *bottom panel*). One representative experiment out of seven experiments performed. (**B**) Analysis of the cell-surface coexpression of HLA-DR and CD69 or CD25 on purified $CD4^+CD3^+$ T cells without any in vitro stimulation (*left panel*). Analysis of ILT2 membrane expression between the same $CD3^+CD4^+$HLA-DR^+, $CD3^+CD4^+CD69^+$, and $CD3^+CD4^+CD25^+$ decidual T cells (*right panel*) using a multicolor flow cytometer (one representative experiment shown). (**C**) mRNA quantitative analysis by TaqMan methodology of ILT2 and KIR2DL4 on freshly isolated MNC and $CD4^+$ T cells purified from the same MNC fraction of elective abortion decidua (*top panel*) and PB from the same two pregnant women (*bottom panel*). Two representative experiments are shown.

pregnant women undergoing normal pregnancy are not similarly activated and do not express ILT2 receptor.

In contrast to decidual $CD4^+$ T cells that spontaneously produce IL-4 but not IFN-γ, decidual macrophages do not spontaneously produce IL-12

Purified decidual $CD4^+CD3^+$ T cells were stimulated or not with anti-CD3 Abs for 24 or 48 h and IL-4 and IFN-γ production measured in the corresponding cell-culture supernatants (Fig. 7A). At both times of incubation, decidual T cells produced IL-4 spontaneously (without any in vitro stimulation). Anti-CD3 Abs stimulation further increased the levels of IL-4 release at both times. In contrast to IL-4, $CD4^+$ decidual T cells never spontaneously produced IFN-γ even after 48-h incubation (Fig. 7A).

Decidual and PB $CD4^+CD3^+$ T cells purified from the same pregnant women were stimulated or not with anti-CD3 Abs for

24 h and IL-4 and IFN-γ production measured in the corresponding cell-culture supernatants (Fig. 7B). In contrast with decidual $CD4^+$ T cells, PB $CD4^+$ cells were not able to produce IL-4 without in vitro stimulation, whereas they did produce IFN-γ spontaneously (Fig. 7B), suggesting a peculiar Th2-type pattern exhibited by decidual $CD4^+$ cells.

Purified decidual $CD14^+$ macrophages were stimulated or not with LPS for 24, 48, 72, or 96 h and IL-12, IL-6, and TNF-α production measured in the corresponding cell-culture supernatants (Fig. 8). Decidual macrophages without any in vitro stimulation spontaneously produce IL-6 and, to a lesser extent, TNF-α and the levels of these cytokines increased after LPS stimulation, indicating that macrophages purified from decidua exhibit the receptor for LPS, TLR4 (43), and are functional. In contrast, IL-12 was never spontaneously produced by decidual macrophages (Fig. 9) whether stimulated or not with LPS (Fig. 8).

Discussion

Several years ago, Kanai et al. (44, 45) investigated the role of sHLA-G1 (now called HLA-G5) on cytokine production by PBMC from nonpregnant women and by decidual MNC. The MNC were cultured with a lymphoblastoid cell line transfectant, which solely expresses membrane-bound (m)HLA-G1 (721 221-G1 cells) or with the parental untransfected cell line (722-221 cells) in the absence and presence of HLA-G5 added to the culture medium. Thus, the effect of HLA-G5 on the MNC was always combined with the effect of the mHLA-G1, leading to a complex experimental model, quite difficult to explain and discuss. The choice of the authors to check the effect of HLA-G5 on MNC, which are a mixture of different immune cells, made the interpretation of the role of HLA-G5 on $CD4^+$ T cells difficult. Moreover, the HLA-G5 used by the authors was, as they called it, a sub–HLA-G5, not the authentic soluble HLA-G5, which had an additional 20 aa at the C terminus of the molecule compared with the sub–HLA-G5. The authors first reported an increased production of IL-4 by peripheral MNC (44) but not by decidual MNC (45) in the presence of 221-G1 cells in contrast to the addition of 221 cells to the MNC. Second, they found a decreased production of IFN-γ and TNF-α by both PB and decidual MNC in the presence of 221-G1. When the sub–HLA-G5 was added to the coculture of MNC and 221-G1 cells, the IL-4 production by both peripheral MNC (44) and decidual MNC (45) was not modified, whereas both IFN-γ and

