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Natural-killer cell ligands at the maternal–fetal interface: UL-16 binding proteins, MHC class-I chain related molecules, HLA-F and CD48

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BACKGROUND: In the early stages of human placentation, the decidua is invaded by fetal extravillous trophoblast (EVT) cells. Interactions between EVT cells and local decidual leukocytes are likely to contribute to immunological accommodation of the semi-allogeneic fetus. METHODS AND RESULTS: Natural-killer group 2 member D (NKG2D) and 2B4 (CD244) are receptors ubiquitously expressed by the distinctive population of CD56 bright, uterine natural-killer cells, which dominate the decidua at the time of implantation. Here, we investigate the UL-16 binding protein (ULBP) and MHC class-I chain related molecule (MIC) ligands of NKG2D, the CD48 ligand of 2B4 and the non-classical HLA class-I molecule, HLA-F, at the maternal–fetal interface of normal pregnancies. For many of these molecules, significant mRNA expression was detected by RT-PCR in decidual and placental tissue throughout gestation. Flow cytometry of isolated cells or immunohistological staining of implantation site sections was then performed. No protein expression of NKG2D ligands or HLA-F could be detected in decidual leukocytes or fetal trophoblast cells from the first trimester. An NKG2D-Fc fusion protein identified no novel ligands for this promiscuous receptor at the maternal–fetal interface. Strong surface protein expression of CD48 by decidual leukocytes but not by trophoblast cells was detected by flow cytometry. Histological staining showed a clear aggregation of CD48$^+$ cells around transformed spiral arteries of the implantation site. CONCLUSIONS: We conclude that the role of NKG2D and 2B4 is not focussed on trophoblast recognition in normal pregnancy, but is more likely involved in cross-talk among maternal cells of the placental bed.

Keywords: UL-16 binding protein; HLA-F; trophoblast; decidual leukocytes; natural-killer cells

Introduction

Placentation in humans is a complex process in which invading extravillous trophoblast (EVT) cells move through the decidua to transform spiral arteries and establish an increased blood supply to the fetus (Moffett and Loke, 2006). EVT cells display an unusual array of HLA class-I (HLA-I) molecules: HLA-C, HLA-E and HLA-G (King *et al*., 2000; Apps *et al*., 2007, 2008). As they migrate through the uterine wall, EVT cells come into close contact with maternal decidual leukocytes composed mainly of natural-killer (NK) cells and macrophages with a small population of T cells. The dominant decidual leukocytes at the time of implantation are a distinctive population of uterine NK (uNK) cells that have a phenotype and function distinct from cells in peripheral blood (Moffett-King, 2002). There is growing evidence that uNK cells play a crucial role in regulating the degree of trophoblast invasion and therefore maternal blood flow to the placenta and developing fetus (Hiby *et al*., 2004; Hanna *et al*., 2006). uNK cells have killer cell immunoglobulin-like receptors (KIR), leukocyte immunoglobulin-like receptors (LILR) and CD94/NKG2 family receptors capable of recognizing all three trophoblast HLA-I molecules (King *et al*., 2000; Apps *et al*., 2007; Sharkey *et al*., 2008). uNK also express additional activating and inhibitory receptors including NK group 2 member D (NKG2D) and 2B4 (CD244) whose potential interactions with trophoblast ligands have not been explored (Hanna *et al*., 2006; Vacca *et al*., 2006; Zhang *et al*., 2007).

NKG2D is a C-type lectin-like molecule that is an activating receptor expressed by NK cells and T cell subsets (Raulet, 2003). Unlike many NK receptors, the ligands for NKG2D (NKG2DL) are diverse and numerous. In humans there are two

families structurally related to HLA-I molecules: MHC class-I related (MIC) and UL-16 binding proteins (ULBP) (Eagle and Trowsdale, 2007). Certain NKG2DLs are constitutively expressed by cells at high risk of infection, such as the gut epithelium (Groh *et al*., 1996). NKG2DL expression can be induced in other tissues by stresses such as infection or transformation (Groh *et al*., 2001; Gasser *et al*., 2005). EVT cells are of extra-embryonic origin and express epithelial cell markers. By virtue of their endogenous retroviral expression and invasive behaviour, they also have similarities to infected and tumour cells. In addition, NKG2DLs have been found to be expressed by leukocytes such as activated antigen-presenting cells and provide a mechanism of interaction with NK cells (Draghi *et al*., 2007).

2B4 (CD244) is a receptor from the family of signalling lymphocyte-activation molecules (SLAM) which encode a ligand binding immunoglobulin (Ig)V domain distal to a single IgC2 domain (Ma *et al*., 2007). 2B4 is expressed by NK cells and T cell subsets (Valiante and Trinchieri, 1993) and binds CD48 (Brown *et al*., 1998), a glycosylphosphatidylinositol-linked CD2-related molecule expressed widely on haematopoietic cells but not investigated in the decidua (Korínek *et al*., 1991; Yokoyama *et al*., 1991). Although 2B4–CD48 interactions influence activation of leukocyte effector function, the outcomes are not clearly understood. In human NK cells, 2B4 can activate cytotoxicity and interferon (IFN)-γ production through SLAM-associated protein (SAP), an SH2 domain-containing adaptor molecule which results in severe immunodeficiency when defective (Tangye *et al*., 2000). In contrast, in the absence of SAP, 2B4 delivers inhibitory signals in murine and human NK cells (Parolini *et al*., 2000; Lee *et al*., 2004).

Trophoblast cells may express additional molecules, which are potential ligands for NK cell receptors. HLA-F is a non-classical HLA-I molecule with a restricted distribution of tissue expression (Lepin *et al*., 2000). Endoplasmic reticulum export trafficking is distinct from other HLA-I molecules but no specific functions are known (Boyle *et al*., 2006). Existing studies with the first widely available anti-HLA-F monoclonal antibody (mAb), 3D11, produced conflicting results regarding surface or intracellular HLA-F expression by trophoblast cells (Ishitani *et al*., 2003; Nagamatsu *et al*., 2006; Shobu *et al*., 2006).

Therefore, the NKG2D and 2B4 receptors expressed by uNK cells could bind ligands on EVT cells and influence NK cell function. We have now investigated the expression of NKG2DLs, CD48 and HLA-F on normal trophoblast and also on maternal cells present in the placental bed.

Materials and Methods

Cell lines and primary tissue

B lymphoblastoid 721 221 cells and the same line transfected with HLA-F were obtained from Dr N. Holmes (Lury *et al*., 1990). U373 cells and the same line transfected with MICB were obtained from Dr H. Reyburn (Valés-Gómez *et al*., 2006). HLA-I negative African Green Monkey CV-1 cells, human choriocarcinoma cell lines JEG-3 and JAR, as well as 293T, Jurkat, HT1080, H9 and Hela cell lines were all purchased from the American Type Culture Collection (ATCC reference numbers CCL-70, HTB-36, HTB-144, CRL-11268, TIB-152, CCL-121, HTB-176 and CCL-2, respectively). Using the primers shown (forward, reverse) extracellular regions of the ULBP1 (5′-TATAAGCTTGGCTGGTCCCGGGCA-3′, 5′-CTCGAA TTCTCATCTGCCAGCTAG-3′), ULBP3 (5′-TATAAGCTTTTCG ACTGGTCCGGG-3′, 5′-GATGAATTCTCAGATGCCAGGGAG-3′) and MICA (5′-TATAAGCTTCCCCACAGTCTTCGT-3′, 5′-TATG AATTCCTGGACCCTCTGCAG-3′) genes were each cloned into a mammalian expression vector p3xFLAG-CMV-9 (Sigma–Aldrich). A similar ULBP2 fragment in this vector was obtained from Bacon *et al*. (2004). CV-1 cells were individually transfected with these NKG2DL constructs using Lipofectamine 2000 (Invitrogen). Sequencing of the newly constructed plasmids used for these transfectants matched the Genebank sequences of accession numbers AB052907 (ULBP1), AB052908 (ULBP3) and L14848 (MICA) between the regions defined by the primers above.

