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Tubal versus uterine placentation: similar HLA-G expressing extravillous cytotrophoblast invasion but different maternal leukocyte recruitment

Key words:

Fallopian tubes; HLA-G; HLA-G monoclonal antibodies; immunohistochemistry; NK cell receptors; placenta

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Abstract: The nonclassical HLA-G class I gene is expressed by extravillous cytotrophoblast that invades decidua in uterine pregnancy, suggesting that it may contribute to the immunological mechanisms that protect the fetus against maternal alloimmune response and/or pathogen infections. We first addressed the question of whether HLA-G expression was dependent on maternal tissue environment by comparing uterine and ectopic tubal pregnancies. Using HLA-G-specific mAb on placental cryosections, we found by immunohistochemistry that all subtypes of extravillous cytotrophoblast similarly expressed HLA-G in pregnant tubes, demonstrating that its expression was independent of the site of implantation. We next compared by immunohistochemistry the phenotype of maternal leukocytes recruited in both pregnant tissues. In contrast to decidua, pregnant tubes were characterized firstly, by the lack of natural killer (NK) cells and of cells expressing CD94 receptor specific for HLA-E, secondly, by a prominent increase of CD8+ T cells, dendritic cells, and macrophages, the latter co-expressing the LIR1/ILT2 killer immunoglobulin-like receptor (KIR), and finally, by the presence of cells expressing LIR2/ILT4 KIR or BY55 NK receptors, known to bind to HLA-G. Such cell types may favor a unique innate defense in pregnant tubes. These observations also suggest that trophoblast HLA-G expression does not influence the recruitment of particular maternal leukocytes in pregnant tissues.

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During pregnancy, trophoblast cells expressing paternal alloantigens invade maternal uterine tissue to form the placenta (1, 2). They are faced to the following dilemma: firstly, they should escape a likely maternal anti-paternal alloimmune response, possibly directed by decidual cytotoxic CD8+ T and/or natural killer (NK) cells, secondly, in case of viral uterine infection, extravillous cytotrophoblast cells (Evct), that constitute one of the first layers of trophoblast which could be infected, should be recognized and eliminated either by maternal NK cells or self-HLA class I-restricted, anti-viral

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Fig. 1. Schematic diagram comparing a first trimester pregnancy in human uterus and in Fallopian tube. A) Uterine placentation is characterized by interstitial implantation and decidualization of endometrium, attachment of chorionic villi to the maternal decidua basalis and establishment of a functional blood flow. In tubal implantation, the overall structure of placentation is the same as in uterus, but the ectopic placental bed structure is different (see below). Fallopian tube lies in a peritoneal fold along the superior margin of the broad ligament, the mesosalpinx. It can be divided into intrauterine part, isthmus, ampulla and infundibulum. **B) and C) enlarged diagrams corresponding to areas enclosed in boxes shown in A):** transverse sections illustrating the major uterine and Fallopian tube implantation sites. **B) Intrauterine pregnancy.** Anchoring and floating villi bath in maternal blood of the intervillous space. They are constituted of a central mesenchyme separated from the maternal blood by a double layer of inner villous cytotrophoblast and outer syncytiotrophoblast. At the base of cell columns which fix the anchoring villi to the decidua, villous cytotrophoblast becomes proliferative and then invades the decidua basalis (interstitial trophoblast), and breach maternal

uterine spiral arteries (endovascular trophoblast). These extravillous cytotrophoblast (Evct) cells are therefore in contact with maternal leukocytes, whereas chorionic villus syncytiotrophoblast is in contact with maternal blood leukocytes. **C) Ectopic tubal pregnancy.** Floating and anchoring chorionic villi exhibit a similar structure with the same mesenchymal differentiation, and normal syncytiotrophoblast and villous cytotrophoblast layers. Anchoring villi similarly attach to the connective tissue components, showing a slightly more prominent development of trophoblastic shell. Invading Evct derived from proliferating trophoblast at the tips of the cell column infiltrate and extensively colonize muscular wall as well as maternal vessels of tubal tissue. Spiral arteries, important for uterine placentation, are absent in Fallopian tube. However, it is well vascularized, maternal vessels providing blood supply into the intervillous space. Therefore, Evct invasion of the tubal wall follows a very similar pattern in both tubal and uterine pregnancies, as already described (21). The wall of the Fallopian tube consists of different layers including muscularis, Lamina propria or connective tissue and serosa. 1, 2, 3 and 4 inserts correspond to the sites of sections shown in Figs. 2–5.

peptide T cells, in order to protect the fetus against an harmful viral spreading (3, 4). Among the different mechanisms the conceptus has developed to fulfill these dual functions, one can think that the particular pattern of HLA class I expression exhibited by Evct may be of importance. In contrast to most other somatic tissues, these invasive trophoblast cells do not constitutively express polymorphic HLA-A and HLA-B MHC class Ia surface proteins (5), thus protecting them from attack by maternal T-cell-mediated alloreaction. Conversely, they express the non-polymorphic HLA-G (5, 6), and HLA-E (7) class Ib and, to a lower level, HLA-C (8).

Several functional properties of HLA-G have been recently demonstrated (reviewed in 9). HLA-G was shown to protect cells from lysis by maternal NK cells in a functional assay (reviewed in 10). Protection of trophoblast cells from NK cell attack is crucial for the outcome of pregnancy since many maternal NK cells have been identified in the decidua at the implantation site (reviewed in 3). HLA-G1, which is the only isoform stably expressed at the cell surface (11), is indeed recognized by different Ig-like receptors expressed by subpopulations of T cells, NK cells, B cells, myelomonocytic cells or dendritic cells, namely LIR1/ILT2, LIR2/ILT4, KIR2DL4 and BY55 (reviewed in 6). HLA-G presents nonamer peptides to HLA-G-restricted T cells (12, 13). Therefore, capability of HLA-G to exert viral peptide presentation function is very likely. Finally, the soluble form HLA-G1 was recently shown to trigger Fas/Fas ligand-mediated apoptosis in activated CD8⁺ cells by interacting with CD8 (14).

