Published in final edited form as:

Placenta. 2008 January ; 29(1): 58–70. doi:10.1016/j.placenta.2007.10.001.

Nonclassical MHC-E (Mamu-E) expression in the rhesus monkey placenta

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Abstract

The aim of this study was to characterize the expression of the rhesus HLA-E ortholog Mamu-E, particularly at the maternal-fetal interface. Mamu-E expression was confirmed by locus-specific RT-PCR in the placenta as well as in peripheral blood mononuclear cells (PBMC) and other organs. We evaluated the utility of antibodies recognizing HLA-E (MEM-E/06 against native HLA-E, MEM-E/02 against denatured HLA-E) to detect Mamu-E by flow cytometry/immunofluorescence, Western blot, and immunohistochemistry (IHC). Western blot analysis of cells and selected transfectants confirmed the recognition of Mamu-E but not Mamu-AG by antibodies MEM-E/06 and HC10 but not MEM-E/02. Immunohistochemical staining of frozen sections of rhesus placenta with the MEM-E/06 antibody demonstrated expression in most populations of rhesus monkey trophoblast cells, including villous cytotrophoblasts (strong positive staining), apical membrane of syncytiotrophoblasts (light to moderate staining) and extravillous cytotrophoblasts (moderate to strong staining, especially endovascular trophoblasts in early pregnancy). Expression was not trophoblast cell-specific, especially at term, when endothelial cells in both the chorionic plate and placental villi showed strong staining for Mamu-E. Staining of rhesus extravillous trophoblast cells suggested the co-expression of Mamu-E and Mamu-AG (the rhesus HLA-G homolog) on these cells. MEM-E/06 was shown also to react with differentiating rhesus placental syncytiotrophoblasts in primary culture, detecting intracellular and weak surface expression of Mamu-E. We conclude that the gestation-dependent co-expression of Mamu-E with Mamu-AG in villous and extravillous trophoblast cells suggests important and perhaps complementary but distinct roles of these two nonclassical MHC class I loci in pregnancy at the maternal-fetal interface. In addition, the MEM-E/06 antibody will be useful for the detection of Mamu-E at the maternal-fetal interface in the rhesus monkey.

Keywords

HLA-E; Mamu-E; rhesus monkey; pregnancy; trophoblast; placenta

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Introduction

MHC class I molecules are cell-surface glycoproteins that play a central role in immune recognition and alloreactivity by presenting peptides to cytotoxic T lymphocytes. Highly polymorphic class I HLA-A, -B, and -C molecules are broadly expressed in somatic cells. Another group of MHC class I molecules, referred to as nonclassical MHC class I molecules, exhibits limited polymorphism and includes HLA-E, -F and -G. HLA-G is unique in its predominant expression in placental trophoblasts, including alternatively spliced transcripts (1–3). HLA-G has long been assumed to be one of the major factors in maternal-fetal immune tolerance particularly in light of the absence in trophoblasts of expression of classical HLA-A and HLA-B molecules (4–6). Surface expression of HLA-F was also observed in extravillous trophoblasts in addition to EBV-transformed lymphoblastoid cell lines, whereas protein synthesis without expression at the cell surface was revealed in a number of tissues and cell lines (7,8).

HLA-E, like the other nonclassical MHC class I molecules, has very limited allelic variation, however it is not characterized by limited distribution. HLA-E mRNA transcripts are present in a variety of human tissues examined (9), however the surface expression of HLA-E protein is generally weak and mainly restricted to lymphocytes, monocytes/macrophages, endothelial cells (10) and some tumor cells (11,12). However, in addition to HLA-G and HLA-F, HLA-E expression also was found in trophoblast cells (13,14), implying an important role of MHC class I molecules with low polymorphism in maternal-fetal cellular interactions.

The binding of endogenously generated peptides is required for proper cell surface expression of nearly all MHC class I molecules. The analysis of peptides bound to HLA-E revealed that the majority of them are derived from the leader sequences of other HLA class I molecules, so HLA-E could serve as a principal indicator of their expression (15). Other peptides reported to be presented by HLA-E include those derived from the leader sequences of the stress-associated molecule hsp60, as well as some viral and bacterial molecules (16–18). HLA-E is recognized by heterodimeric CD94/NKG2 receptors on NK cells and a subset of T cells. The interaction with inhibitory CD94/NKG2A or activating CD94/NKG2C might depend on the bound peptide sequence and lead to the modulation of NK cell and T cell function (19–21).