Decidual and placental tissue was obtained at term or from elective terminations of normal pregnancies between 6 and 12 weeks gestation. Ethical approval for the use of these tissues was obtained from the Cambridge Local Research Ethics Committee. Decidual leukocytes and trophoblast cells were isolated as previously described (Trundley *et al*., 2006). Briefly, decidual tissue was disaggregated with collagenase and mononuclear cells purified on Lymphoprep. Trophoblast was released from chorionic villi by trypsin digestion and macrophages were depleted by adherence to plastic. Freshly isolated cells are predominantly of a villous trophoblast (VT) phenotype. After culture overnight on fibronectin, 50–80% of the cells become HLA-G$^+$, characteristic of EVT cells.

mAbs and fusion proteins

mAbs to ULBP, MIC, HLA-F and CD48 molecules are detailed in Table I. A mAb to NKG2D (M585) was also obtained from Cosman *et al*. (2001) (Amgen Inc.). Well-established mAbs to HLA-DR (L243), HLA-I (W6/32), 2B4 (C1.7), cytokeratin (MNF116) and the Flag epitope (M2) were purchased from Becton Dickinson, Serotec, Beckman Coulter, Dako and Sigma–Aldrich, respectively. Isotype control IgG1, IgG2a, IgG2b, IgG3 and IgM mAbs were obtained from Oxford Biotechnology or BD Pharmingen. Binding of all these unlabelled mAbs was detected by phycoerythrin (PE)-conjugated secondary reagents to IgG (Sigma–Aldrich) or total mouse Ig (Dako). Conjugated mAbs used were CD3- fluorescein isothiocyanate (FITC) (SK7), CD14-FITC (MϕP9), HLA-DR-FITC (L243), CD56-Alexa 488 (B159), LILRB3-PE-Cy5 (ZM3.8), and control IgG1- or IgG2a-FITC (all BD Biosciences); HLA-G-FITC (clone G233 developed in our own laboratory (Loke *et al*., 1997) or clone MEM-G/9 from Abcam) and epidermal growth factor receptor (EGF-R)-FITC (EGFR1) from Insight Biotechnology. A sequenced NKG2D-IgG Fc fusion protein construct in the mammalian expression vector pcDNA3, obtained from Dr H. Reyburn (Valés-Gómez *et al*., 2003), was transiently transfected into 293T cells using Lipofectamine 2000 (Invitrogen). Fusion protein produced was quantified by Easy-Titer (Pierce) and in reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis resolved as a single band at ∼70 kDa. Binding of this fusion protein was detected by a PE-conjugated anti-human IgG secondary antibody (Sigma–Aldrich).

RT-PCR

Fragments of decidual or placental tissue were stored at −80°C before total RNA isolation (Qiagen RNAeasy), DNA digestion (Qiagen DNase) and complementary DNA (cDNA) synthesis (Invitrogen Superscript II) following the manufacturers instructions. For each RT-PCR 50 ng of cDNA, 0.5 μM primers (Sigma–Genosys), 200 μM dNTP (Roche), between 2 and 3 mM magnesium chloride and 0.6 U of Amplitaq Gold DNA polymerase with its potassium chloride buffer (Applied Biosystems) were incubated on a Peltier Thermal Cycler. After an initial incubation at 95°C for 8 min, cycles of 95°C for 25 s, 50–70°C for 45 s and 72°C for 30 s were repeated

Table I. Monoclonal antibodies used to investigate expression of potential natural-killer cell ligands.

Antigen	Clone name	Experimental conditions		Source	Reference for production and characterization
		Flow cytometry	Immunohistochemistry		
ULBP1	M295	12 μg/ml	5 μg/ml	Dr D. Cosman (Amgen)	Cosman *et al*. (2001)
ULBP1	AUMO1	hybridoma supernatant diluted 1:3	hybridoma supernatant diluted 1:4	Dr A. Steinle	Welte *et al*. (2003)
ULBP2	M311	12 μg/ml	5 μg/ml	Dr D. Cosman (Amgen)	Cosman *et al*. (2001)
ULBP2	BUMO1	hybridoma supernatant diluted 1:3	hybridoma supernatant diluted 1:4	Dr A. Steinle	Welte *et al*. (2003)
ULBP3	M550	16 μg/ml	5 μg/ml	Dr D. Cosman (Amgen)	Cosman *et al*. (2001)
ULBP3	166510	8 μg/ml	5 μg/ml	R&D Systems	R&D datasheet
MICA	M673	16 μg/ml	5 μg/ml	Dr D. Cosman (Amgen)	Cosman *et al*. (2001)
MICA	AMO1	14 μg/ml	5 μg/ml	Immatics Biotechnologies	Welte *et al*. (2003)
MICB	BMO1	hybridoma supernatant diluted 1:3	hybridoma supernatant diluted 1:4	Dr A. Steinle	Welte *et al*. (2003)
MICB	BMO2	hybridoma supernatant diluted 1:3	hybridoma supernatant diluted 1:4	Dr A. Steinle	Welte *et al*. (2003)
HLA-F	FG-1	15 μg/ml	5 μg/ml	Dr E. Lepin	Lepin *et al*. (2000)
CD48	Tü145	14 μg/ml	na	BD Pharmingen	Hadam (1989)
CD48	6.28	10 μg/ml	5 μg/ml	Developmental Studies Hybridoma Bank	Yokoyama *et al*. (1991)

ULBP, UL-16 binding protein; MIC, MHC class-I chain related.

for between 30 and 35 cycles. PCR products were resolved by agarose electrophoresis (1% agarose in TBE (10 mM Tris (pH 7.4) and 1 mM EDTA (pH 8) in dH_20), with 0.5 μg/ml ethidium bromide (Sigma–Aldrich)) and a 1 kb reference ladder (Invitrogen). Gels were visualized using a FluorChem 9900 UV imaging system.

RT-PCR conditions were optimized to distinguish between NKG2DLs and HLA-I molecules of up to 97% nucleotide sequence identity. The primers and reaction conditions are detailed in Table II, those for MIC amplification were based on Welte *et al*. (2003). Primer pairs span intronic sequences to avoid amplification of genomic DNA. To confirm specific detection of the intended gene, several PCR products from selected amplification reactions were cloned into pCR4-TOPO vectors and DH5α E. Coli transformed using the TOPO cloning system (Invitrogen). Plasmids were extracted (Qiagen Miniprep) and sequencing using T7 (5′-TAATACGACTCACTATAGGG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) primers. At least four PCR products from two or more tissues were sequenced for each of the NKG2DLs and HLA-I genes investigated. All of the 50 PCR products sequenced were the intended target.

Flow cytometry

Flow cytometry conditions to detect NKG2DLs for which transcripts had been found were established using U373 cells stably expressing MICB, or ULBP1-3 and MICA expressed in HLA-I negative CV-1 cells (Table I). Staining of the flag epitope is included to indicate transfection efficiencies. For staining of primary cells, first Fcγ receptors were blocked by incubating with human IgG (Sigma–Aldrich). Unlabelled antibodies or fusion protein was added and detected with fluorochrome-conjugated secondary reagents. In blocking experiments, fusion protein was incubated with anti-NKG2D (M585) or isotype control antibody prior to staining. Free secondary antibody binding sites were blocked with the appropriate species Ig, before staining with directly conjugated mAb to identify each leukocyte or trophoblast cell population. VTs are HLA-I negative but are identified as the only cells in our trophoblast preparations to express EGF-R (Jokhi *et al*., 1994). EVT cells are the only cell type confirmed to express the non-classical HLA-G molecule, which is recognized specifically by our mAb G233 (Loke *et al*., 1997). Cells were analysed using a FACscan flow cytometer and CellQuest software (Becton Dickinson).

Immunohistochemistry

Acetone-fixed, frozen sections of human tissue or smears of cultured cells were blocked by incubating with serum of the species in which the secondary antibody was raised. Primary antibodies described above, were detected with biotinylated secondaries and streptavidin–horse–radish peroxidase (both Vector Laboratories) and developed with diaminobenzidine substrate (Sigma–Aldrich) as previously described (Jokhi *et al*., 1993). Sections were counterstained in Carazzi's haematoxylin and mounted with glycerol/gelatin. For double staining,

Table II. Primers and PCR conditions for gene expression analysis.

Target transcript	Forward primer	Reverse primer	Amplicon size (bp)	Annealing temperature (°C)	[$MgCl_2$] (mM)	Number of cycles	Positive control cell line
ULBP1	5′-GATGGGTCGACACACACTG-3′	5′-AGAGGGTGGTTTTGTTGGA-3′	560	64	2.5	30	Jurkat
ULBP2	5′-GCTACCAAGATCCTTCTGT-3′	5′-GTCAAAGAGGAGGAAGAACTGC-3′	441	62	3.0	30	Jurkat
ULBP3	5′-GCGATTCTTCCGTACCTGCTA-3′	5′-TGGTGGCTATGGCTTTGGGTT-3′	640	67	2.0	30	HT1080
ULBP4	5′-TATCCCTGACTTCTAGCCCT-3′	5′-GCCACTCACCATTTTGCCAC-3′	749	60	2.5	33	HT1080
RAET1G	5′-AGCCCCGCGTTCCTTCTA-3′	5′-TGTATACAAGGCAAGAGGGC-3′	935	54	3.0	30	HT1080
RAET1L	5′-CGCCATCCCAGCTTTG-3′	5′-TCAGATGCCAGGGAGGAT-3′	730	58	2.0	35	Hela
MICA	5′-CCTTGGCCATGAACGTCAGG-3′	5′-CCTCTGAGGCCTCRCTGCG-3′	177	60	2.0	33	HT1080
MICB	5′-ACCTTGGCTATGAACGTCACA-3′	5′-CCCTCTGAGACCTCGCTGCA-3′	179	60	3.0	33	Jurkat
HLA-G	5′-GACTCGGCGTGTCCGAGGAT-3′	5′-GACCGCAGCTCCAGTGACT-3′	795	56	2.0	30	JEG-3
HLA-F	5′-GTGGCCCTGAGGAACCTG-3′	5′-TGAGAGTAGCTCCCTCTGTTTCT-3′	740	60	2.5	33	H9
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′	5′-TCCACCACCCTGTTGCTGTA-3′	~450	56	2.5	30	any

R in a primer sequence indicates a mixture of A or G nucleotides in a ratio of approximately 1:1. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. RAET1, retinoic acid early transcript 1.

sections were additionally labelled with mAb to CD56 developed using FastRed substrate (Sigma–Aldrich).