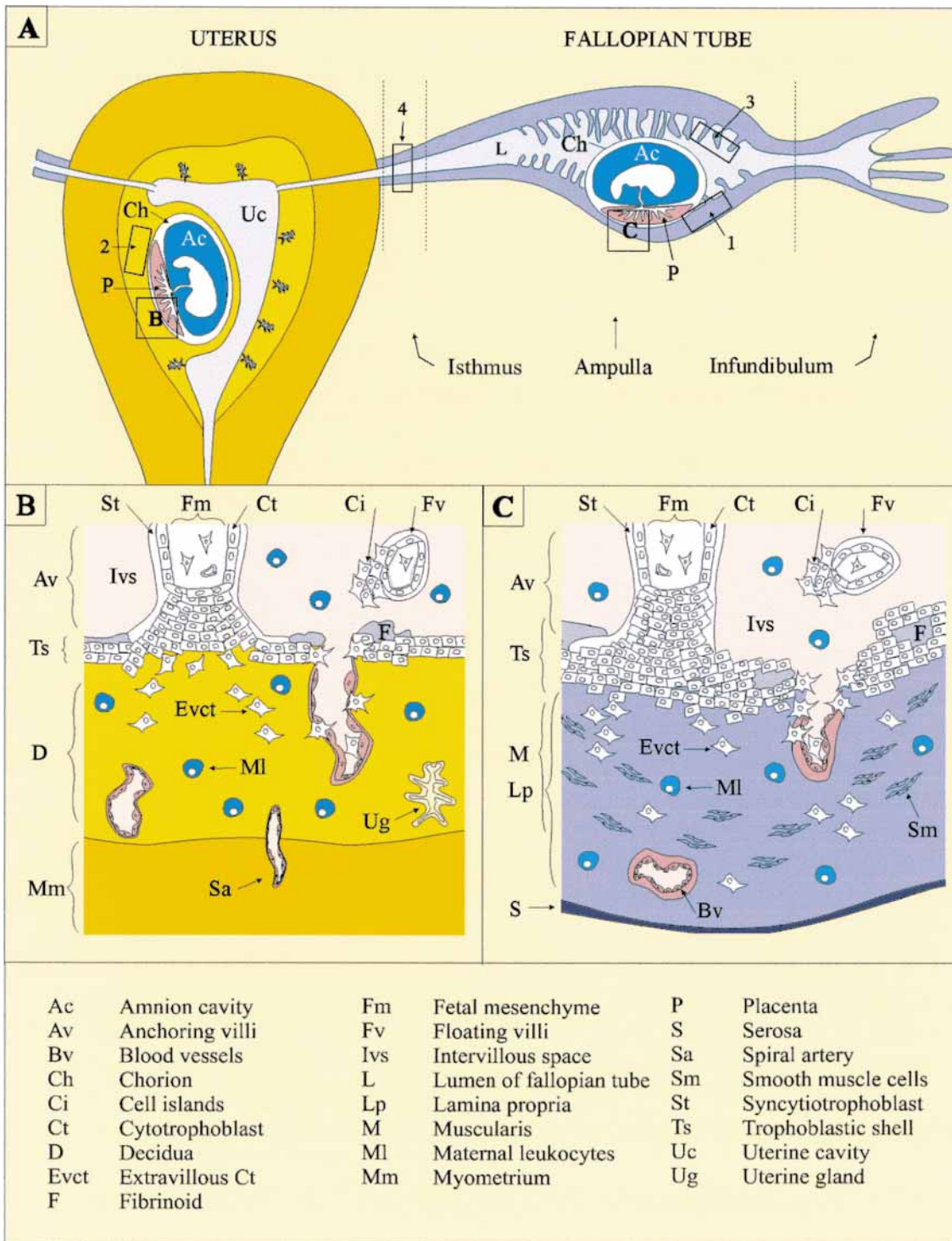
In order to evaluate the *in vivo* importance of HLA-G in the outcome of pregnancy, we asked the following questions: first, is trophoblast HLA-G expression induced by maternal environment? Second, do HLA-G expressing trophoblast cells participate to the

recruitment of defined maternal leukocytes, NK cells in particular, at the sites of implantation? Such questions have been addressed in the present study by comparing HLA-G expression in normal uterine and in ectopic tubal pregnancies (Fig. 1) and by examining the phenotype of maternal leukocytes recruited in both pregnant tissues. By immunohistochemical analysis, we found that Evct, both in uterine and in tubal pregnancies, similarly express HLA-G. This suggests that HLA-G expression is endogenously regulated through transcription factors and therefore depends on the state of differentiation but not on the maternal environment. When pregnancies develop outside the natural uterine tissue, in Fallopian tubes, our immunohistochemical study revealed a different leukocyte phenotype profile, including a lack of NK cells and a presence of CD8⁺ cells, dendritic cells and cells expressing different Ig-like receptors recognizing HLA-G. This further suggests that HLA-G does not participate to the recruitment of maternal leukocytes to the sites of implantation.

Material and methods

Tissues

Seven surgically removed Fallopian tubes containing ectopic pregnancies were examined. The clinically estimated ages of the tubal pregnancies ranged from 8 to 10 weeks postconception. No sign of salpingitis or infection such as cellular infiltrates into stroma and lumen was detectable by histopathology evaluation. Normal non-pregnant control Fallopian tubes were either obtained from two non-menopausal women undergoing hysterectomy due to benign cause



or from a woman having evolutive intrauterine pregnancy of 12 weeks: at the time of elective abortion, a tubal sterilisation was performed by partial salpingectomy. Fragments of chorionic villi and samples from decidua were obtained from three women under-

going elective terminations of 10 to 12 weeks normal pregnancies with no known abnormality. Tissues were obtained from patients at La Grave hospital, Toulouse, France and la Citadelle hospital, Liège, Belgium. Tissues were dissected to include implantation sites

of either tubal or uterine pregnancies, as well as tubal wall of isthmic and ampullar regions (tubal pregnancies) and decidua basalis (uterine pregnancies). They were placed into O.C.T. embedding compound (Miles Incorporation, Elkhart, IN, USA) and immediately snap frozen and stored in liquid nitrogen until cryostat sectioning. All procedures and consent forms were approved by the local ethical committees.

Antibodies

Characteristics and source of the mAbs used in this study are listed in Table 1.

Immunohistochemistry

Serial cryostat sections (5 µm) from the tissues described above were air-dried onto frosted slides (Erie Scientific Company, Portsmouth NH, USA) for 2 h, fixed in acetone at room temperature for 5 min and rehydrated with phosphate-buffered saline (PBS) followed by an incubation for 15 min with Ab diluent (Dako, Glostrup, Denmark) containing 10% normal human serum. Sections were then incubated with primary mAb at appropriate dilution for 30 min. After washing with PBS/0.01% Tween 20 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) the slides were incubated with biotinylated goat anti-mouse IgG secondary Ab (LSAB 2 kit; Dako) for 10 min and rinsed with PBS/Tween 20. Slides were then incu-