NK cells are the most abundant (up to 80%) class of lymphocytes in the human and primate uterine endometrial mucosa in early pregnancy (22,23). Within two distinct subsets of NK cells identified by the level of cell surface expression of CD56, CD56^{bright} cells are characterized by higher expression of CD94/NKG2. These CD56^{bright} NK cells demonstrating greater cytokine production are likely to play an immunoregulatory role, in contrast to highly cytotoxic CD56^{low} NK cells (24). Recent studies have emphasized the unique nature of decidual NK cells which share characteristics of both peripheral NK cells subsets, showing the CD56^{bright} phenotype as well as a high level of expression of perforin and granzymes (25,26). King and co-workers (13) reported that most human decidual NK cells bind to HLA-E tetrameric complexes. The interaction between HLA-E and decidual NK cells in the maternal-fetal dialogue is likely to be more complex than simple inhibition of their cytotoxicity potential.

Non-human primates represent an important model for understanding the role of nonclassical MHC class I molecules in the placenta. The rhesus monkey (*Macaca mulatta*) MHC includes classical A and B loci, as well as nonclassical class I genes – rhesus orthologs for HLA-E, -F, -G, termed Mamu-E, Mamu-F and Mamu-G (27). We have previously demonstrated that Mamu-G is a pseudogene (28) and another nonclassical MHC class I locus, Mamu-AG, displays many features of HLA-G (29), and is expressed in the rhesus monkey placenta (30, 31). Analysis of MHC-E genes in different non-human primates has revealed that this locus is the most conserved of all known primate MHC class I loci, including extraordinary

conservation of the codons encoding the peptide binding region (32). Overall, Mamu-E exhibits 92% mRNA sequence identity, and 88% amino acid sequence similarity (33) with HLA-E. Regarding the expression of this molecule in monkeys tissues, Mamu-E mRNA has been detected in the rhesus placenta (33), but there are no published data on the demonstration of MHC-E protein in primate trophoblasts and other cells. In the current study, we have characterized Mamu-E protein expression in the rhesus monkey placenta at different stages of gestation.

Materials and methods

Animals and surgery

Female rhesus monkeys (*Macaca mulatta*) used for timed matings were from the colony maintained at the Wisconsin National Primate Research Center. Placental tissues were obtained by fetectomy at selected stages of pregnancy, with day 0 (d0) = the day after the LH surge. All surgical procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and under the approval of the University of the Wisconsin Graduate School Animal Care and Use Committee.

Trophoblast cell isolation and culture

Rhesus monkey trophoblast cells from d36 placentas were obtained from minced villous tissue dissected free of the amniotic membranes and decidua (34). Digestion was performed with trypsin/DNase enzyme solution. The suspension of dissociated cells was fractionated by centrifugation on a discontinuous 5 to 50% percoll gradient. The middle band (from the 30–40% percoll layer) was removed and washed. For the culture experiments, cells were plated at 0.5×10^6 cells/ml in Dulbecco's Minimum Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS). In some experiments interferon- γ (IFN- γ) was added for 18 hours at 25 ng/ml (Pharmingen). Cultured trophoblasts were isolated for flow cytometry analysis by brief trypsinization.

Rhesus PBMC were obtained from heparinized blood after separation on gradient of Ficoll-Paque Plus solution (Amersham); in some experiments PBMC were cultured with IFN- γ (25ng/ml) in RPMI-1640 medium, 10% FCS for 18 hours.

Cell lines and transfections

MHC class I deficient and positive B-lymphoblastoid cell lines 721.221 and 721 respectively, were maintained in RPMI-1640 medium supplemented with 15% heat-inactivated FCS. COS-7 (African green monkey kidney) cells were cultured in DMEM supplemented with 10% heat-inactivated FCS and used for transfection experiments performed with FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions.