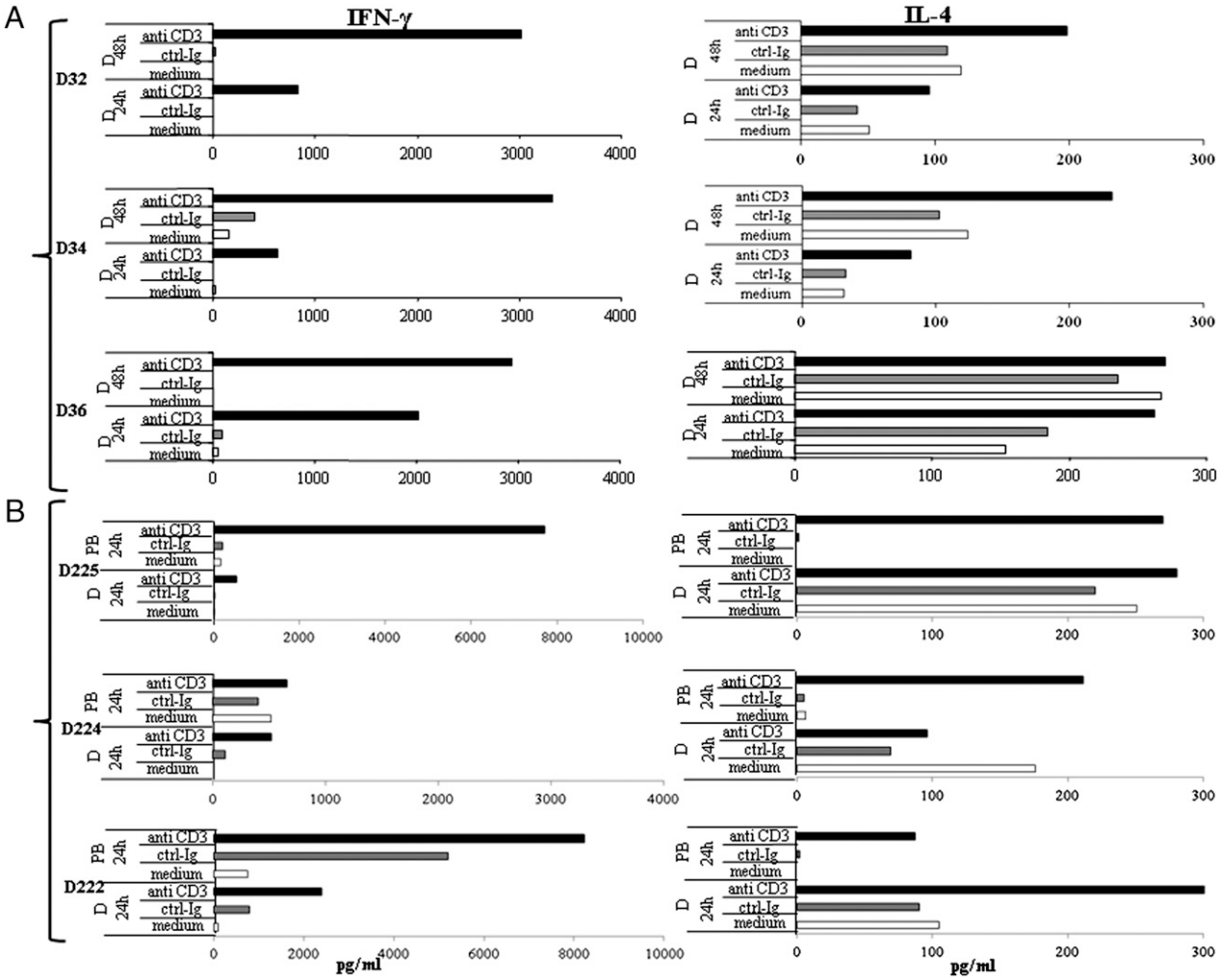

FIGURE 7. Decidual $CD4^+$ T cells spontaneously produce IL-4, but not IFN-γ . (**A**) Freshly isolated decidual $CD4^+$ T cells (D) purified by MACS negative selection were cultured with immobilized anti-CD3 mAb (black bar), IgG isotype control (gray bar), or medium alone (white bar). After 24 or 48 h of incubation, the concentrations of IFN-γ or IL-4 in the culture supernatants (triplicates) were measured using the CBA kit. Results obtained from three different deciduas (D32, D34, and D36) are shown. (**B**) Freshly isolated decidual (D) and PB $CD4^+$ T cells from the same pregnant women and purified by MACS negative selection were cultured with immobilized anti-CD3 mAb (black bar), IgG isotype control (gray bar), or medium alone (white bar). After 24 h of incubation, the concentrations of IFN-γ or IL-4 in the culture supernatants (triplicates) were measured using the CBA kit. Results obtained from three different deciduas (D222, D224, and D225) are shown.