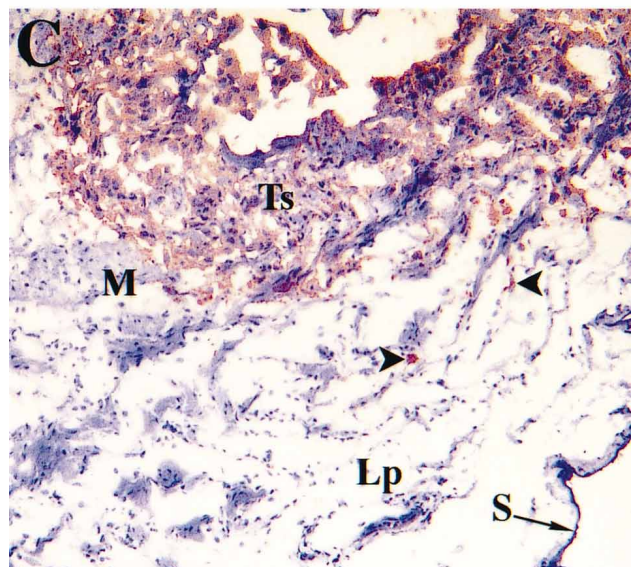
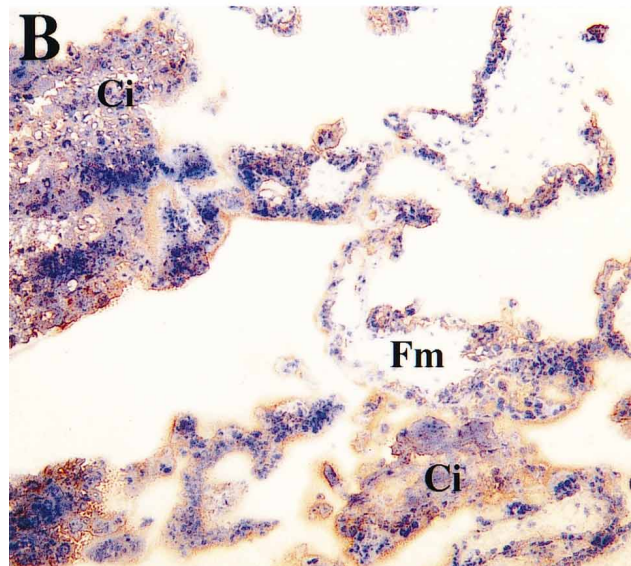
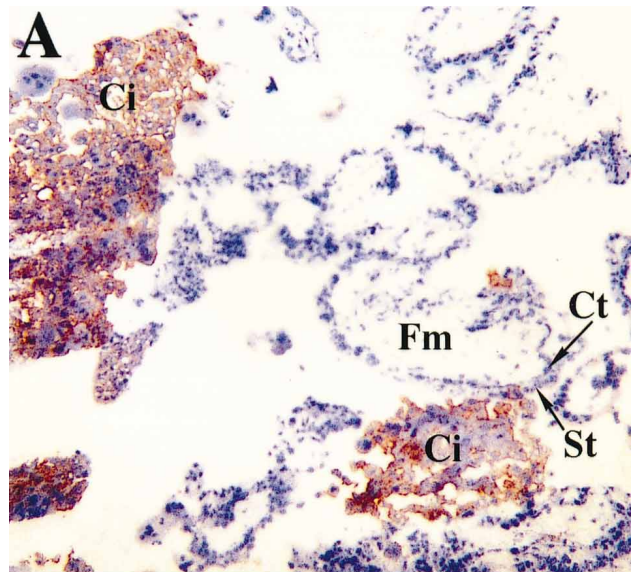
Primary monoclonal antibodies used in the study*

Table 1

Clone	Isotype	Ligand	Source
OV-TL 12/30	IgG1	Cytokeratin 7	Dako, Glostrup, Denmark
3B4	IgG2a	Vimentin	Dako
87G	IgG2a	HLA-G	gift of Dr. D. Geraghty, Seattle, WA, USA
MEM-G/1	IgG1	HLA-G	Exbio, Praha, Czech Republic
W6/32	IgG2a	HLA-G,-A,-B,-C,-E	Coulter-Immunotech
HCA2	IgG1	HLA-G,-A,-E (free hc)	gift of Pr. H. Ploegh, Boston, MA, USA
HC10	IgG2a	HLA-B,-C (free hc)	gift of Pr. H. Ploegh
L31	IgG1	HLA-C (free hc)	gift of Dr. A. Beretta, Paris, France
O10	IgG1	CD1a	INSERM U448, Paris**
4A7.6	IgG2a	CD1b	INSERM U448, Paris**
L161	IgG1	CD1c	INSERM U448, Paris**
0516	IgG2a	CD4	INSERM U448, Paris**
CD8x8	IgG2b	CD8	INSERM U448, Paris**
BE48	IgG2a	CD8	INSERM U448, Paris**
GPE3	IgG1	CD14	INSERM U448, Paris**
CR16	IgG1	CD16	INSERM U448, Paris**
CR20	IgG1	CD20	INSERM U448, Paris**
M-A251	IgG1	CD25	Pharmingen, San Diego, CA, USA
C-218	IgG1	CD56	gift of Dr. A. Moretta, Genova, Italy
HB-15A	IgG2b	CD83	Coulter-Immunotech
HP-3B1	IgG2a	CD94	gift of Dr. M. Lopez-Botet, Madrid, Spain
BMA 031	IgG2b	pan TCR α/β	Coulter-Immunotech
IMMU 389	IgG1	TCR V δ 2	Coulter-Immunotech
B8.12.2	IgG2b	HLA-DR	Coulter-Immunotech
M407	IgG1	LIR1/ILT.2	gift of Dr. D. Cosman, Immunex, Seattle, WA, USA
42D1	Rat IgG1	LIR2/ILT4	gift of Dr. M. Colonna, Basel, Switzerland
TM-60	IgG1	BY55	INSERM U448, Paris**

* Each of these mAbs was used at 3–10 µg/mL final dilution; ** mAbs produced locally; hc, heavy chain

Fig. 2. Expression of HLA-G by extravillous cytotrophoblast in a tubal pregnancy: immunohistochemical staining. Cryosections were incubated with primary mAb, biotinylated goat anti-mouse IgG Ab, streptavidin-peroxidase and AEC+ substrate and counterstained with hemalum. **A) placental section in the ampullary region (see insert 1 of Fig. 1A and its enlargement in Fig. 1C): staining with 87G HLA-G mAb.** Cell islands (Ci) of floating villi are strongly stained, whereas both villous cytotrophoblast (Ct) and syncytiotrophoblast (St) as well as fetal mesenchyme (Fm) of chorionic villi are negative. **B) Serial section stained with cytokeratin 7 mAb, a trophoblast-specific marker.** Extravillous cytotrophoblast (Evct) in cell island (Ci) is stained, demonstrating the trophoblast origin of HLA-G expressing cells. Both villous cytotrophoblast and syncytiotrophoblast are also stained, but not fetal mesenchyme (Fm). **C) Another section showing a tubal placental bed stained with 87G HLA-G mAb.** Evct HLA-G-positive cells are prominent in the trophoblastic shell (Ts) and show invasion (arrowheads) in muscularis (M) and lamina propria (Lp), near the serosa (S). ($\times 125$).



bated with peroxidase blocking solution (Dako) for 15 min at room temperature and incubated with streptavidin-peroxidase (LSAB 2 kit; Dako) for 10 min. The peroxidase staining was developed with 3-amino-9-ethylcarbazole (AEC+, Dako). After extensive washes in distilled water, slides were counterstained with Mayer's hemalum solution (Merk, Darmstadt, Germany) and mounted in Faramount (Dako). Negative control slides were incubated with a mixture of mouse IgG1, IgG2a and IgG2b isotypic control mAbs (Dako). Photographs were taken with a Zeiss Axioskope microscope (Zeiss, Oberkochen, Germany).