A cDNA encoding Mamu-E was generated via RT-PCR using RNA isolated from the rhesus placenta and was used to prepare a chimeric cDNA containing the leader segment of *Mamu-A01* (a widely expressed classical MHC class I allele in the WNPRC colony) (35) and the extracellular domains of *Mamu-E*. The leader peptide will insure proper folding and processing of recombinant Mamu-E in the endoplasmic reticulum of transfectant cells. An enterokinase (EK) cleavage site and hemagglutinin (HA) protein tag were then added to the 3' terminus. The resulting cDNA was subcloned into pcDNA3.1 (Invitrogen) and sequenced to confirm all functional domains. A cDNA for soluble (s) Mamu-AG/EK/HA has been previously described (31). Plasmids were linearized with *Bgl*II and used for transfections at 3:2 FuGENE 6 reagent:DNA ratio (vol:wt).

Western blots

Cells were lysed in 10mM Tris-Cl pH 8.0, containing 0.14 M NaCl, 0.025% NaN₃, 2% Triton X-100, and protease inhibitors (Complete Mini, Roche, Indianapolis, IN). Proteins were separated on a 10% Tris-HCl polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham, Piscataway, NJ). To block nonspecific binding the membrane was incubated with 5% nonfat dry milk/TBS solution for 2 hours. Protein was detected by incubation overnight with MEM-E/02, MEM-E/06 (0.3 µg/ml) (Exbio, Praha, Czech Republic), HC10 (2 µg/ml, Harlan, Indianapolis, IN) or anti-HA (0.5 µg/ml, Roche, Indianapolis, IN) monoclonal antibodies (mAb), followed by HRP-labeled rat anti-mouse mAb (Amersham) at 1:3000 dilution for 1 hour, and visualized with the enhanced chemiluminescence Western Blot analysis system (ECL) (Amersham).

Purification of recombinant (r) proteins (rMamu-E and rMamu-AG)

Batch immunoaffinity purification of rMamu-E and rMamu-AG was performed with an anti-HA affinity matrix (Roche, Indianapolis, IN). 0.8–1.0 ml of crude cell lysate and 150 µl of anti-HA affinity matrix were incubated on a shaker for 2 hours at 4°C. The matrix was pelleted at full speed in a microcentrifuge for 5–10 seconds and washed 3 times with wash buffer (20 mM Tris, pH 7.5; 0.1 M NaCl; 0.1 M EDTA; 0.05% Tween-20). For protein elution 300 µl of 50 mM glycine, pH 2.5; 0.1% Triton X-100; 0.15 M NaCl was added to the matrix. After 20 minutes incubation at room temperature (RT) on a shaker, the matrix was pelleted and supernatant was collected into a tube with 0.5 volumes of 1 M Tris, pH 9.0 to adjust pH.

ELISA detection of rMamu-E and rMamu-AG

50 µl of PBS containing 0.5 µg of recombinant protein or an equal volume of elution buffer (for evaluation of mAb binding to the plastic) was coated into wells of 96-well assay plates (Costar) overnight at 4°C. The wells were blocked with skim milk (3%) reconstituted in PBS containing 0.01% Tween-20 (PBS-T) for 1 hour at RT. Wells were washed three times with PBS-T and 50 µl of antibodies MEM-E/06 (1.3 µg/ml), HC10 (4 µg/ml) or 16G1 (5 µg/ml, a generous gift from D. Geraghty) were added to the wells and incubated at RT for 1 hour. Isotype-matched mouse IgG diluted to the same protein concentration as the test antibody was added to the control wells for evaluation of non-specific binding of mAb to recombinant protein. After three washes with PBS-T, 50 µl of sheep anti-mouse IgG HRP conjugate (Amersham) diluted 1:3000 in PBS-T was added as second antibody to the wells and allowed to react for 1 hour at RT. The reaction product was developed with 3, 3', 5, 5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA (Sigma), for 20 min and was terminated by addition of 50 µl of stop reagent (Sigma). The resultant yellow color was read at 450 nm by an ELISA reader (Spectra Max 3400, Molecular Devices).