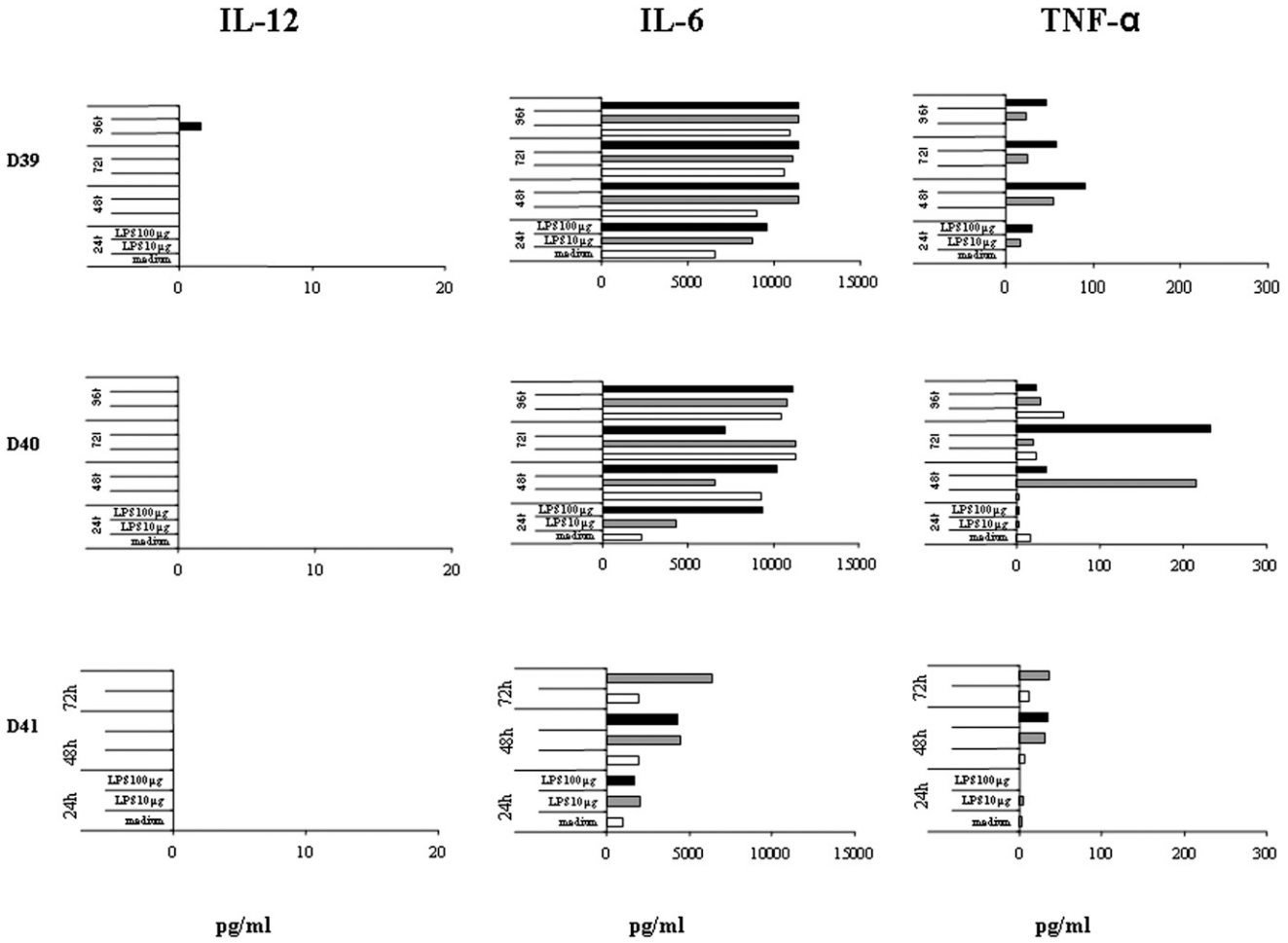

FIGURE 8. Decidual macrophages do not spontaneously produce IL-12. Freshly isolated decidual $CD14^+$ cells were cultured for 24, 48, 72, or 96 h in the absence or presence of LPS (10 or 100 μg/ml) or normal culture medium. The concentrations of IL-12, IL-6, or TNF-α in the culture supernatants (triplicates) were measured using the CBA kit. Results obtained from three different deciduas (D39, D40, and D41) are shown.

TNF-α increased in the presence of PB MNC (44) and decreased in the presence of decidual MNC (45). Thus, mHLA-G1 seemed to increase IL-4 and decrease IFN-γ production, whereas HLA-G5 seemed to have no effect on IL-4 production by MNC after the modulation by mHLAG1. The authors did not explain how the MNC could be activated to respond to mHLAG1 and then to sub–HLA-G5 and to express the receptor for HLA-G. They did not investigate which receptor for HLA-G was expressed on MNC nor which cells among decidual and PB MNC responded to mHLA-G1 to produce IL-4.

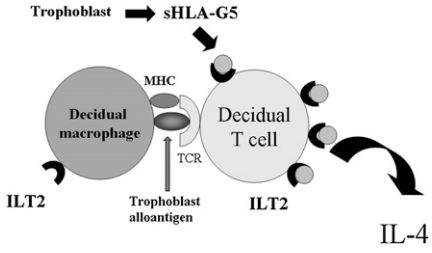

FIGURE 9. HLA-G5, via ILT2 receptor differential expression on decidual T cells and macrophages, induces IL-4 secretion by human decidual $CD4^+$ T cells in successful pregnancy. When macrophages present the alloantigens from trophoblast to the T cells, ILT2, the receptor for HLA-G5, is upregulated on the activated $CD4^+$ T cells and extensively downregulated on the activated macrophages. Decidual $CD4^+$ T cells expressing higher levels of ILT2 can bind efficiently HLA-G5 produced by trophoblast and switch toward IL-4–producing T cells.