Double-labeling immunofluorescence

Tissues were treated as described above. For double immunostaining, sections were first incubated with Ab diluent blocking solution (Dako) containing 10% normal human serum for 15 min at room temperature, then with primary Ab at 4°C for 30 min, washed in PBS/0.01% Tween 20 for 30 min and incubated with the secondary biotinylated anti-mouse IgG Ab (30 min, 4°C). After washing in PBS (30 min, 4°C) sections were incubated with rhodamine-conjugated streptavidine (3.4 $\mu\text{g}/\text{mL}$; Coulter-Immunotech, Marseille, France). Following another washing step, slides were incubated with FITC-conjugated mAb for 30 min at 4°C. After extensive washing in PBS for 2 h, sections were mounted in Moviol containing 2.5% 1,4-diazobicyclo-octane (DABCO; Sigma) as a fading retardant. Controls were done by incubation with isotypic control mouse Ig (mixture of IgG1, IgG2a and IgG2b, Dako, Trappes, France), followed by biotinylated anti-mouse IgG Ab and rhodamine-conjugated streptavidine or by FITC-conjugated mouse IgG (Coulter Immunotech, Marseille, France). Results were examined using a laser scanning confocal microscope (Zeiss).

Statistical analysis

A Mann and Whitney test was used.

Results

Invasive extravillous cytotrophoblast in tubal pregnancies express HLA-G

A comparative immunohistochemical study, using different HLA-G mAbs, was performed on frozen sections of implantation sites and chorionic villi taken from pregnant first trimester Fallopian tube and uterus (see Fig. 1). 87G mAb strongly stained Evct present in cell islands sprouting from floating villi in Fallopian tube (Fig. 2A). In contrast, villous cytotrophoblast and syncytiotrophoblast of chorionic villi were unreactive. Trophoblast nature of these HLA-G expressing cells was identified by the use of trophoblast specific, cytokeratin 7 mAb (15): in a serial section, this mAb specifically labeled the cells that were also HLA-G positive (Fig. 2B). By contrast, these cells did not react with vimentin mAb (Table 2). Cytotrophoblastic shell as well as Evct infiltrating the tubal muscularis were also stained with 87G (Fig. 2C), as were Evct invading maternal blood vessels (data not shown). No trophoblast giant cells could be seen in pregnant tubes. Two other HLA-G mAbs, namely MEM-G/1 and HCA2, gave a similar staining pattern (Table 2). Some mesenchymal cells in the chorionic villi also appeared positive with these latter mAbs. By comparison, W6/32 mAb, which recognizes a framework determinant on all HLA class heavy chains associated with $\beta 2m$ (16), also stained Evct as well as most mesenchy-

mal cells, whereas cytotrophoblast and syncytiotrophoblast remain negative (Table 2). It was previously reported that, in addition to HLA-G, Evct invading the decidua also expressed HLA-C (8). HLA-G positive Evct cells in pregnant tubes similarly expressed HLA-C, as demonstrated by their staining with HC10 and L31, HLA-C mAbs (Table 2). Incubations of the same sections with isotypic Ig control Abs always gave negative stainings (data not shown). The same results were reproduced on three different tubal pregnancies.

By comparison, immunohistochemical study performed with the same mAbs on uterine first trimester placenta similarly detected expression of HLA-G and HLA-C in evct (data not shown), confirming previous studies (3, 5, 17, 18). Our report therefore clearly demonstrates that the different Evct subpopulations invading a pregnant Fallopian tube strongly express HLA-G, like in normal uterine pregnancy.

Lack of CD56+, maintenance of CD14+ and CD4+ and increasing number of CD8+, CD1+ and γ/δ T cells in pregnant Fallopian tubes

In order to evaluate whether HLA-G expressing cells recruited particular types of maternal cells, we then compared the maternal leukocyte populations present in uterine or tubal pregnancies. We performed an immunohistochemical study on decidua or pregnant tubal cryosection by the means of a series of markers, namely CD56 (NK-cell marker), CD14 (macrophage), α/β or γ/δ T-cell receptors, CD4, CD8 (T cell), CD1a, CD1b, CD1c or CD83 (mostly dendritic cell), CD20 (mostly B cell), CD16 (NK cells, monocytes, macrophages, granulocytes), HLA-DR (mostly antigen presenting cells) and CD25 (activated T, B cells, macrophages). Results are illustrated in Fig. 3,

Expression of HLA-G in Fallopian tube placenta

mAb	Specificity	Trophoblast cell subpopulations			Fetal mesenchyme
		Evct	vct	st	
87G	HLA-G	+++	-	-	-
MEM-G/1	HLA-G	++	-	-	-/+
HCA2	HLA-G,-A,-E (free hc)	++	-	-	-/+
W6/32	HLA-G,-A,-B,-C,-E	+++	-	-	+ / +++
HC10	HLA-B,C (free hc)	++	-	-	- / ++
L31	HLA-C (free hc)	++	-	-	- / ++
OV-TL 12/30	cytokeratin 7	+++	+++	+++	-
3B4	vimentin	-	-	-	++

Staining intensity: -, negative; -/+, some cells negative others positive; +, faintly positive; ++, positive; +++, strongly positive; hc, heavy chains; Evct, extravillous cytotrophoblast; vct, villous cytotrophoblast; st, syncytiotrophoblast