Results

The receptors NKG2D and 2B4 are expressed by decidual leukocytes

Flow cytometry of decidual leukocytes isolated from normal first trimester pregnancies confirmed expression of both NKG2D and 2B4 on all $CD56^+$ and around 50% of $CD3^+$ cells (Fig. 1). This is consistent with previous reports for decidual leukocytes (Hanna *et al.*, 2006; Vacca *et al.*, 2006; Zhang *et al.*, 2007) and the pattern of expression for these receptors in peripheral blood (Valiante and Trinchieri, 1993; Raulet, 2003). Interestingly, NKG2D labelled with a higher intensity on uNK than peripheral blood NK cells from the same individual (data not shown (DNS)) consistent with reported mRNA level expression studies (Koopman *et al.*, 2003).

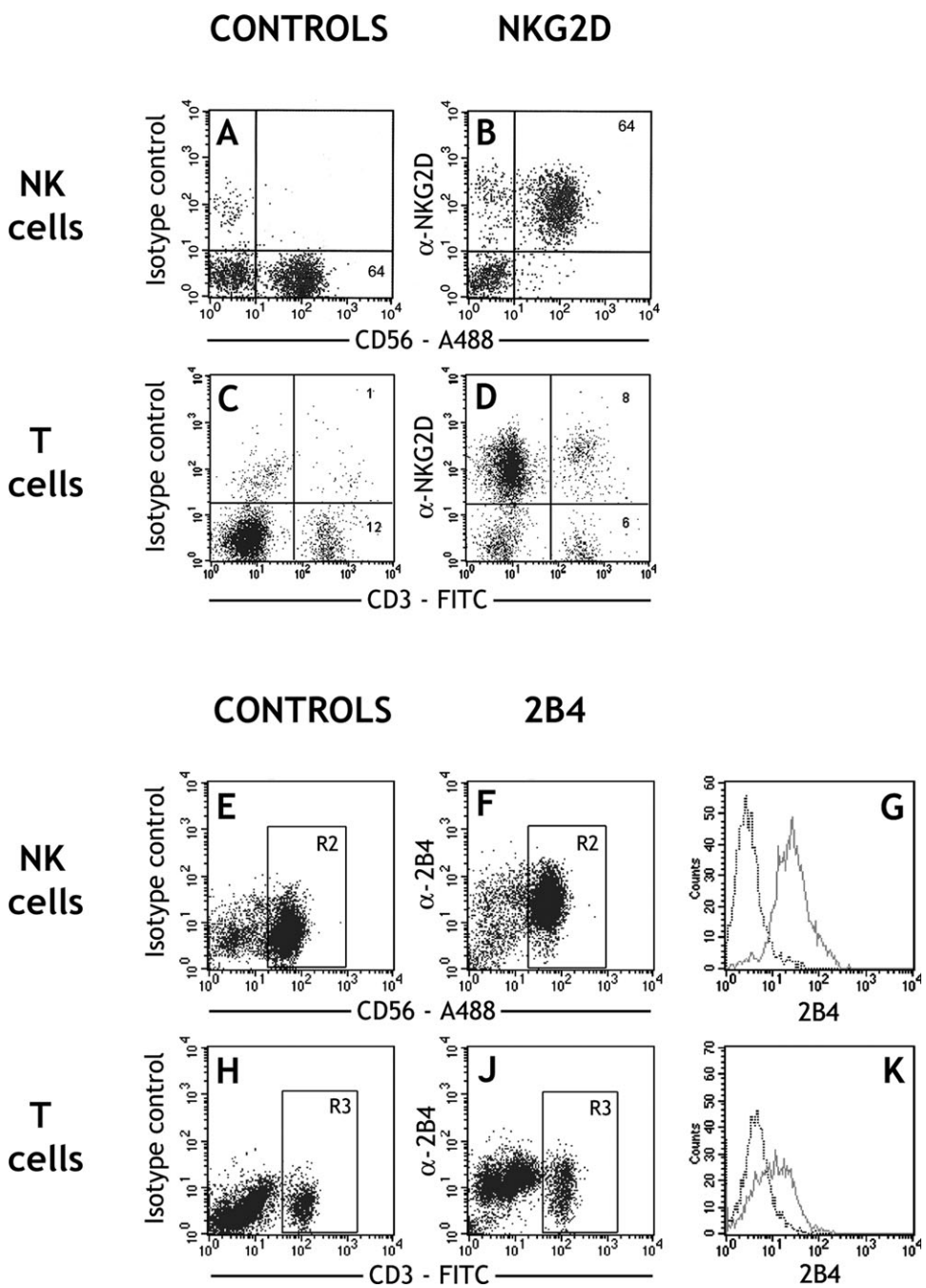

Figure 1: Expression of NKG2D and 2B4 receptors by decidual leukocytes.
Freshly isolated first-trimester decidual leukocytes from normal human pregnancies were stained for NKG2D (**A**–**D**) or 2B4 (CD244) (**E**–**K**), with $CD56^+$ or $CD3^+$ cells identified by directly conjugated monoclonal antibody (mAb). For each dot plot, leukocytes were gated by scatter properties (not shown). For traces G and K, the additional gates shown R2 ($CD56^+$ cells) or R3 ($CD3^+$ cells) were used to select NK and T lymphocytes, respectively. Broken traces represent isotype control and continuous traces the anti-2B4 mAb staining. These results are representative of decidual leukocytes isolated from at least four individuals. FITC, fluorescein isothiocyanate; A488, alexa fluor 488.

NKG2DL mRNA expression at the maternal–fetal interface

NKG2DL mRNA expression was investigated using RT-PCR capable of discriminating between the closely related MIC and ULBP family members. Samples of decidua and placental tissue from normal pregnancies in their first trimester and at term were screened, as well as freshly isolated trophoblast (which is predominantly villous) and isolated trophoblast cells cultured overnight (which differentiate to an HLA-G^+ extravillous phenotype (Trundley *et al*., 2006)) (Fig. 2). Detection of glyceraldehyde 3-phosphate dehydrogenase and HLA-G transcripts verifies cDNA synthesis and confirms the presence of EVT cells, respectively. ULBP1 was detected in each of the three cultured EVT cell preparations and first trimester placental samples as well as two of the three term placental samples. In contrast, ULBP1 was not detected in freshly isolated VT, decidua or peripheral blood mononuclear cells (PBMC). ULBP2, MICA and MICB transcripts were widespread in decidual, placental and trophoblast preparations. ULBP3 was detected in all placental samples, but less frequently in trophoblast. ULBP4 and retinoic acid early transcript (RAET) 1G expression was detected mainly in decidual tissue. RAET1L was not detected in any primary tissue. Only low levels of ULBP3 and MIC transcripts were detected in peripheral blood. This rules out the possible detection of expression in decidual samples as a result of contamination by peripheral blood. ULBP1-3, MICA and MICB transcripts were also detected in the JEG-3 choriocarcinoma cell line (DNS). In summary, significant mRNA expression of ULBP1-3, MICA and MICB molecules was detected in trophoblast and decidual cells.

Surface protein expression of NKG2DL investigated by flow cytometry

We next investigated protein expression of ULBP1-3, MICA and MICB on cells from the implantation site using flow cytometry. Individual transfectants were produced to establish the flow cytometry conditions for detection of each of these NKG2DLs (Fig. 3A–C). Using these flow cytometry conditions, the choriocarcinoma cell lines JEG-3 and JAR were initially tested. They only expressed ULBP1 surface protein (Fig. 3D and E). Next, freshly isolated first trimester trophoblast cells were analysed. Cells of villous and extravillous phenotype in our primary cell preparations were positively identified by labelling for EGF-R and HLA-G, respectively (Jokhi *et al*., 1994; Loke *et al*., 1997). Neither of these trophoblast sub-populations bound mAbs to ULBP1-3, MICA or MICB (Fig. 3F and G). As a positive control for HLA-I-like molecule staining, labelling was performed in parallel with the mAb W6/32 (Parham *et al*., 1979). Shown at the bottom of Fig. 3G, HLA-I negative VT remain in the lower right quadrant whereas EVT and stromal cells/leukocytes contaminating our primary cell preparations stain with W6/32 in the upper right and left quadrants, respectively.