Flow cytometry

The following mAbs were used in the analysis: W6/32-fluorescein isothiocyanate (FITC) mAb against HLA class I antigens (Sigma), MEM-E/06 combined with secondary R-Phycoerythrin (PE)-conjugated rat anti-mouse IgG1 mAb (BD Pharmingen), FITC-conjugated anti-pan cytokeratin (Sigma). Cells were resuspended in PBS with 2% heat inactivated FCS and incubated with rat IgG (Sigma) for 15 min to block secondary mAb non-specific binding sites, then incubated with MEM-E/06 or an isotype control mAb for 30 min. All incubations were performed at 4°C. After 2 washes cells were labeled with secondary mAb for 30 min, washed twice and then fixed in 2% paraformaldehyde in PBS. For intracellular staining cells were fixed and permeabilized first using the Cytotfix/Cytoperm kit (BD Pharmingen) according to the manufacturer's protocol and then labeled for the intracellular antigen. For MEM-E/06 and cytokeratin double staining the surface antigen was labeled first with MEM-E/06, then after

fixation and permeabilization an additional blocking step was performed with mouse IgG (Sigma) before labeling with FITC-anti-cytokeratin. For each sample 10,000 cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and CellQuest Software. Data analysis was done using the FlowJo software 4.6 (Tree Star Inc., Ashland, OR).

Immunofluorescence

Isolated primary rhesus cytotrophoblasts were plated for 24 h as previously described (34). Cultured cells were rinsed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min and incubated with blocking buffer (5% BSA in PBS) overnight at 4°C. The next day cells were washed with PBS and incubated for 1 hour at 4°C with MEM-E/06 (10 µg/ml), FITC-anti-cytokeratin (12 µg/ml) or isotype control mAbs (IgG1 and IgG1-FITC) in blocking buffer with or without 0.2% Triton X-100. Cells were washed again with PBS and wells stained with MEM-E/06 and isotype control mAb were additionally incubated with secondary rat anti-mouse FITC-conjugated mAb (BD Pharmingen) (5 µg/ml) for 1 hour at 4°C and rewashed. Wells were air dried and mounted with Vectashield Mounting Medium with DAPI (4',6-diamidino-2-phenylindole) for nuclear staining (Vector Laboratories). Images were taken with a Leica DM-IRB microscope.

Immunohistochemistry

Placental tissues collected at surgery were immediately prepared for frozen sections. The tissues were fixed for 4 h in 2% paraformaldehyde, washed with PBS, dehydrated in 9% and 25% sucrose, embedded in OCT mounting medium (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen. Sections of 6 µm were cut and stained with MEM-E/06 mAb at 2.5 µg/ml, anti-human cytokeratin mAb (CAM 5.2; Becton Dickinson) at 62.5 µg/ml and the 25D3 mAb at 10 µg/ml (36). Concentration matched mouse IgG1 and IgG2 (Sigma-Aldrich Inc, St. Louis, MO) correspondingly served as negative controls.

Results

Localization of Mamu-E at the maternal-fetal interface in rhesus monkey

Whereas the expression of Mamu-E mRNA has been recognized in rhesus monkey placenta, a lack of specific antibodies prevented defining the localization of Mamu-E protein. An evaluation of recently described antibodies against HLA-E (MEM-E/06 against native HLA-E, MEM-E/02 against denatured HLA-E) (37) revealed the reactivity of MEM-E/06 with rhesus monkey PBMC by flow cytometry analysis (data not shown). We conducted immunohistochemical analysis for Mamu-E expression in rhesus placentas. In parallel with MEM-E/06 mAb staining, the expression of Mamu-AG molecules with the 25D3 mAb at the maternal-fetal interface was evaluated (Fig. 1). Analysis of floating villi of rhesus placenta on day 36 of gestation. revealed strongly positive staining for Mamu-E in villous cytotrophoblasts, and lower staining in syncytiotrophoblasts with MEM-E/06 mAb (Fig. 1C). In comparison, the anti-MamuAG 25D3 mAb, strongly bound to villous syncytiotrophoblasts (Fig. 1D), particularly at the apical membrane. In the border between the maternal decidua and trophoblast cells of the trophoblastic shell (Fig. 1E–H), MEM-E/06 mAb showed light to negative staining of extravillous trophoblasts of the cytotrophoblastic shell (arrowheads, Fig. 1G) while endovascular trophoblasts (arrows) were strongly positive for MEM-E/06 (Fig. 1G). These latter cells were also Mamu-AG and cytokeratin-positive, as were the cells of the trophoblastic shell (Fig. 1F, 1H). In both the villous stroma (Fig. 1C0, as well as the maternal decidua (Fig. 1G), there was apparent faint-to-moderate (villi) staining with the MEM-E/06 mAb, which could represent cell surface expression of Mamu-E on the surfaces of these stromal cells, or cross-reactivity in non-trophoblast cells with classical MHC class I loci. MEM-E/06 has been described to also react with some classical HLA alleles (37).