Table 2

Table 2

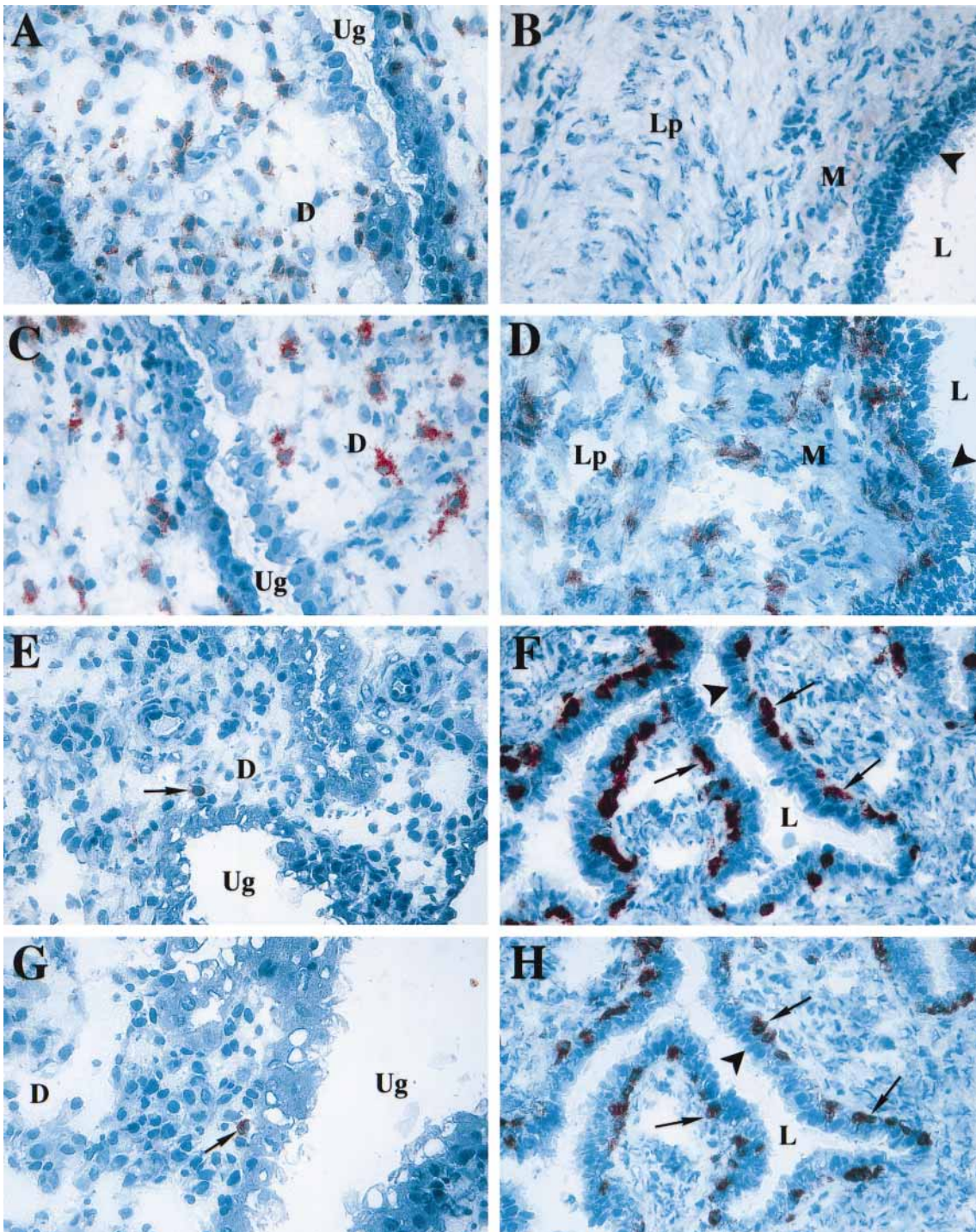


Fig. 3. Expression of CD56 (A and B), CD14 (C and D), CD8 (E and F) and CD1a (G and H): comparative immunohistochemical analysis (immunoperoxidase staining) in first trimester decidua basalis (A, C, E, G, insert 2 of Fig. 1A) and pregnant Fallopian tubes (B, D, ampulla, F, H, isthmus, inserts 3 and 4 of Fig. 1A, respectively). CD56 staining reveals many positive leukocytes in the decidua (A) but not in Fallopian tubes (B). Only few cells which exhibit

smooth muscle morphology appear very faintly stained. Almost equal CD14 positive cell density is observed in both tissues (C and D). Very few CD8 and CD1a stained cells are detectable in the decidua basalis (E and G, respectively) whereas many of them are present in tubal epithelium of the isthmus (F and H, arrows). D, decidua; Ug, uterine glands; Lp, Lamina propria; L, lumen; M, muscularis; arrow heads: Fallopian tube epithelium. ($\times 228$).

Maternal leukocytes in pregnant endometrium and Fallopian tubes: comparative immunohistochemical analysis**Table 3**

Intrauterine pregnancy				Fallopian tube pregnancy		
Phenotypic markers	Localization	Cell density	Staining intensity	Localization	Cell density	Staining intensity
CD56	db-int	<u>+++</u>	<u>+++</u>		–	–
CD14	db-int	<u>+++</u>	<u>+++</u>	isth, amp – lp, m	++	<u>+++</u>
CD4	db-int	+	+++	isth, amp – lp, m	+	<u>+++</u>
CD8	db-int	+	++	isth, amp – lp, m, ep	<u>++</u>	<u>+++</u>
CD1a	db-ep	–/+	<u>+++</u>	isth, amp – lp, m, ep	<u>+++</u>	<u>+++</u>
CD1b		–	–	isth, amp – lp, m, ep	<u>++</u>	<u>+++</u>
CD1c	db-ep	–/+	<u>+++</u>	isth, amp – lp, m, ep	<u>++</u>	<u>+++</u>
CD83		–	–	isth, amp – lp, m, ep	<u>–/+</u>	<u>++</u>
CD20		–	–		–	–
CD25		–	–		–	–
CD16	db-int	+	<u>+++</u>	isth, amp – lp, m	+	<u>+++</u>
TCR α/β	db-int	–	–	isth, amp – lp, m	<u>±</u>	<u>++</u>
TCR $V\delta 2$	db-int	+	+	isth, amp – lp, m	+	<u>++</u>
HLA-DR	db-int	<u>+++</u>	<u>+++</u>	isth, amp – lp, m, ep	<u>+++</u>	<u>+++</u>
LIR1/ILT2	db-int	–/+	–/+	isth, amp – lp, m, ep	<u>–/+++</u>	<u>++</u>
LIR2/ILT4		–	–	isth, amp – m	<u>–/+</u>	<u>±</u>
BY55		–	–	isth, amp – m	<u>–/+</u>	<u>±</u>
CD94	db-int	<u>+++</u>	<u>+++</u>		–/+	+

Cell density: –, negative; –/+, very few; +, few; +++, many; Staining intensity: –, negative; +, faintly positive; ++, positive; +++, strongly positive; Localization: db, decida basalis; lp, lamina propria; m, mucosa; int, interstitial; ep, epithelial; isth, isthmus; amp, ampulla

Major differences between intrauterine and Fallopian tube pregnancy are underlined

summarized in Table 3 and quantified in Fig. 4. The decidua contains a large number of CD56+ NK cells (Fig. 3A). In contrast, no CD56+ leukocyte was detected in Fallopian tubes (Fig. 3B). The decidua also contains many CD14+ cells (Fig. 3C), whereas less are detected in tubal epithelium (Fig. 3D). A higher number of CD14+ cells was observed in the isthmus as compared with the ampullar region of the tube. There were few CD4 weakly positive cells in decidua and tube (Table 3).