Our experimental model used rHLA-G5 and Ag (SK or TT)-specific $CD4^+$ T cell lines. Our present investigations clearly indicated that HLA-G5 significantly increased IL-4 protein production as well as its mRNA expression, but not that of IL-5, IL-10, or IFN-γ by Ag-specific $CD4^+$ T cells. The production of IL-4 was a specific effect of HLA-G5 and not of any other soluble classical class I molecule, as sHLA-B7 showed no effect on the T cell IL-4 production, whereas it increased IFN-γ and decreased IL-5 production.

The possible functional relationship between soluble HLA-G and T cell–IL-4 production was also suggested by the presence of sHLA-G in serum of allergic patients (46, 47). Allergy is a disorder characterized by an increased ability of B cells to produce IgE to certain groups of ubiquitous Ags (allergens). IgE Ab synthesis results from the production of IL-4 by the Th2 cells, which induces the production of IgE by B cells, whereas Th1 cells produce IFN-γ that suppresses IgE synthesis (48). Higher levels of sHLA-G in the serum of patients with allergic rhinitis and in allergic children with asthma compared with healthy donors have been reported (46, 47). In contrast, sublingual immunotherapy, which was associated with a Th1-type response in patients with allergic rhinitis, was associated with reduced serum levels of sHLA-G (49). These findings highlight a possible functional relationship between HLA-G5 and IL-4 production by Th2 cells.

The surprising production of IL-4 by T cells in response to HLA-G5, whereas macrophages produced IL-12, an inducer of Th1 cells under the same conditions, could be explained by the fact that when Ag-specific T cell lines were generated, the bulk cultures included T cells and APCs (monocytes/macrophages), both of which express receptors for HLA-G5 (20). HLA-G5 has a much higher affinity for ILT2 than LILRB2 (23, 24), and ILT2 is extensively expressed on T cells and macrophages (20–22). We found that when an Ag-specific T cell line was generated in the first 24 h when APCs present the Ag to the T cells, ILT2 was upregulated on the activated T cells and extensively downregulated on the activated macrophages, which became unable to bind HLAG5 efficiently and to produce levels of IL-12 necessary to induce the great majority of T cells to switch toward IFN-γ–producing T cells. In contrast, the Ag-activated T cells, expressing higher levels of ILT2, could bind efficiently HLA-G5 added to the culture and switch toward IL-4–producing T cells. Therefore, via ILT2 receptor differential expression on decidual T cells and macrophages, HLA-G5 could be able to induce IL-4 secretion by $CD4^+$ T cells.

We verified our hypothesis in situ at the feto–maternal interface where decidual macrophages and T cells are continuously exposed to HLA-G5 produced locally. We analyzed the spontaneous expression of ILT2 by decidual macrophages and decidual T cells immediately after their purification without any additional in vitro stimulation. We found that freshly isolated decidual $CD3^+CD4^+$ T cells expressed high levels of CD25, CD69, and HLA-DR markers of activation, suggesting that decidual $CD4^+$ T cells were activated at the feto–maternal interface. Interestingly, the percentage of activated $CD69^+CD25^+CD4^+$ T cells and $CD69^+HLA\text{-}DR^+CD4^+$ T cells, which were extremely low in the PB of pregnant women cultured alone, dramatically increased in PB $CD4^+$ T cells from the same pregnant women when they were cocultured with an enriched trophoblast cells fraction. These latter results indicated that the trophoblast cells alloantigens could be responsible for the activated status of decidual $CD4^+$ T cells. More importantly, we found that decidual activated $CD3^+CD4^+$ T cells expressed spontaneously the receptor for HLA-G5, ILT2, because mRNA for ILT2 was found in the freshly purified decidual $CD3^+CD4^+$ T cell population. By comparison, mRNA for KIR2DL4, another HLA-G–specific receptor (50), which is expressed preferentially by decidual NK cells (22, 35), was not found in the decidual $CD3^+CD4^+$ T cell fraction, but only in the decidual MNC fraction, in which NK cells account for ~70% of CD45-positive cells (42). Accordingly, we have previously demonstrated that many decidual NK cells from first-trimester pregnancy express KIR2DL4 protein at their cell surface (35). This finding was confirmed in other reports (22, 51). Interestingly, Li et al. (22) also demonstrated that decidual $CD14^+$ cells were KIR2DL4 negative.