On day 50 of gestation (Fig. 2), rhesus villous cytotrophoblasts were only slightly positive for MEM-E/06 (Fig. 2C), in distinction from Mamu-AG, which remained strongly expressed in the syncytium (Fig. 2D). In addition, individual extravillous cytotrophoblasts, notably in the border of the cytotrophoblastic shell, were positively stained by MEM-E/06 (arrows, Fig. 2G), while strong staining was revealed with 25D3 mAb (Fig. 2H) and with anti-Cytokeratin mAb (Fig. 2F).

The staining pattern was noticeably different in term (day 165 of gestation) placenta. Trophoblast cell-specific staining of anti-Cytokeratin mAb (Fig. 3B) and syncytiotrophoblast-restricted staining of 25D3 mAb (Fig. 3D) were revealed, along with strongly positive staining with the MEM-E/06 mAb (Fig. 3C) in the vascular endothelial and stromal cells of the placental villi. Staining of trophoblasts at term with MEM-E/06 was low to undetectable (Fig. 3C).

Specificity of MEM-E/06 is confirmed by recognition of recombinant Mamu-E protein

To evaluate the specificity of trophoblast cell MHC recognition by MEM-E06, cells transfected with a Mamu-E expression plasmid were prepared. A hybrid cDNA containing the Mamu-E extracellular domains fused to the Mamu-A01 leader peptide was cloned into an expression vector containing sequences encoding an in-frame HA tag at the 3' end of the Mamu-E cDNA. The leader peptide will insure proper Mamu-E folding and expression. COS-7 cells were transiently transfected with the construct and analyzed by flow cytometry. The efficiency of Mamu-E expression after transfection to COS-7 was about 15% (Fig. 4A). Transfected cells containing rMamu-E were detected when permeabilized cells were used for the analysis. Despite overexpression of rMamu-E in the cytoplasm, rMamu-E was not detected on the surface of non-permeabilized cells (data not shown).

Cell lysates of COS-7-Mamu-E and naïve COS-7 cells were analyzed then by Western blot (Fig. 4B–D). Recombinant HA-tagged Mamu-E (~34 kDa) was recognized by the anti-HA mAb, as well as by the HC10 mAb raised against free class I heavy chains (Fig. 4B). HC10 mAb also showed immunoreactive bands of ~40 kDa, the native MHC heavy chains of transfected and naïve COS-7 cells, confirming the truncated nature of the recombinant protein (no transmembrane or cytoplasmic domains are included in the cDNA Mamu-E sequence used for transfection). Next, MEM-E/02 and MEM-E/06 mAbs were used in parallel analysis of Mamu-E transfectants with negative and positive controls for MHC class I molecules (721.221 and 721 human B-lymphoblastoid cells). Fig. 4C demonstrates that MEM-E/06 but not MEM-E/02 bound strongly to recombinant protein in the cell extract of COS-7-Mamu-E. MEM-E/02 mAb effectively detected HLA-E in 721 human B lymphoblastoma cells (Fig. 4C). MEM-E/06 mAb also detects a protein in cell lysates from rhesus monkey PBMC that co-migrates with HLA-E in 721 cells (Fig. 4D). Protein expression was modestly elevated in PBMC after interferon- γ (IFN- γ) treatment. Importantly, as shown in Fig. 4D, the MEM-E/06 mAb recognizes recombinant Mamu-E but not recombinant Mamu-AG protein in crude cell lysates obtained from Mamu-E and Mamu-AG transfected COS-7 cells.

To confirm the specificity of MEM-E/06 binding, an immunoaffinity purification of HA-tagged rMamu-E and rMamu-AG was performed using anti-HA affinity matrix. Eluted recombinant proteins were analyzed by ELISA with HC10, MEM-E/06, and mAb 16G1 against soluble HLA-G (38) (Fig. 5). rMamu-E coated wells were positive with HC10 and highly positive with MEM-E/06 mAbs, while rMamu-AG coated wells were readily detected with HC10 and 16G1 mAbs, as we have previously shown for this soluble isoform (31).