When CD8 and CD1a markers were examined, it appeared that no or very few cells were positively stained in decidual tissue (Fig. 3E, G, respectively), whereas tubal epithelia did contain many of them (Fig. 3F, H, respectively). CD1a+ cells were mainly located at the base of epithelial cells (Fig. 3F). In the isthmus, a high number of CD1a-, CD1b- and CD1c-positive cells was also found (Table 3). Another dendritic marker (CD83) stained only very few cells in the isthmus and the ampulla (Table 3) exhibiting typical dendritic morphology. We observed that the number of CD1a-, CD1b-, CD1c-positive cells was higher in the isthmus than in the ampulla.

Table 3 summarizes expression of other markers: T-cell receptor (TCR) α/β expressing cells, absent in decidua, were detected in low numbers in tubes. Some $\gamma\delta$ -positive T cells, identified by the $V\delta 2$ staining, were observed in decidua but more were present in pregnant tubes. Immunostaining with CD20 B-cell marker or CD25, interleukin (IL)-2 receptor activation marker was negative in either tissue. A low number of CD16+ cells was found in uterus and Fallopian tube. The number of CD16+ cells was more important in the subepithelial regions of the ampulla than in the isthmus. The number and staining intensity of HLA-DR+ cells were almost identical in both uterus and tubes (Table 3). HLA-DR staining was particularly intense in mucosal epithelial cells lining the lumen of Fallopian tubes. By double labeling, we found that some CD1a+ cells also expressed HLA-DR (data not shown). There was no difference for either of the markers analysed above between the tubal implantation site and the tubal mucosa away from the implantation site.

We then analysed whether expression of these different leukocyte markers was similar in non pregnant Fallopian tubes obtained

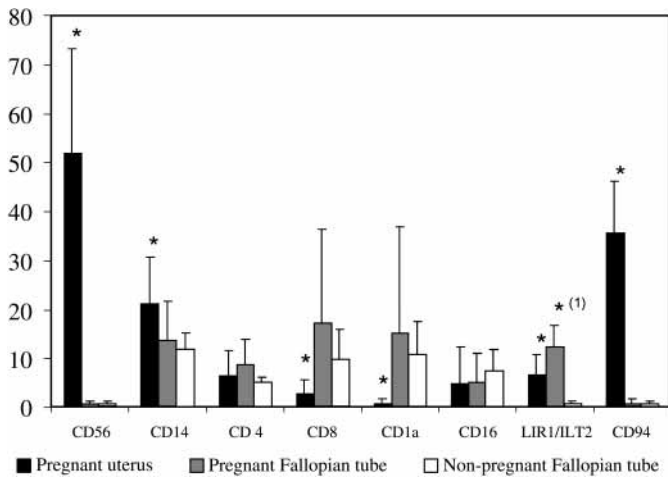


Fig. 4. Comparison between the maternal leukocyte populations in pregnant uterus and pregnant Fallopian tubes (implantation regions) as well as between pregnant and non pregnant Fallopian tubes: statistical analysis. Frozen sections of decidua and pregnant tubes were labelled with CD56, CD14, CD4, CD8, CD1a, CD16, LIR1/ILT2, or CD94, followed by an avidin-biotin immunoperoxidase staining system. Columns represent the mean percentage of positive cells for a given marker obtained from 4,000 cell counts on ten different fields of 218 μm^2 of tissue (mean of three different samples \pm SD). The non parametric Mann-Whitney U test was used to make statistical comparison between pregnant uterus (decidua basalis) and Fallopian tube (ampullar/or isthmic regions). (1): Statistical comparison between pregnant and non pregnant Fallopian tube. * $p < 0.05$.

from a woman with uncomplicated uterine pregnancy or from a non pregnant woman undergoing hysterectomy: no significant difference was observed between the three kinds of tubes (Fig. 4).

Altogether, these results showing that tubal leukocyte phenotype profile was very different from the one observed in decidua, suggest that HLA-G expressing cells did not recruit particular maternal cell types.

Several HLA-G recognizing Ig-like receptors are up-regulated in Fallopian tubes

Knowing that HLA-G is the ligand of several Ig-like receptors that are known to modulate cell lysis and also to induce particular cytokine secretion reviewed in (6), we then investigated whether some maternal leukocytes potentially in contact with evct, did express some of these receptors. We performed a comparative immunohistochemical analysis of three of these receptors, namely LIR1/ILT2, LIR2/ILT4 killer Ig-like receptors (KIR) and BY55 NK receptor, in decidua and pregnant tube cryosections, using specific mAbs. Whereas very few and faintly positive LIR1/ILT2 expressing cells were detected in decidua basalis of intrauterine pregnancy (Fig. 5A),

a higher number of stained cells was observed in the intra- and subepithelial regions of pregnant Fallopian tube (Fig. 5B). There was no preferential localization at the implantation site, since positively stained cells were also observed in other parts of the tube, mainly in the isthmus and also, but in lower number, in the ampullar epithelial region (Table 3). When pregnant and non-pregnant Fallopian tubes were examined, it appeared that the number of LIR1/ILT2 positive cells was significantly enhanced in pregnant tubes (Fig. 4). Few LIR2/ILT4 and BY55 slightly positive cells were also detected in pregnant tubes but not in decidua (Table 3).

By comparison, we also studied expression of another receptor, CD94, known to associate with NKG2 lectin-like subunits to form an heterodimer and to recognize a nonclassical MHC class I ligand, HLA-E (19, 20). Whereas many CD94 strongly stained cells were observed in the decidua (Fig. 5C), very few were detected in pregnant tube (Fig. 5D), showing that this lectin-like receptor was also differentially expressed, according to normal or ectopic sites of implantation. These results are summarized in Table 3 and quantified in Fig. 4.

LIR1/ILT2+ cells co-express CD14

In order to define the phenotype of LIR1/ILT2 expressing cells found in relatively great number in pregnant tubes, we performed a two-color laser scanning microscopy analysis. Since NK cells and B cells were almost absent in tubes, and that CD1a expressing cells were mostly concentrated on epithelium whereas LIR1/ILT2-positive cells were scattered in all parts of the tube, suggesting that these two markers were not expressed by the same cells, we used CD14 in combination with LIR1/ILT2 mAbs. Such a dual labeling revealed that LIR1/ILT2 co-localized with CD14 macrophage marker (Fig. 6). All isotypic controls were negative (data not shown).