Our isolation of primary trophoblast cells includes a trypsin digestion to dissociate cells from chorionic villi (Trundley *et al*., 2006). Exposure of transfected cell lines to similar

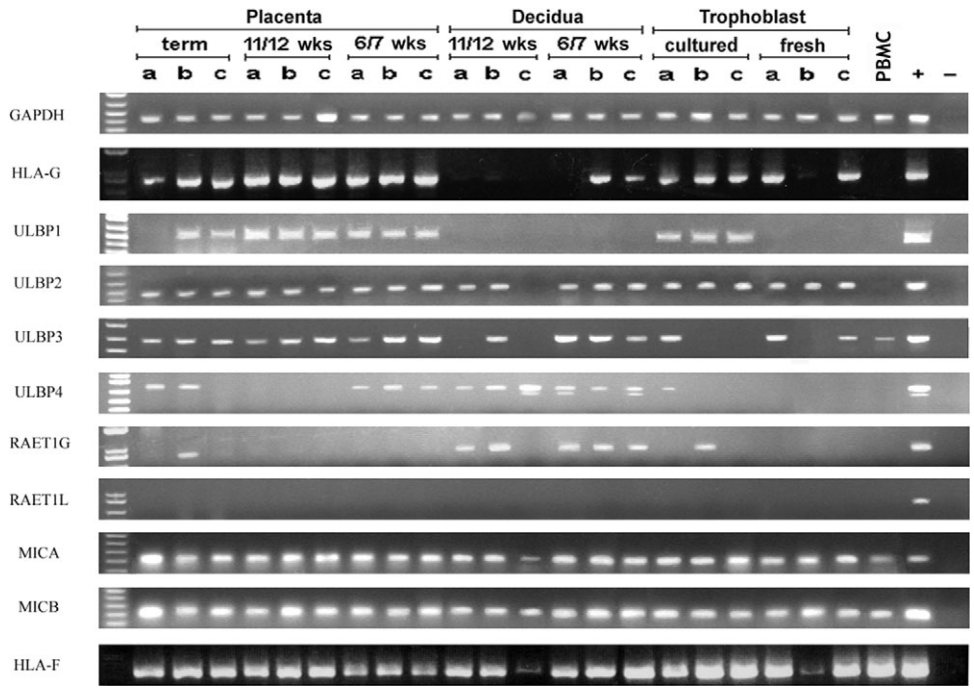

Figure 2: mRNA expression of NKG2D ligands and non-classical HLA-I genes at the human implantation site.
Triplicate samples of placental and decidual tissues were obtained at term, 11–12 and 6–7 weeks gestation from normal human pregnancies. Trophoblast cells were also isolated from early placental samples (see Materials and Methods). cDNA from these tissues was screened by RT-PCR for each NKG2DL, HLA-G and HLA-F. PCR products from each reaction were cloned, sequenced and in every case the intended transcript had been amplified. ULBP, UL-16 binding protein; MIC, MHC class-I chain related; GAPDH, glyceraldehyde 3- phosphate dehydrogenase; RAET1, retinoic acid early transcript 1; PBMC, peripheral blood mononuclear cells.

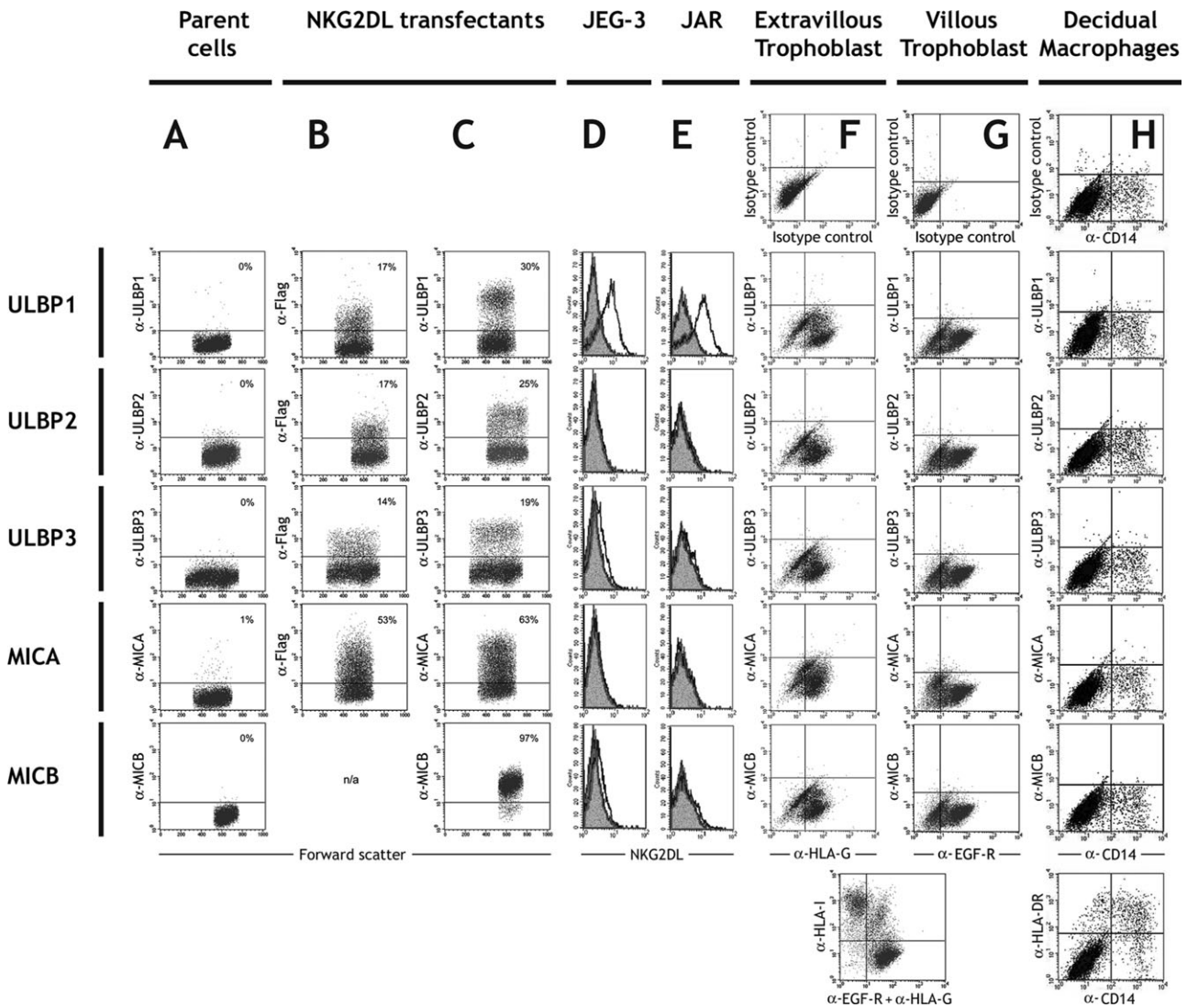

Figure 3: NKG2DL expression investigated by flow cytometry.
(**A**–**C**) A panel of transfected cells was stained with isotype control mAb (A), anti-flag mAb to indicate expression of tagged NKG2DL constructs in CV-1 cells (**B**) and anti-NKG2DL mAb (C). The proportion of cells staining (positive) is indicated by the percentage shown in each plot. (**D** and **E**) NKG2DL expression was investigated on the choriocarcinoma cell lines JEG-3 (D) and JAR (E). Binding of mAbs detecting each NKG2DL (open traces) and an isotype control (filled traces) is shown. (**F** and **G**) NKG2DL expression by fetal trophoblast cells isolated from normal, first trimester pregnancies. EVT cells are identified by labelling HLA-G (F), and VT cells by staining EGF-R (G). No NKG2DL mAbs bound either cell type, compared with an isotype control (top). Binding of the mAb W6/32 recognizing all HLA-I molecules is shown as a positive control (bottom). (**H**) NKG2DL expression by maternal decidual macrophages isolated from normal, first trimester pregnancies. Myelomonocytic cells are identified by CD14 labelling, but no NKG2DL mAbs bind compared with an isotype control (top). Binding of HLA-DR (bottom) represents a positive control. Primary cells from at least 10 individuals were independently analysed, with no NKG2DL expression detected throughout. Multiple mAbs for each NKG2DL are listed in Table I, the same binding patterns were observed with both mAbs to each NKG2DL throughout our study.

trypsin or collagenase incubations substantially decreased labelling of MIC and ULBP. This effect is not observed with HLA-I molecules (DNS). We therefore analysed trophoblast cells cultured overnight to check the possibility that surface NKG2DLs might be reconstituted and then detected. In these experiments, trophoblast cells were dissociated after culture by a brief incubation with EDTA. Neither VT nor EVT cells, identified by EGF-R and HLA-G labelling as before, bound mAb to ULBP1-3, MICA or MICB (DNS). In other tissues, surface expression of ULBP and MIC molecules is induced or up-regulated by stress stimuli such as infection or DNA damage (Groh *et al*., 2001; Gasser *et al*., 2005). We used the stress stimuli of irradiation or toll-like receptor (TLR) stimulation, but no induction of NKG2DL expression was observed (DNS).