Flow cytometry and immunofluorescence analysis of Mamu-E expression in rhesus monkey primary cytotrophoblast culture reveal low surface but detectable intracellular expression

Freshly isolated villous cytotrophoblasts were stained with MEM-E/06 mAb (Fig. 6A) and evaluated by flow cytometry. The analysis demonstrated very weak staining of freshly isolated cells (0 h). Since we previously reported that rhesus MHC class I molecules are upregulated with trophoblast cell differentiation and syncytiotrophoblast formation in vitro (30,36), we also performed an analysis of trophoblasts after 24–48 hours of culture. The intensity of MEM-E/06 staining of the trophoblasts increased during the first 24 hours of culture and slightly decreased after 48 hours. However, Mamu-E expression detected with MEM-E/06 mAb remained quite low in comparison with parallel staining performed with W6/32 mAb, which recognizes all rhesus MHC class I molecules (39) (Fig. 6A) and 25D3 mAb against Mamu-AG molecules (36). Comparative flow cytometry analysis with MEM-E/06 of permeabilized versus non-permeabilized trophoblast cells revealed a higher intracellular Mamu-E pool, rather than surface expression (Fig. 6B). Immunofluorescence microscopic analysis also allowed Mamu-E detection in cultured trophoblasts (Fig. 6D). Whereas surface staining with MEM-E/06 was found very weak and close to isotype control staining (only isotype control staining presented, Fig. 6C), permeabilized trophoblast showed detectable intracellular Mamu-E expression (Fig. 6D). Parallel anti-cytokeratin staining was performed to verify the purity of the trophoblast cell culture (Fig. 6E). As with rhesus PBMC (Fig. 4D), treatment of primary villous trophoblast cell cultures with IFN- γ increased both cell-surface as well as cytoplasmic Mamu-E expression, as detected by flow cytometry (Fig. 6F).

Discussion

In the present study, using freshly obtained rhesus monkey peripheral blood cells, cells transfected with recombinant rhesus MHC molecules, and rhesus placental cells and tissue, we have investigated the expression of Mamu-E. In the rhesus placenta the pattern of expression of Mamu-E was found to be very similar to HLA-E expression in the human placenta (14). MEM-E/06 staining revealed strong cytotrophoblast expression but syncytial surface expression of Mamu-E molecules was low in comparison to the strong surface expression of Mamu-AG. Moreover, the intensity of trophoblast MEM-E/06 staining declined toward term, with very low to undetectable staining in the trophoblasts, although staining was very intense in the villous stroma. Identical results were seen by King and co-workers (13) with purified human trophoblast cell suspensions using HLA-E-specific RT-PCR. In term villous stroma MEM-E/06 staining was mainly restricted to the endothelial cells. Recent data from Coupel et al. (10) also demonstrates this “endothelial” pattern of the HLA-E expression in different normal non-lymphoid human tissues.

Surface expression of HLA-E in cells is dependent on co-expression of other HLA class I molecules to provide a leader peptide nonamer for appropriate folding and assembly of the HLA-E complex (15,40). In the human placenta, HLA-G and HLA-C have been found to be expressed by trophoblast cells and can be considered as the source of these peptides (13,41). Although HLA-F is also expressed in the placenta, it has not formally been shown to be able to donate leader peptide to HLA-E (8). HLA-G is expressed in the placenta as multiple isoforms in extravillous and likely villous compartments as well (2,3,38,42), including soluble molecules, which also can supply HLA-E with peptide derived from the leader sequence. In the rhesus monkey placenta, Mamu-AG is the main and likely only source of peptides for Mamu-E cell surface expression in trophoblast cells because of the lack of the MHC-C locus in rhesus monkeys (43). Mamu-AG shares many of the novel biochemical and molecular features of HLA-G (44); furthermore, Mamu-AG mRNAs are alternatively spliced in a manner similar to HLA-G mRNAs (29), giving rise to several isoforms. Consistent with our previous reports, the 25D3 mAb identified Mamu-AG on syncytiotrophoblasts, extravillous