Discussion

In the present study, we demonstrated by immunohistochemistry that evct similarly expressed HLA-G in tubal or uterine placenta, but we observed that maternal tubal and decidual leukocytes populations significantly differ in their composition. The following conclusions can be drawn from these informative observations: i) HLA-G is expressed by invasive evct, independently of the maternal tissue environment; ii) HLA-G is unlikely to control the recruitment of maternal leukocytes to the site of implantation; iii) NK cells might not be essential for proper implantation and outcome of pregnancy; iv) finally, tubal leukocyte composition may favor unique innate

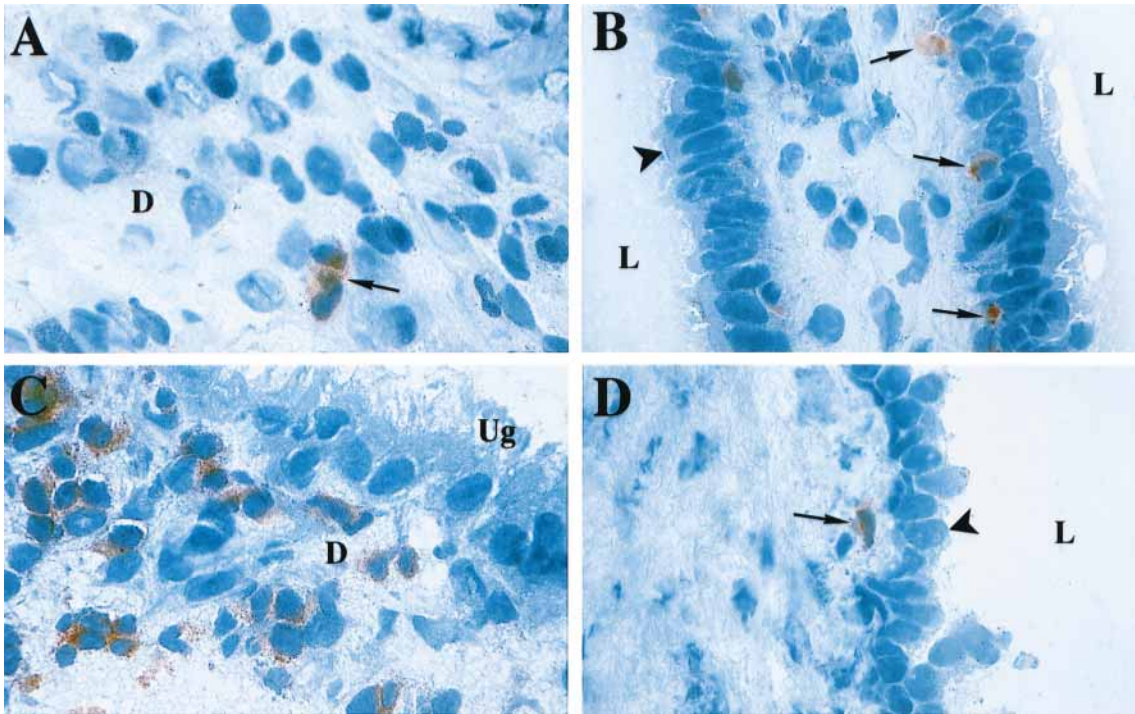


Fig. 5. Comparative immunohistochemical analysis of LIR1/ILT2 (A and B) and CD94 (C and D) expression in first trimester human decidua (A and C, insert 2 of Fig. 1A) and pregnant Fallopian tubes (B and D, insert 3 of Fig. 1A): same immunoperoxidase staining system as in Figs. 2 and 3. Very few LIR1/ILT2 expressing cells

are present in the decidua (A), whereas some intraepithelial cells are stained in tubal epithelium (arrows) (B). In contrast, many CD94 stained cells are present in the decidua basalis (C), whereas very few are detectable in tubal epithelium (D). D, decidua; L, lumen; Ug, uterine gland. Arrow heads: Fallopian tube epithelium. (×570).

defense and local mucosal immunity without compromising potential pregnancy.

HLA-G is expressed by invasive Evct, independently of the maternal tissue environment. On the contrary, we observed that non invasive trophoblast subpopulations, namely villous cytotrophoblast and syncytiotrophoblast, did not express HLA-G in either tissue. Using different HLA-G mAbs, we have observed that the different subpopulations of evct in pregnant Fallopian tubes were strongly stained and followed a pattern very similar to the one observed in pregnant uterus that is, in trophoblastic cell islands and trophoblastic shell as well as on evct penetrating the muscularis and maternal vessels. These observations suggest that HLA-G expression is not dependent upon the maternal microenvironment which differs in many ways between uterus and tubes, including tissue structure (21), leukocyte populations (22 and this study), cytokine or hormone secretion (23). Indeed HLA-G expression was described at much earlier stages of development, in human pre-implantation blastocysts (24), not yet in contact with maternal tissue. HLA-G may thus favor attachment of blastocyst to the maternal tissue either uterus, Fallopian tube or likely any other potential ectopic sites.

HLA-G is unlikely to control the recruitment of maternal leukocytes to the site of implantation. Different observations made in this study support this hypothesis. Firstly, despite the presence of similar HLA-G expressing Evct in both pregnant tissues, decidual and tubal leukocyte composition significantly differ. In addition to the up-regulation of some Ig-like cell receptors, we observed a shift of leukocyte distribution in favour of CD8, CD1 and γ/δ expressing cells, as well as a lack of CD56+ and CD94+ cells in Fallopian tubes. Secondly, we did not notice a preferential localization of these leukocytes at areas of strong HLA-G-positive trophoblast shell invasion in tubal pregnancies. CD8, CD1, γ/δ , as well as LIR1/ILT2-, LIR2/ILT4- and BY55-positive cells, were detected both in ampulla and isthmus, therefore far away from the site of implantation.

NK cells might not be essential for proper implantation and outcome of pregnancy. No evidence was found in our study for the presence of CD56+ cells in pregnant tubes resembling the major decidual leukocyte population reproducibly detected in pregnant uterus ((3, 5, 25) and this study). Such a lack of tubal CD56 expressing cells was also observed in previous studies (22, 25–27). Al-