In addition, ILT2 expression was significantly decreased on decidual macrophages compared with PB monocytes obtained from the same pregnant women, suggesting that decidual macrophages have been activated at feto–maternal interface, and according to what was shown in Fig. 4, ILT2 receptor expression was downmodulated at the cell surface of the activated macrophages. Thus, in vivo, within the decidua basalis of early pregnancy where decidual macrophages and $CD4^+$ T cells are activated, macrophage ILT2 expression was downregulated, whereas decidual $CD4^+$ T cells expressed higher levels of ILT2. At the feto–maternal interface, where macrophages and T cells are continuously exposed to HLA-G5 locally produced, we analyzed the cytokine profile of these cells. We showed that many decidual $CD4^+CD3^+$, which exhibit an activated phenotype and expressed ILT2, produced spontaneously IL-4 (without any in vitro stimulation). In contrast, IFN-γ was never produced spontaneously by decidual $CD4^+$ T cells but only after stimulation by anti-CD3 Ab. We also found that decidual $CD14^+$ macrophages, which also exhibit an activated phenotype, and a downregulation of ILT2 cell-surface expression, produced spontaneously (without any in vitro stimulation) IL-6 and TNF-α according to Li et al. (22), but they did not spontaneously produce IL-12 and thus were not able to induce decidual T cells to produce IFN-γ spontaneously. Accordingly, it has been reported that other decidual APCs, such as decidual dendritic cells, secreted less IL-12 than blood monocyte-derived dendritic cells and induced Th2 cells when cocultured with naive $CD4^+$ T cells (52).

These results showed that HLA-G5, produced locally by extravillous cytotrophoblast could be responsible, at least in part, for the decidual T cells-IL-4 production necessary for a successful pregnancy (Fig. 9). Interestingly, it has been found recently that reduced or aberrant HLA-G expression seemed to be associated with certain complications of pregnancy, including the risk of miscarriage or preeclampsia (53–59). However, when soluble HLA-G isoforms were analyzed late in pregnancy, a low level of sHLA-G5 seemed to be associated with uncomplicated pregnancy, whereas in preeclampsia, the levels of sHLA-G5 were higher (60, 61). The opposite was found for the concentrations of sHLA-G1 isoform shedding from mHLA-G that were lower in women with severe PE compared with normal uncomplicated pregnancy. Altogether, these results suggest that both forms of sHLA-G may be important in the outcome of pregnancy.

We found that the HLA-G5–induced IL-4 production by decidual $CD4^+$ T cells was dependent on the differential expression of ILT2 on decidual $CD4^+$ T cells and macrophages, which could be critical in some pathologies of pregnancy, such as recurrent spontaneous abortion, in which a defective IL-4 production by the $CD4^+$ decidual T cells was observed (4, 6, 10, 62).

ILT2, which contains an immunoreceptor tyrosine-based switch motif, functions in this study as an activating receptor, although it also exerts inhibitory functions. The activating role of ILT2 has been also suggested by Li et al. (22). These authors isolated human decidual $CD14^+$ macrophages from terminated first-trimester pregnancies and cross-linked them with anti-ILT2 Ab. This resulted in upregulation of IL-6, IL-8, and TNF-α transcripts. In addition, they found that cytokine secretion from macrophages was blocked by the appropriate anti-receptor mAb as well as by anti–HLA-G mAb.

Our results demonstrated a possible protective role of the HLA-G5 for the outcome of pregnancy, via the induction of T cell-IL-4 production. This could be partly responsible for the absence of maternal rejection of the fetus during pregnancy and thus for the state of tolerance toward the semiallogeneic conceptus. Our data provide new insights into the role played by HLA-G5 at the feto–maternal interface in triggering decidual T cell production of IL-4 in this process.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Moffett-King, A. 2002. Natural killer cells and pregnancy. *Nat. Rev. Immunol.* 2: 656–663.
2. Tilburgs, T., S. A. Scherjon, B. J. van der Mast, G. W. Haasnoot, M. Versteeg-V D Voort-Maarschalk, D. L. Roelen, J. J. van Rood, and F. H. Claas. 2009. Fetal-

maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. *J. Reprod. Immunol.* 82: 148–157.
3. Wegmann, T. G., H. Lin, L. Guilbert, and T. R. Mosmann. 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol. Today* 14: 353–356.
4. Lin, H., T. R. Mosmann, L. Guilbert, S. Tuntipopipat, and T. G. Wegmann. 1993. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. *J. Immunol.* 151: 4562–4573.
5. Piccinni, M. P., and S. Romagnani. 1996. Regulation of fetal allograft survival by a hormone-controlled Th1- and Th2-type cytokines. *Immunol. Res.* 15: 141–150.
6. Piccinni, M. P., L. Beloni, C. Livi, E. Maggi, G. Scarselli, and S. Romagnani. 1998. Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. *Nat. Med.* 4: 1020–1024.
7. Piccinni, M. P., C. Scaletti, A. Vultaggio, E. Maggi, and S. Romagnani. 2001. Defective production of LIF, M-CSF and Th2-type cytokines by T cells at fetomaternal interface is associated with pregnancy loss. *J. Reprod. Immunol.* 52: 35–43.
8. Piccinni, M. P. 2010. T cell tolerance towards the fetal allograft. *J. Reprod. Immunol.* 85: 71–75.
9. Saito, S., N. Tsukaguchi, T. Hasegawa, T. Michimata, H. Tsuda, and N. Narita. 1999. Distribution of Th1, Th2, and Th0 and the Th1/Th2 cell ratios in human peripheral and endometrial T cells. *Am. J. Reprod. Immunol.* 42: 240–245.
10. Michimata, T., M. Sakai, S. Miyazaki, M. S. Ogasawara, K. Suzumori, K. Aoki, K. Nagata, and S. Saito. 2003. Decrease of T-helper 2 and T-cytotoxic 2 cells at implantation sites occurs in unexplained recurrent spontaneous abortion with normal chromosomal content. *Hum. Reprod.* 18: 1523–1528.
11. Graca, L., S. P. Cobbold, and H. Waldmann. 2002. Identification of regulatory T cells in tolerated allografts. *J. Exp. Med.* 195: 1641–1646.
12. Piccinni, M. P., M. G. Giudizi, R. Biagiotti, L. Beloni, L. Giannarini, S. Sampognaro, P. Parronchi, R. Manetti, F. Annunziato, C. Livi, et al. 1995. Progesterone favors 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.* 155: 128–133.
13. Hunt, J. S., and D. L. Langat. 2009. HLA-G: a human pregnancy-related immunomodulator. *Curr. Opin. Pharmacol.* 9: 462–469.
14. Carosella, E. D., B. Favier, N. Rouas-Freiss, P. Moreau, and J. Lemaoult. 2008. Beyond the increasing complexity of the immunomodulatory HLA-G molecule. *Blood* 111: 4862–4870.
15. Apps, R., L. Gardner, and A. Moffett. 2008. A critical look at HLA-G. *Trends Immunol.* 29: 313–321.
16. Shiroishi, M., K. Kuroki, T. Ose, L. Rasubala, I. Shiratori, H. Arase, K. Tsumoto, I. Kumagai, D. Kohda, and K. Maenaka. 2006. Efficient leukocyte Ig-like receptor signaling and crystal structure of disulfide-linked HLA-G dimer. *J. Biol. Chem.* 281: 10439–10447.
17. Solier, C., M. Aguerre-Girr, F. Lenfant, A. Campan, A. Berrebi, V. Rebmann, H. Grosse-Wilde, and P. Le Bouteiller. 2002. Secretion of pro-apoptotic intron 4-retaining soluble HLA-G1 by human villous trophoblast. *Eur. J. Immunol.* 32: 3576–3586.
18. Morales, P. J., J. L. Pace, J. S. Platt, T. A. Phillips, K. Morgan, A. T. Fazleabas, and J. S. Hunt. 2003. Placental cell expression of HLA-G2 isoforms is limited to the invasive trophoblast phenotype. *J. Immunol.* 171: 6215–6224.
19. Ishitani, A., N. Sageshima, N. Lee, N. Dorofeeva, K. Hatake, H. Marquardt, and D. E. Geraghty. 2003. Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition. *J. Immunol.* 171: 1376–1384.
20. Morales, P. J., J. L. Pace, J. S. Platt, D. K. Langat, and J. S. Hunt. 2007. Synthesis of beta(2)-microglobulin-free, disulphide-linked HLA-G5 homodimers in human placental villous cytotrophoblast cells. *Immunology* 122: 179–188.
21. Apps, R., L. Gardner, A. M. Sharkey, N. Holmes, and A. Moffett. 2007. A homodimeric complex of HLA-G on normal trophoblast cells modulates antigen-presenting cells via LILRB1. *Eur. J. Immunol.* 37: 1924–1937.
22. Li, C., B. L. Houser, M. L. Nicotra, and J. L. Strominger. 2009. HLA-G homodimer-induced cytokine secretion through HLA-G receptors on human decidual macrophages and natural killer cells. *Proc. Natl. Acad. Sci. USA* 106: 5767–5772.
23. Gonen-Gross, T., H. Achdout, T. I. Arnon, R. Gazit, N. Stern, V. Horejsí, D. Goldman-Wohl, S. Yagel, and O. Mandelboim. 2005. The CD85J/leukocyte inhibitory receptor-1 distinguishes between conformed and beta 2-microglobulin-free HLA-G molecules. *J. Immunol.* 175: 4866–4874.
24. Shiroishi, M., K. Tsumoto, K. Amano, Y. Shirakihara, M. Colonna, V. M. Braud, D. S. Allan, A. Makadzange, S. Rowland-Jones, B. Willcox, et al. 2003. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. *Proc. Natl. Acad. Sci. USA* 100: 8856–8861.
25. Ellis, S. A., I. L. Sargent, C. W. Redman, and A. J. McMichael. 1986. Evidence for a novel HLA antigen found on human extravillous trophoblast and a choriocarcinoma cell line. *Immunology* 59: 595–601.
26. Kovats, S., E. K. Main, C. Librach, M. Stubblebine, S. J. Fisher, and R. DeMars. 1990. A class I antigen, HLA-G, expressed in human trophoblasts. *Science* 248: 220–223.
27. Carosella, E. D. 2000. [HLA-G: fetomaternal tolerance]. *C. R. Acad. Sci. III* 323: 675–680.
28. Le Bouteiller, P., and I. L. Sargent. 2000. HLA class I molecules in the placenta: which ones, where and what for? A workshop report. *Placenta* 21(Suppl A): S93–S96.
29. Ober, C., and C. L. Aldrich. 1997. HLA-G polymorphisms: neutral evolution or novel function? *J. Reprod. Immunol.* 36: 1–21.