Another possibility is that NKG2D on uNK cells interacts with its ligands on maternal decidual cells. For example, antigen presenting cells have been reported to express NKG2DLs when infected with influenza (Draghi *et al*., 2007). Anti-NKG2DL mAbs showed no labelling of decidual

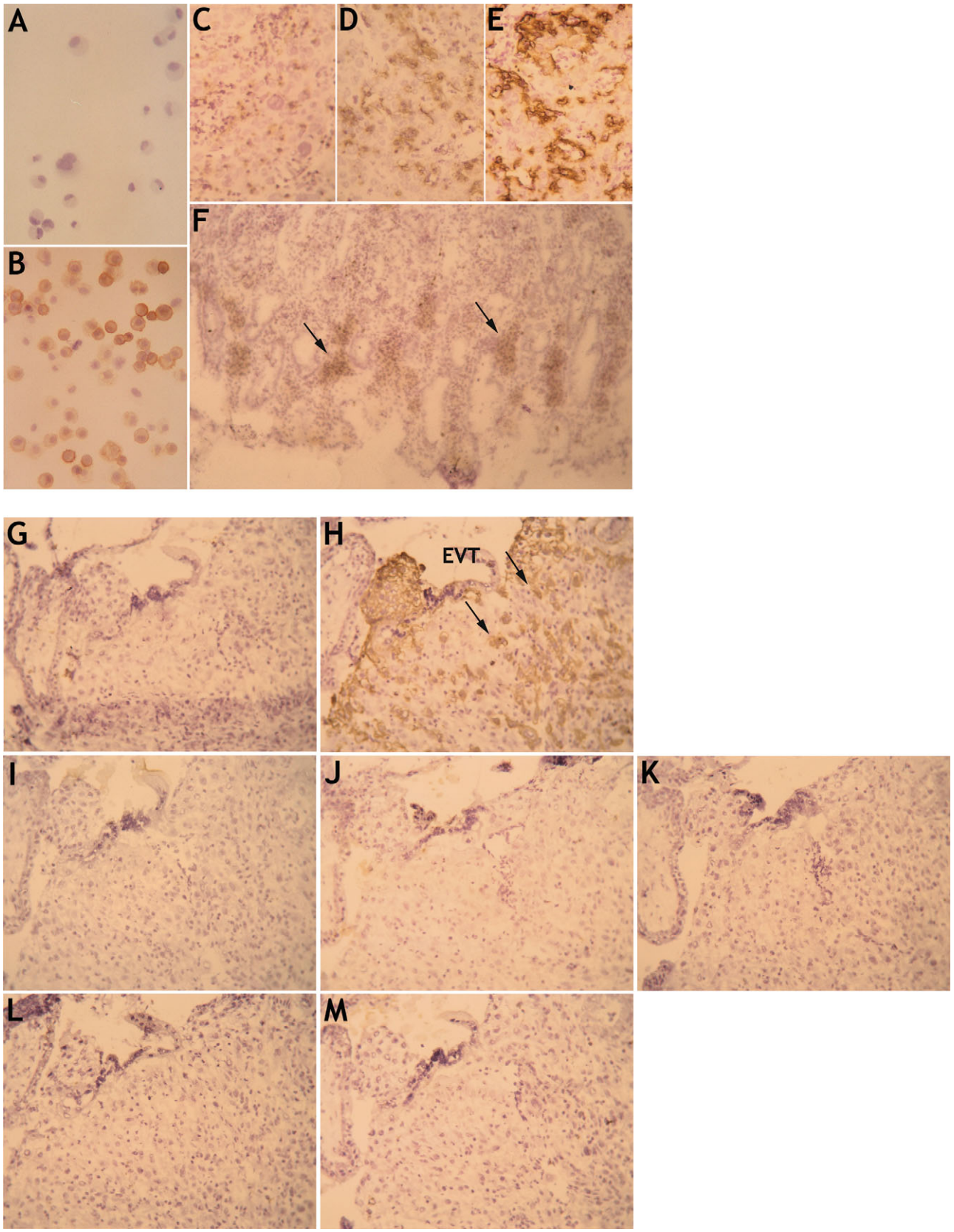

Figure 4: NKG2DL expression investigated by immunohistochemistry.
(**A** and **B**) As positive controls, acetone fixed and frozen smears of cells individually transfected with ULBP1-3, and MICA and MICB were stained. Representative labelling with anti-MICB mAb to parent (A) and MICB-transfected U373 (B) cells is shown. (**C–F**) A section of breast carcinoma is shown stained with isotype control mAb (C), anti-MICA mAb (**D**) and for cytokeratin which is strongly positive (**E**). A section of gastric mucosa also stains with mAb to MICA (F). (**G–M**) Serial sections of a normal human implantation site at 8 weeks gestation labelled with isotype control mAb (G), anti-HLA-G mAb G233 to identify EVT cells (**H**), and mAb to ULBP1 (**I**), ULBP2 (**J**), ULBP3 (**K**), MICA (**L**) and MICB (**M**). No binding to any cell type was detected in sections from three independent donors. A and B are at $\times 400$ magnification, C–M are $\times 200$.

$CD14^+$ $HLA\text{-}DR^+$ myelomonocytic cells, or any other of the cells identified by a cocktail of leukocyte lineage markers either freshly isolated with collagenase or after overnight culture (Fig. 3H and DNS). Stress stimuli were also investigated as above but no inducible NKG2DL expression was detected (DNS).

NKG2DL protein expression investigated by immunohistochemistry

To avoid the difficulties of enzymic digestion stripping NKG2DLs from the cell surface and to detect any intracellular proteins present, we performed immunohistochemistry staining of acetone-fixed implantation site sections. The mAbs used were able to detect their antigens in cytospin smears of transfectants and on tissue sections of gastric mucosa and breast carcinoma (Fig. 4A–F) (Eagle and Trowsdale, 2007). Serial sections of the implantation site (identified by $HLA\text{-}G^+$ EVT) from a normal human pregnancy at 8 weeks gestation were then screened for NKG2DL expression (Fig. 4G–N). No labelling with mAbs to the ULBP1-3, MICA or MICB molecules was observed by trophoblast or any other cells at the implantation site.

Investigation of novel NKG2DLs with an NKG2D-Fc fusion protein

As NKG2D is a promiscuous receptor with polygenic and polymorphic ligands, it is possible that novel NKG2DLs are present at the maternal–fetal interface. An NKG2D-Fc fusion protein was used that specifically bound to MICB on transfected cells (Fig. 5A) (Sutherland *et al.*, 2002). No binding of NKG2D-Fc was observed to freshly isolated or overnight cultured $HLA\text{-}G^+$ or $EGF\text{-}R^+$ trophoblast cells (Fig. 5B–H and DNS).

HLA-F expression at the maternal–fetal interface

There are conflicting reports about the expression of HLA-F on trophoblast cells (Ishitani *et al.*, 2003; Nagamatsu *et al.*, 2006; Shobu *et al.*, 2006). Using RT-PCR, we detected HLA-F transcripts in all decidua, placenta and trophoblast samples (Fig. 2). We next used a new well-characterized mAb FG-1 to screen for protein expression (Lepin *et al.*, 2000). Using flow cytometry and immunohistochemistry, the mAb FG-1 was confirmed to detect HLA-F on transfected cells (Fig. 6A and DNS). No HLA-F expression was detected on freshly isolated trophoblast cells or decidual leukocytes by flow cytometry (Fig. 6B–J). By immunohistochemistry only very weak staining of FG-1 was seen (Fig. 6K and L). The cells staining were not EVT, but possibly uNK cells identified by labelling serial sections for FG-1, HLA-G and CD56 (Fig. 6M–O). The weak staining with mAb FG-1 was restricted to those uNK cells near the site of trophoblast invasion.

CD48 is expressed by decidual cells at the maternal–fetal interface

As all uNK cells express 2B4 (Fig. 1E–K), we investigated expression of its ligand CD48 at the maternal–fetal interface using two different mAbs, clones Tü145 and 6.28. In flow cytometry, fetal $HLA\text{-}DR^+$ Hofbauer cells bound mAb Tü145 but not mAb 6.28, whereas $HLA\text{-}G^+$ extravillous or $EGF\text{-}R^+$ VT cells did not bind either of the anti-CD48 mAbs (Fig. 7A–F). In leukocytes isolated from the decidua, CD48 was strongly detected on all $CD3^+$ T cells by both mAbs and $CD56^+$ cells only by the mAb 6.28. $HLA\text{-}DR^+$ or $LILRB3^+$ myelomonocytic cells in the decidua did not bind either CD48 mAb relative to isotype controls (Fig. 7G–L and DNS). Histological staining of normal first trimester decidual sections with mAb 6.28 revealed striking aggregation of positive cells around modified spiral arteries at the implantation site, which showed some co-localization with $CD56^+$ cells in double labelling (Fig. 7M–O).