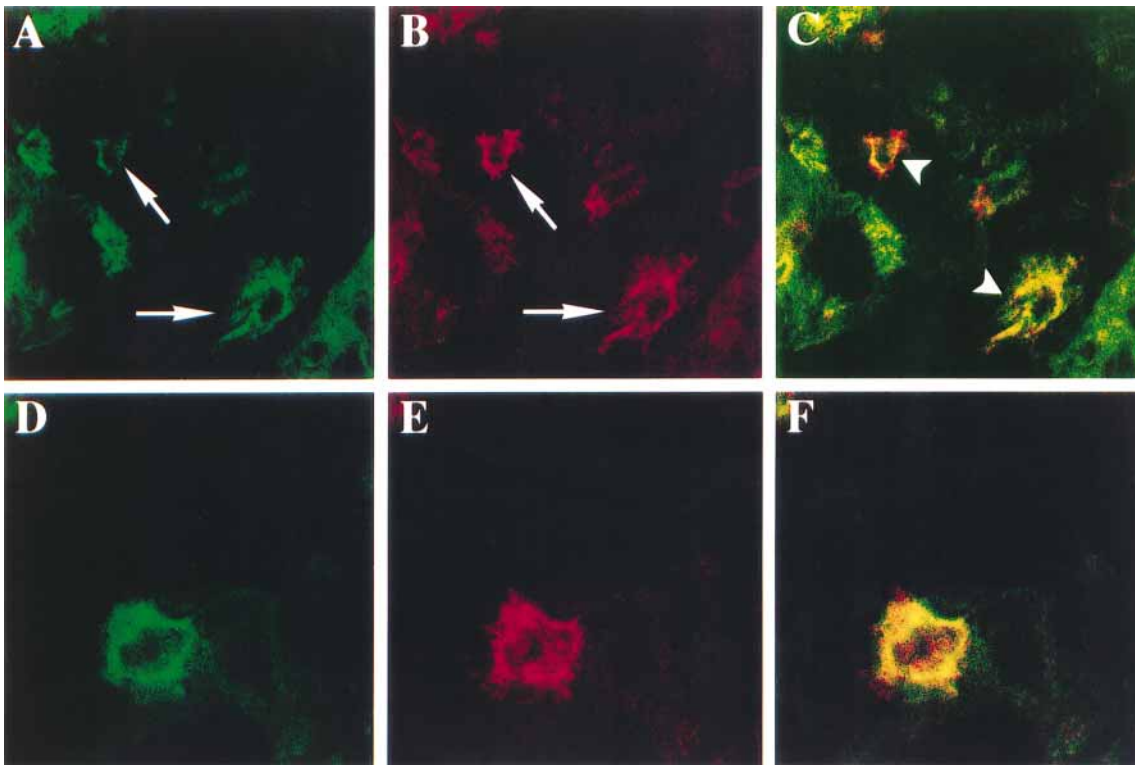


Fig. 6. Colocalization of CD14 and LIR/ILT2 in pregnant Fallopian tubes. Pregnant tubal sections were fixed in acetone and incubated with FITC-labelled CD14 (A and D, green) or with LIR1/ILT2, followed by biotinylated anti-mouse IgG and rhodamine-conjugated streptavidin (B and E, red) and were imaged by confocal laser scanning microscopy. (C and F) An

overlay of A and B or D and E, respectively, where yellowish coloring (arrow heads) indicates colocalization of CD14 and LIR1/ILT2. The pictures are representative of three independent experiments. Upper panels ($\times 630$). Lower panels ($\times 2016$).

though we cannot exclude that few CD56^{dull} cells were detectable in our immunohistochemical study, these observations suggest that NK cells might not be essential for normal implantation and successful outcome of pregnancy. Despite some vascular abnormalities and small placental size which have been detected in pregnant mice depleted of NK cells (28), there is so far no direct proof that the absence of NK cells prevents pregnancy success.

Tubal leukocyte composition may favor unique innate defense and local mucosal immunity without compromising potential pregnancy. Fallopian tubes have been considered as part of the mucosal immune system, due in particular to the expression of IgA receptors (29). Our observations fit with this interpretation. Firstly, we observed that two types of professional antigen presenting cells were particularly abundant in tubes: macrophages and dendritic cells, which are known to participate to the immediate defense against bacteria at vital tissues sites. Macrophages were identified by the CD14 marker (3). In addition to their antigen presenting function, placental macrophages were shown to actively participate in anti-inflammatory process characterized by a Th2-associated effector function with a high capacity of phagocytosis (25). CD14 was re-

cently shown to mediate clearance of apoptotic cells without inciting inflammation (30). Functions of CD14+ tubal macrophages are likely to be the same. Dendritic cell phenotype was deduced by the following markers: CD1a, CD1b, CD1c, CD83 and HLA-DR (31, 32) CD1 expressing cells recruit $\gamma\delta$ T cells (which were also detected in tubes) as well as NK T cells and present unique microbial lipids and glycolipids and therefore diversify the antigenic repertoire presented to T cells (33). Cells expressing these different markers were all localized in the same areas, mostly intraepithelial, and were particularly abundant in isthmus at the uterine entrance. Secondly, tubal leukocyte phenotypic pattern resembles the one of intestinal intraepithelial lymphocytes (IEL) which is CD8+, CD1a+, CD56- and BY55+ (34). BY55 is a GPI-anchored protein with a single Ig-like domain highly homologous to the pregnancy-specific glycoprotein and weakly homologous to KIR, mostly to KIR2DL4 (34). It has been shown that HLA-G is one of the BY55 ligands and that optimal binding required a prior aggregation of MHC class I complexes (35). The triggering of MHC class I molecules on human T-cell clones expressing BY55 was shown to deliver a potent proliferative signal in the presence of soluble CD3 mAb (35). Such effector

cell proliferation could thus contribute to an efficient immune response even in the absence of CD28 co-stimulation. In addition to BY55, we demonstrated in this study that some tubal IEL also expressed LIR1/ILT2 (36), in greater number in pregnant than in non pregnant tubes and than in decidua, as well as LIR2/ILT4 Ig-like receptors (37), both of them being ligands of HLA-G (38) and expressed in particular on blood monocytes (39). By using double immunofluorescence staining, we found in this study that LIR1/ILT2 receptor was expressed by CD14+ cells in tubes, which contrasted with decidua in which cells expressing LIR1/ILT2 were shown to be confined to CD56+ (40). Such inhibitory receptors are known to regulate NK- and T-cell cytotoxicity as well as to inhibit or down modulate monocyte activation signals (41). In addition, it was recently shown that HLA-G tetramers with self peptide bound to CD14+ blood monocytes expressing LIR2/ILT4 and also LIR1/ILT2 (38). When specific mAb will be available, it would be interesting to examine *in situ* expression of another Ig-like cell receptor, namely

KIR2DL4, capable of sensing the HLA-G expression, and previously reported to be expressed on isolated decidual NK cells (40).

In conclusion, during uterine pregnancy, the presence in decidua of many NK cells and cells expressing CD94 receptor specific for HLA-E suggest a prominent function of this latter nonclassical HLA class I molecule. HLA-G, through its leader sequence, may positively regulate expression of HLA-E (42). During tubal pregnancy, HLA-G may modulate the myelomonocytic cell behaviour. The presence of HLA-G-specific receptors on this cell type may influence the inflammatory response to pathogens and cytotoxicity as well as cytokine production and therefore participate to the first line of defense against tubal infections. Tubal leukocytes should protect female reproductive tract without compromising fetal survival in case of pregnancy. Constant expression of HLA-G by Evct in both tubal and uterine placentation strongly suggests important functions of this molecule at the feto-maternal interface during the establishment of pregnancy at either site of implantation.

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