30. King, A., T. D. Burrows, S. E. Hiby, J. M. Bowen, S. Joseph, S. Verma, P. B. Lim, L. Gardner, P. Le Bouteiller, A. Ziegler, et al. 2000. Surface expression of HLA-C antigen by human extravillous trophoblast. *Placenta* 21: 376–387.
31. Hunt, J. S., D. K. Langat, R. H. McIntire, and P. J. Morales. 2006. The role of HLA-G in human pregnancy. *Reprod. Biol. Endocrinol.* 4(Suppl 1): S10.
32. Fournel, S., M. Aguerre-Girr, A. Campan, L. Salauze, A. Berrebi, Y. C. Lone, F. Lenfant, and P. Le Bouteiller. 1999. Soluble HLA-G: purification from eukaryotic transfected cells and detection by a specific ELISA. *Am. J. Reprod. Immunol.* 42: 22–29.
33. Fons, P., S. Chabot, J. E. Cartwright, F. Lenfant, F. L'Faqihi, J. Giustiniani, J. P. Herault, G. Gueguen, F. Bono, P. Savi, et al. 2006. Soluble HLA-G1 inhibits angiogenesis through an apoptotic pathway and by direct binding to CD160 receptor expressed by endothelial cells. *Blood* 108: 2608–2615.
34. Piccinni, M. P., D. Bani, L. Beloni, C. Manuelli, C. Mavilia, F. Vocioni, M. Bigazzi, T. B. Sacchi, S. Romagnani, and E. Maggi. 1999. Relaxin favors the development of activated human T cells into Th1-like effectors. *Eur. J. Immunol.* 29: 2241–2247.
35. El Costa, H., A. Casemayou, M. Aguerre-Girr, M. Rabot, A. Berrebi, O. Parant, M. Clouet-Delannoy, L. Lombardelli, N. Jabrane-Ferrat, D. Rukavina, et al. 2008. Critical and differential roles of NKp46- and NKp30-activating receptors expressed by uterine NK cells in early pregnancy. *J. Immunol.* 181: 3009–3017.
36. Barakonyi, A., K. T. Kovacs, E. Miko, L. Szereday, P. Varga, and J. Szekeres-Bartho. 2002. Recognition of nonclassical HLA class I antigens by gamma delta T cells during pregnancy. *J. Immunol.* 168: 2683–2688.
37. Pröll, J., A. Bensussan, F. Goffin, J. M. Foidart, A. Berrebi, and P. Le Bouteiller. 2000. Tubal versus uterine placentation: similar HLA-G expressing extravillous cytotrophoblast invasion but different maternal leukocyte recruitment. *Tissue Antigens* 56: 479–491.
38. Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25: 169–193.
39. Maecker, H. T., and J. Trotter. 2006. Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry A* 69: 1037–1042.
40. Manetti, R., P. Parronchi, M. G. Giudizi, M. P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J. Exp. Med.* 177: 1199–1204.
41. Maggi, E., P. Parronchi, R. Manetti, C. Simonelli, M. P. Piccinni, F. S. Rugiu, M. De Carli, M. Ricci, and S. Romagnani. 1992. Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones. *J. Immunol.* 148: 2142–2147.
42. Bulmer, J. N., P. J. Williams, and G. E. Lash. 2010. Immune cells in the placental bed. *Int. J. Dev. Biol.* 54: 281–294.
43. Allhorn, S., C. Böing, A. A. Koch, R. Kimmig, and I. Gashaw. 2008. TLR3 and TLR4 expression in healthy and diseased human endometrium. *Reprod. Biol. Endocrinol.* 6: 40.
44. Kanai, T., T. Fujii, S. Kozuma, T. Yamashita, T. Miki, A. Kikuchi, and Y. Taketani. 2001. Soluble HLA-G influences the release of cytokine from allogenic peripheral blood mononuclear cells in colture. *Mol. Hum. Reprod.* 7: 195–200.
45. Kanai, T., T. Fujii, S. Kozuma, A. Miki, T. Yamashita, H. Hyodo, N. Unno, S. Yoshida, and Y. Taketani. 2003. A subclass of soluble HLA-G1 modulates the release of cytokines from mononuclear cells present in the decidua additively to membrane-bound HLA-G1. *J. Reprod. Immunol.* 60: 85–96.
46. Tahan, F., and T. Patiroglu. 2006. Plasma soluble human leukocyte antigen G levels in asthmatic children. *Int. Arch. Allergy Immunol.* 141: 213–216.
47. Ciprandi, G., B. M. Colombo, P. Contini, P. Cagnati, A. Pistorio, F. Puppo, and G. Murdaca. 2008. Soluble HLA-G and HLA-A,-B,-C serum levels in patients with allergic rhinitis. *Allergy* 63: 1335–1338.
48. Romagnani, S. 1994. Regulation of the development of type 2 T-helper cells in allergy. *Curr. Opin. Immunol.* 6: 838–846.
49. Ciprandi, G., P. Contini, A. Pistorio, G. Murdaca, and F. Puppo. 2009. Sublingual immunotherapy reduces soluble HLA-G and HLA-A,-B,-C serum levels in patients with allergic rhinitis. *Int. Immunopharmacol.* 9: 253–257.
50. Rajagopalan, S., and E. O. Long. 2012. KIR2DL4 (CD158d): An activation receptor for HLA-G. *Front Immunol* 3: 258.
51. Xu, X., Q. Fu, Q. Zhang, M. Zhao, Z. Gao, X. Liu, Y. Liu, and X. Hu. 2013. Changes of human decidual natural killer cells cocultured with YFP-*Toxoplasma gondii*: implications for abnormal pregnancy. *Fertil. Steril.* 99: 427–432.
52. Miyazaki, S., H. Tsuda, M. Sakai, S. Hori, Y. Sasaki, T. Futatani, T. Miyawaki, and S. Saito. 2003. Predominance of Th2-promoting dendritic cells in early human pregnancy decidua. *J. Leukoc. Biol.* 74: 514–522.
53. Cecati, M., S. R. Giannubilo, M. Emanuelli, A. L. Tranquilli, and F. Saccucci. 2011. HLA-G and pregnancy adverse outcomes. *Med. Hypotheses* 76: 782–784.
54. Dahl, M., and T. V. Hviid. 2012. Human leucocyte antigen class Ib molecules in pregnancy success and early pregnancy loss. *Hum. Reprod. Update* 18: 92–109.
55. Kolte, A. M., R. Steffensen, H. S. Nielsen, T. V. Hviid, and O. B. Christiansen. 2010. Study of the structure and impact of human leukocyte antigen (HLA)-G-A, HLA-G-B, and HLA-G-DRB1 haplotypes in families with recurrent miscarriage. *Hum. Immunol.* 71: 482–488.
56. Loisel, D. A., C. Billstrand, K. Murray, K. Patterson, T. Chaiworaphongsa, R. Romero, and C. Ober. 2013. The maternal HLA-G 1597ΔC null mutation is associated with increased risk of preeclampsia and reduced HLA-G expression during pregnancy in African American women. *Mol. Hum. Reprod.* 19: 144–152.