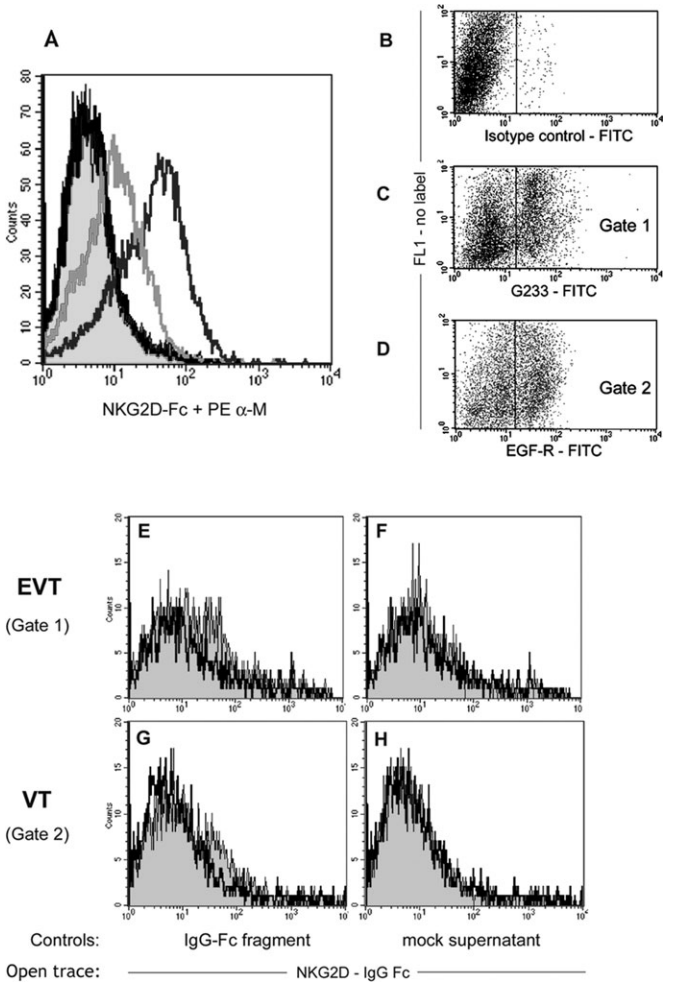

Figure 5: No novel NKG2DLs are detected on human trophoblast cells using an NKG2D-Fc fusion protein.
(**A**) An NKG2D-Fc fusion protein was produced by transient transfection of 293T cells and bound MICB-transfected U373 cells (black trace), compared with control supernatant from 293T cells transfected with an empty plasmid (filled trace) or first incubation with a mAb to NKG2D (grey trace). (**B–H**) NKG2D-Fc binding to freshly isolated first trimester trophoblast cells was tested. Antibodies directly conjugated to FITC were used compared with an isotype control (B), to identify EVT cells by HLA-G labelling (**C**) or VT by EGF-R labelling (**D**). These positive cells in C and D were gated for analysis of NKG2D-Fc fusion protein binding (**E–H**). There was no binding to either trophoblast population of the fusion protein (black trace) compared with controls (filled traces) of an immunoglobulin G-Fc fragment (E and G) or the mock supernatant (F and H) for three independent donors.

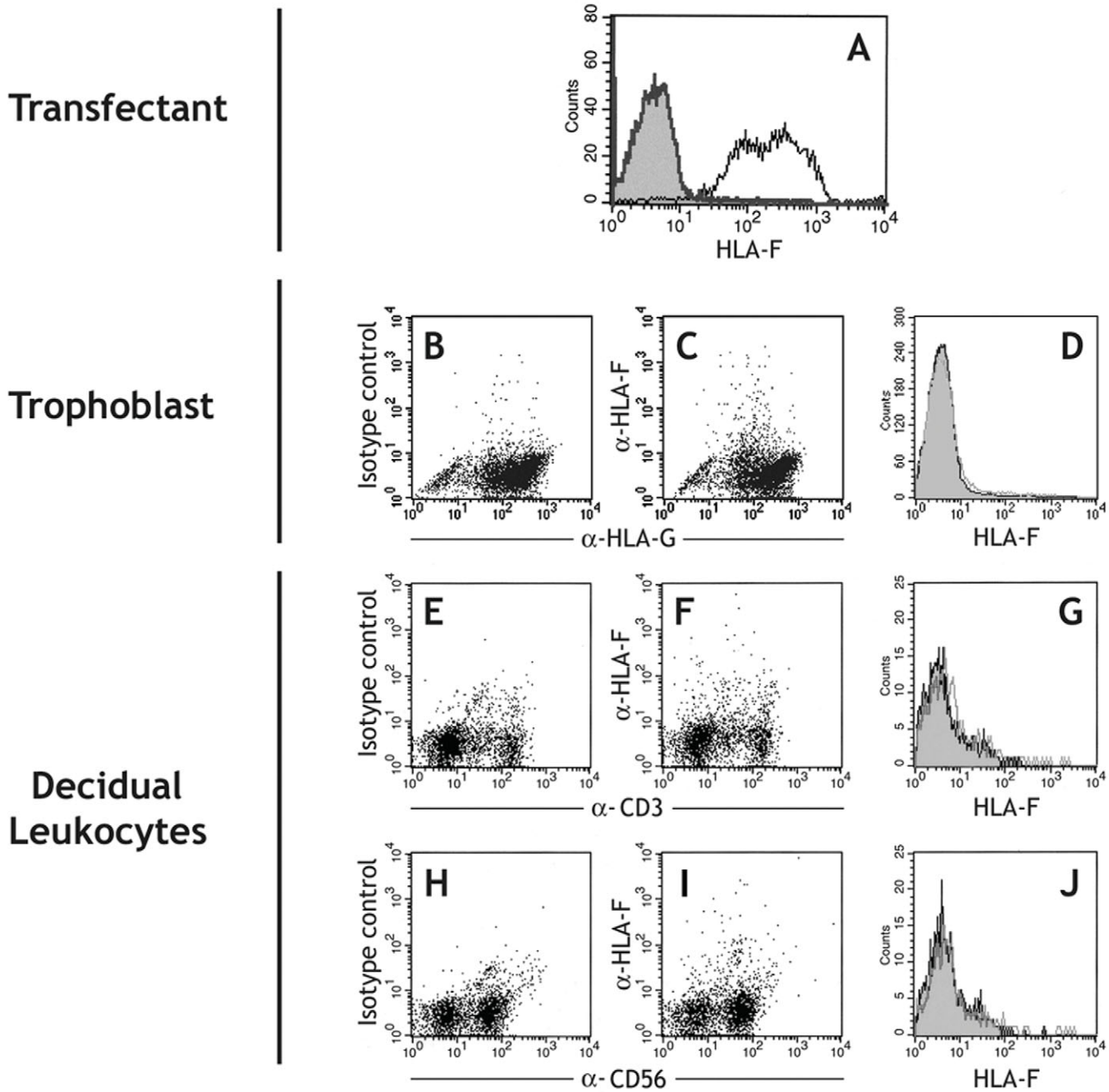

Figure 6: Analysis of HLA-F expression at the first trimester implantation site.
(**A**) mAb FG-1 binds HLA-F transfected (open trace) compared with parent .221 cells (filled trace). (**B**–**D**) This anti-HLA-F mAb shows no binding to trophoblast cells isolated from normal first trimester placenta, with EVT cells identified by labelling of HLA-G. (**E**–**J**) The anti-HLA-F mAb FG-1 shows no binding to decidual leukocytes isolated from normal pregnancies. T lymphocytes (E–G) and NK cells (H–J) are identified by labelling of CD3 and CD56, respectively. Primary cells from at least 10 individuals were independently analysed by flow cytometry. (**K**–**O**) Serial sections of a normal human implantation site at 8 weeks gestation were labelled with isotype control mAb (K), or anti-HLA-F mAb FG-1 (**L**) at $\times$400 magnification. The same section is also shown stained (arrow) with FG-1 (**M**), anti-HLA-G mAb G233 (**N**) and anti-CD56 (**O**) at $\times$200 magnification. The immunohistochemistry figures shown are representative of staining observed in sections from three independent donors.

Discussion

In this study, we analysed the expression of possible ligands for NK receptors on cells at the maternal–fetal interface in the first trimester of pregnancy. The NKG2D receptor was strongly expressed by all uNK cells and 50% of T cells, consistent with increased NKG2D transcription in decidual compared with peripheral NK cells (Koopman *et al*., 2003). Although we found transcripts for several NKG2DLs in tissues from the implantation site, no protein expression was demonstrated by flow cytometry or immunohistochemistry on cells from normal pregnancy. Post-transcriptional control of NKG2DL expression is not unusual. Indeed, MIC

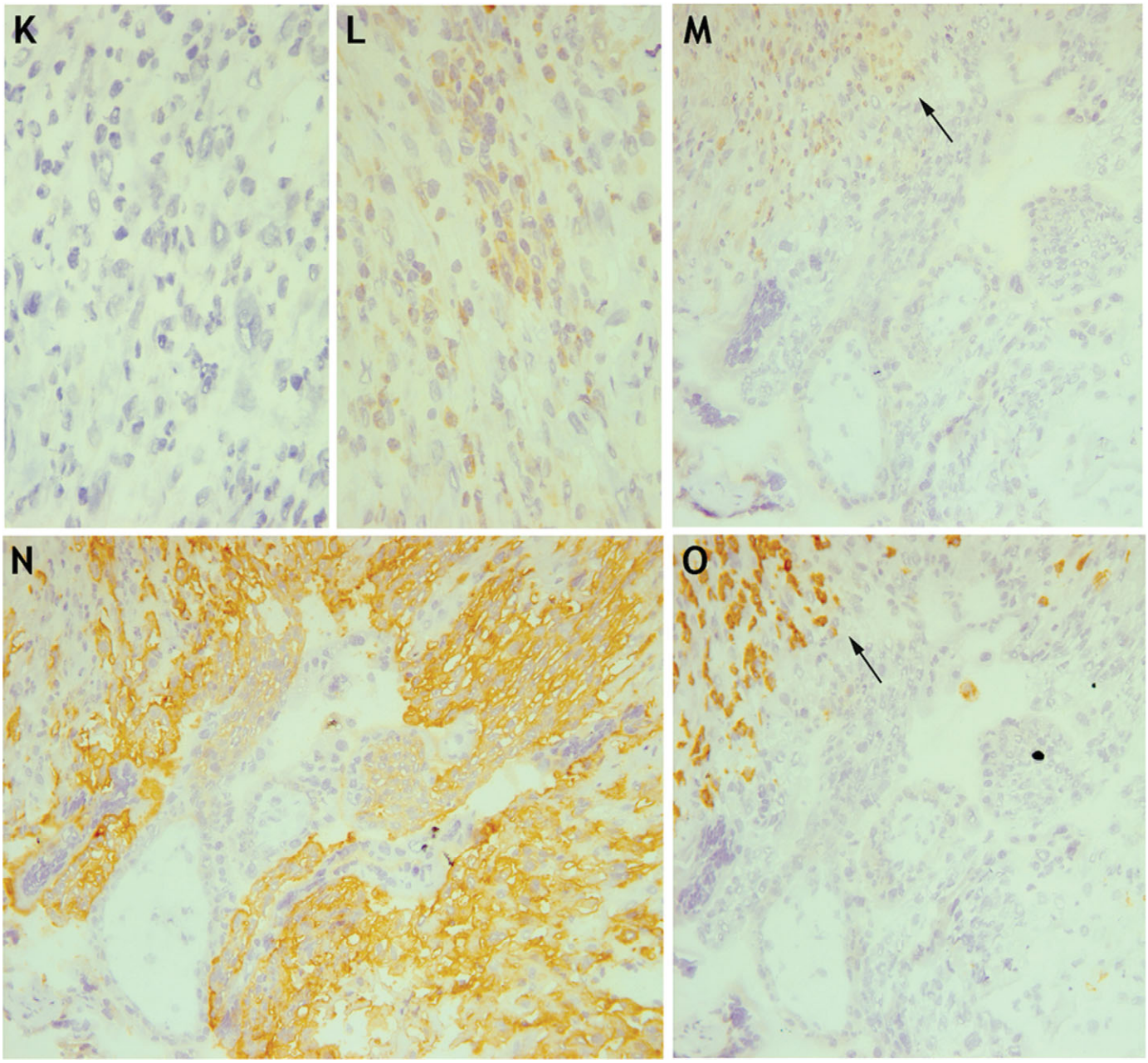

Figure 6: Continued

transcripts are widely expressed but only restricted protein expression is observed and induction of surface ULBP protein expression by bronchial epithelial cells has been shown to occur without protein synthesis (Borchers *et al*., 2006). NKG2DLs are known to be up-regulated by various stress stimuli but after treatment with irradiation or pathogen-associated molecular pattern molecules no expression was found on any cell type. In contrast, and in keeping with their origin from malignant tumours, the choriocarcinoma cell lines JEG-3 and JAR did express ULBP1 surface protein. We also explored whether other, novel NKG2DLs might be found on trophoblast by screening with an NKG2D-Fc fusion protein but found no binding.

NKG2DLs may also be shed from infected or tumour cells and can be detected in the blood of cancer patients (Salih *et al*., 2002, 2006). Constitutive or soluble NKG2DL bind to NKG2D and down-regulate its expression on NK and T cells, abrogating NK activation towards the tumour or infected cell (Doubrovina *et al*., 2003; Oppenheim *et al*., 2005; Wiemann *et al*., 2005). A report that trophoblast cells produce soluble MIC and in this way mediate maternal tolerance to the fetus suggested a similar mechanism might operate in pregnancy (Mincheva-Nilsson *et al*., 2006). In agreement with our study, MIC mRNA was detected in trophoblast. MIC protein expression was demonstrated using a different mAb to that used in our study, E-16, but the specificity of this mAb has