57. Yie, S. M., L. H. Li, Y. M. Li, and C. Librach. 2004. HLA-G protein concentrations in maternal serum and placental tissue are decreased in preeclampsia. *Am. J. Obstet. Gynecol.* 191: 525–529.
58. Hara, N., T. Fujii, T. Yamashita, S. Kozuma, T. Okai, and Y. Taketani. 1996. Altered expression of human leukocyte antigen G (HLA-G) on extravillous trophoblasts in preeclampsia: immunohistological demonstration with anti-HLA-G specific antibody "87G" and anti-cytokeratin antibody "CAM5.2". *Am. J. Reprod. Immunol.* 36: 349–358.
59. Goldman-Wohl, D. S., I. Ariel, C. Greenfield, D. Hochner-Celnikier, J. Cross, S. Fisher, and S. Yagel. 2000. Lack of human leukocyte antigen-G expression in extravillous trophoblasts is associated with pre-eclampsia. *Mol. Human Reprod.* 6: 88–95.
60. Rizzo, R., A. S. Andersen, M. R. Lassen, H. C. Sørensen, T. Bergholt, M. H. Larsen, L. Melchiorri, M. Stignani, O. R. Baricordi, and T. V. Hviid. 2009. Soluble human leukocyte antigen-G isoforms in maternal plasma in early and late pregnancy. *Am. J. Reprod. Immunol.* 62: 320–338.
61. Emmer, P. M., I. Joosten, M. H. Schut, P. L. Zusterzeel, J. C. Hendriks, and E. A. Steegers. 2004. Shift in expression of HLA-G mRNA spliceforms in pregnancies complicated by preeclampsia. *J. Soc. Gynecol. Investig.* 11: 220–226.
62. Jin, L. P., Y. H. Zhou, X. Y. Zhu, M. Y. Wang, and D. J. Li. 2006. Adoptive transfer of paternal antigen-hyporesponsive T cells facilitates a Th2 bias in peripheral lymphocytes and at materno-fetal interface in murine abortion-prone matings. *Am. J. Reprod. Immunol.* 56: 258–266.