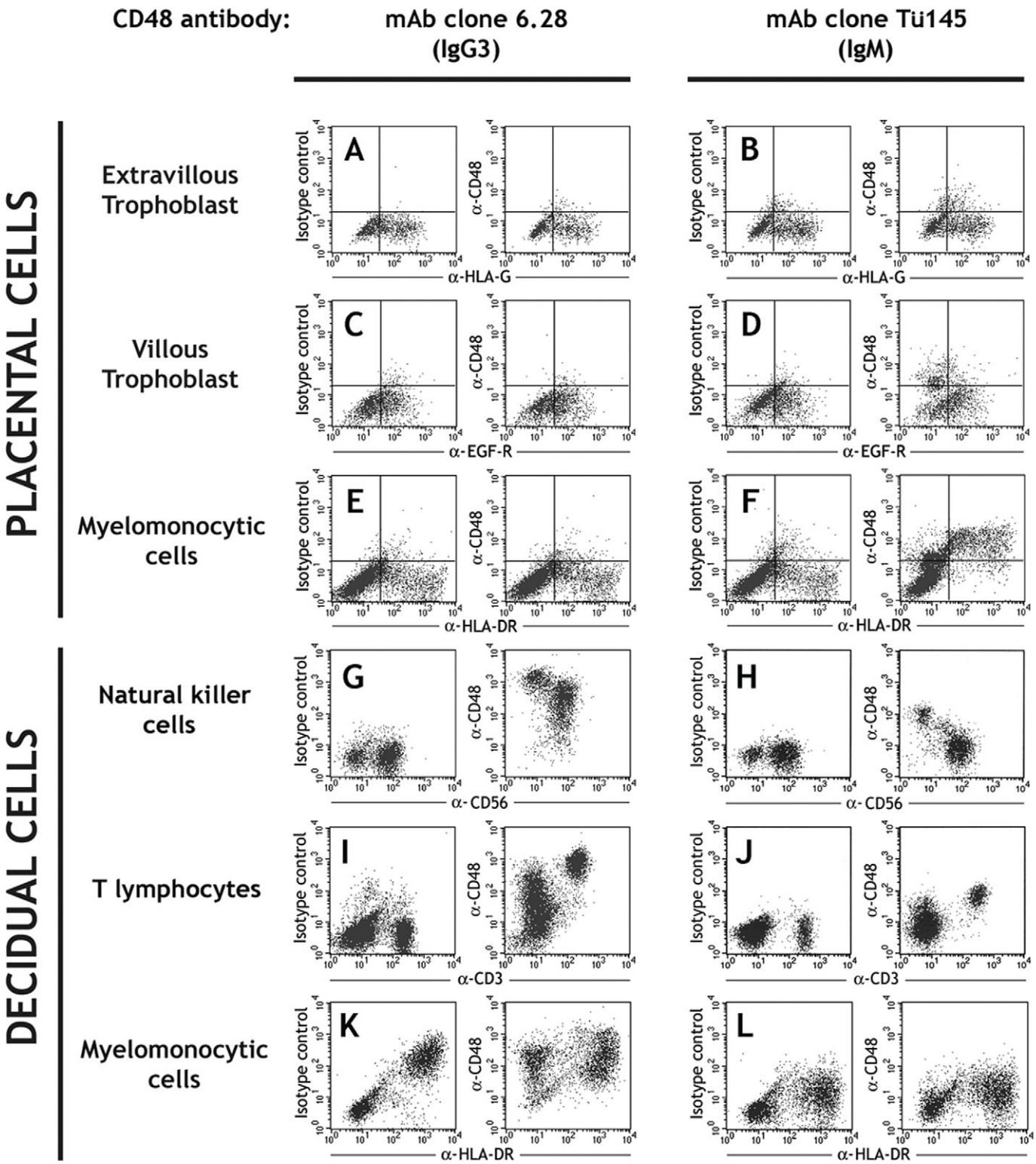

Figure 7: CD48 is expressed by decidual leukocytes but not fetal trophoblast cells.
(**A**–**F**) CD48 expression by fetal cells isolated from normal, first trimester pregnancies. EVT cells are identified by HLA-G labelling (A and B), VT cells by staining EGF-R (**C** and **D**) and myelomonocytic cells by HLA-DR expression (**E** and **F**). Binding of mAbs 6.28 and Tü145, both recognizing CD48, is shown relative to their respective controls. (**G**–**L**) CD48 expression by maternal decidual leukocytes isolated from normal, first trimester pregnancies. NK cells are identified by CD56 expression (G and **H**), T lymphocytes by CD3 staining (**I** and **J**) and myelomonocytic cells by HLA-DR labelling (K and L). Binding of both mAbs 6.28 and Tü145 recognizing CD48 is shown. Primary cells from at least 10 individuals were independently analysed by flow cytometry with each mAb. (**M**–**O**) Immunohistochemical staining of a normal human implantation site at 8 weeks gestation. Compared with an isotype control (M), anti-CD48 mAb 6.28 strongly stains decidual cells aggregated around transformed spiral arteries of the implantation site (**N**) (×200 magnification). A double labelled decidual section at ×400 magnification shows some co-localization (arrows) of mAb 6.28 staining (brown), with $CD56^{+}$ cells (red). The immunohistochemistry figures shown are representative of staining observed in sections from three independent donors.

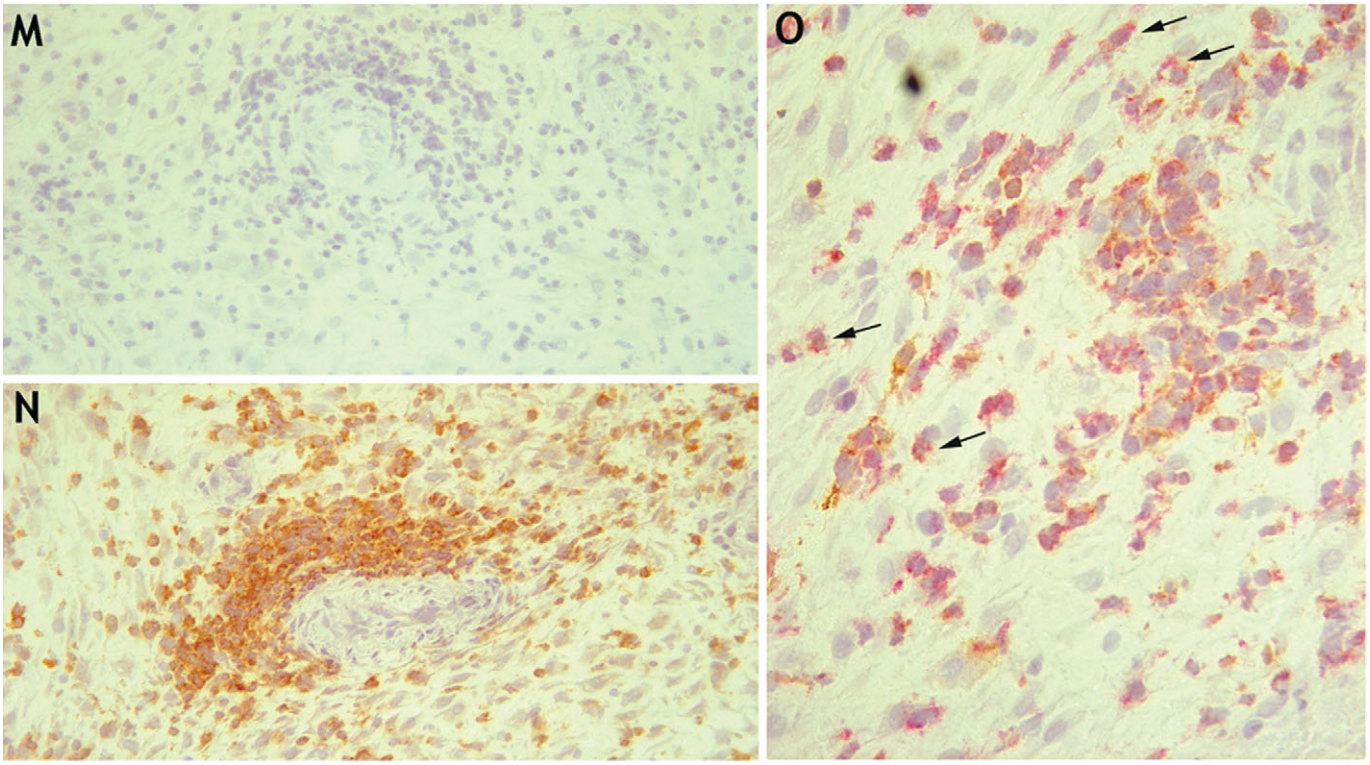

Figure 7: Continued

not been rigorously defined. Furthermore, functional experiments including blocking mAb to soluble MIC were performed without isotype controls. It would seem highly disadvantageous for a pregnant woman if the placenta was a source of soluble NKG2DL with resulting impairment of her systemic immune responses to tumours and infections. We also explored the possibility that NKG2DLs could be expressed by maternal cells in the decidua because following signalling via TLR, NKG2DLs can be up-regulated on antigen-presenting cells (Draghi *et al*., 2007). Macrophages and dendritic cells are abundant at the implantation site but were not found to express any NKG2DLs.

We can conclude that in normal pregnancies NKG2DL proteins are not found on any maternal or fetal cell at the implantation site. The situation might change in pathological pregnancies. Although we tested induction with classical stress stimuli such as TLR stimulation and irradiation, these are unlikely to occur very often in the uterus during pregnancy. Transplacental infections generally do not involve the decidua as the pathogens (e.g. human immunodeficiency virus) cross to the fetus via VT from maternal blood in the intervillous space. NKG2DLs could be up-regulated in response to stresses of pregnancy such as hypoxia or aneuploidy (Weier *et al*., 2005). This could be a mechanism for maternal NK cells to respond to abnormal concepti.

HLA-F is a non-classical HLA-I molecule with a restricted distribution of expression that is mainly not at the cell surface (Wainwright *et al*., 2000). The divergent trafficking of HLA-F suggests any functions of this molecule may be independent of peptide presentation to T cells (Boyle *et al*., 2006). HLA-F tetramers have been shown to bind LILRB1 and 2 which are both expressed by decidual leukocytes (Lepin *et al*., 2000). Previous studies using trophoblast cells are conflicting and have shown either intracellular or surface expression using the mAb 3D11 (Ishitani *et al*., 2003; Nagamatsu *et al*., 2006; Shobu *et al*., 2006). We used a newly available mAb, FG-1, which is highly specific and of high affinity (Lepin *et al*., 2000). With this reagent weak staining was only found for maternal lymphoid cells, probably $CD56^+$ NK cells. We could demonstrate no surface expression of HLA-F by any cells at the implantation site by flow cytometry and all trophoblast populations were also negative by immunohistochemistry. Thus, at present, there is still only convincing evidence that three HLA-I molecules are displayed by human EVT cells: HLA-C, HLA-E and HLA-G (King *et al*., 2000; Apps *et al*., 2007, 2008).

The last NK receptor investigated was 2B4 which was detected on all NK cells and a subset of T cells, confirming previous reports using decidual and blood leukocytes (Vacca *et al*., 2006; Ma *et al*., 2007). CD48, the ligand for both 2B4 and CD2 receptors, was not found to be expressed by trophoblast cells. We used two mAbs to CD48 and found different patterns of binding for these reagents, a finding also observed with PBMC (Korínek *et al*., 1991; Yokoyama *et al*., 1991; Lo *et al*., 1998; Morandi *et al*., 2005; Hernández-Campo *et al*., 2006). In particular, all T cells bound both mAbs to CD48, but $CD56^+$ NK cells bound only the mAb 6.28. In sections, the cells binding anti-CD48 mAb were aggregated around the spiral arteries that are the focus of trophoblast invasion. These results suggest that CD48–2B4 interactions

may occur in the vicinity of spiral arteries at the implantation site, not with trophoblast cells but with other leukocytes. Interestingly, 2B4 ligation can inhibit cytoxicity and IFN-γ production by interleukin-2 stimulated uNK cells (Vacca *et al*., 2006).

Overall, we cannot find evidence for protein expression of ligands for the NK cell receptors NKG2D and 2B4 on normal first trimester trophoblast cells. Both of these receptors are highly expressed by uNK cells and may function in pathological pregnancies. For example, where concepti are affected by karyotypic or other abnormalities, NKG2DLs might be up-regulated and allow the mother to perceive and reject implantation. In cases of intrauterine infection, NKG2DLs may be induced on other maternal cells in the uterus such as myelomonocytic, glandular or stromal cells and modulate uNK cell function.

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