trophoblasts, and did not identify substantial expression in villous cytotrophoblasts in vivo (36). The expression pattern of soluble HLA-G and Mamu-AG (sMamu-AG) can be studied with mAb raised against the retained intron 4, which encodes the unique C-terminal extension (42). We have previously analyzed the pattern of expression of sMamu-AG molecules in rhesus placenta with this 16G1 mAb and have defined the expression of sMamu-AG in villous cytotrophoblast, syncytiotrophoblast and some villous mesenchymal cells (29). Our data from the present study suggests that Mamu-E expression is detected in the villous cytotrophoblast, extravillous trophoblast and is only faintly expressed in the syncytiotrophoblast. Thus, the Mamu-E molecules in the rhesus placenta are likely to be co-expressed with either membrane-bound or soluble Mamu-AG in selected trophoblast populations. The expression of Paan-AG in the baboon placenta is remarkably similar to Mamu-AG (45) and we predict that Paan-E will have a similar distribution to Mamu-E.

Interestingly, the MEM-E/02 mAb, which appears to specifically recognize the denatured HLA-E heavy chain (37), does not recognize rhesus Mamu-E, either in Western blotting or immunohistochemistry analysis. By contrast, MEM-E/06 mAb reacts with rhesus cells and tissue to define the expression of Mamu-E protein. Although the MEM-E/06 mAb is described as exhibiting cross-reactivity with certain HLA class I molecules (HLA-A3, -A11, -B7 and others), it has also been demonstrated that MEM-E/06 does not recognize membrane-bound HLA-G (37). Because rhesus trophoblasts do not express any classical MHC class I molecules we performed additional experiments to define the cross-reactivity of MEM-E/06 with Mamu-AG isoforms, specifically the soluble Mamu-AG5 isoform (31). Using transfected cells, we compared the pattern of reactivity of MEM-E/06 and 16G1 mAbs in Western blotting and ELISA, and revealed that MEM-E/06 mAb does not react with sMamu-AG, demonstrating the effectiveness of this mAb for the specific detection of Mamu-E expression in rhesus trophoblast cells. The high expression of Mamu-AG on villous syncytiotrophoblasts, and the low staining of syncytiotrophoblasts with MEM-E/06, particularly at term, underscores the lack of cross-reactivity with Mamu-AG.

The role of Mamu-E can be considered primarily in light of its interaction with CD94/NKG2 receptors on a majority of NK cells and a subset of CD8+ T cells (46). Five different molecular species of NKG2 form heterodimers with invariant CD94. NKG2A and B have been reported as inhibitory receptors, while NKG2C and possibly NKG2E and H act as activating receptors (47,48). The high degree of homology between the human and rhesus monkey CD94/NKG2 families suggests similar, if not identical, roles for these molecules in NK cell function in nonhuman primates (49,50). Like the human, the rhesus decidua in early pregnancy is enriched with NK cells, which comprise up to 80% of all lymphocytes (22,23). King and co-workers (13) reported that more than 90% of human decidual NK cells are CD94/NKG2A positive and stained with HLA-E tetrameric complexes. The intensity of staining appeared to be considerably higher than that found on some peripheral blood NK cells. It was reported that HLA-E loading with the HLA-G leader peptide yields a binding to the CD94/NKG2A receptor with the highest affinity in comparison with peptides derived from other HLA class I molecules (51). Thus, a majority of decidual NK cells appears to be prepared to trigger an inhibitory signal on contact with trophoblasts expressing HLA-E. On the other hand, it is interesting that a population of decidual NK cells is reported to express activating CD94/NKG2C, as detected by RT-PCR (13). HLA-G leader peptide loading allows binding to C94/NKG2C receptors with an affinity sufficient to trigger cytotoxicity by the NK cells (52), and could be considered a possible mechanism restricting trophoblast invasion into maternal tissue.

It is likely that the combination of Mamu-AG and Mamu-E loaded with Mamu-AG-derived leader sequence also provides unique signals for modulating local uterine immune responses in the rhesus monkey. The NK cell population in rhesus decidua is remarkable, in that peripheral blood NK cells do not express CD56 marker at all, whereas in the decidua, a significant

proportion of NK cells has a CD56^{bright} phenotype (23). Additional studies will be necessary to investigate the outcome of interaction of Mamu-E-positive trophoblasts with these cells. CD56^{bright} NK cells are reported to be immunoregulatory cells, producing a high level of cytokines, in contrast to highly cytotoxic CD56^{low} (24). The likelihood that the modulation of the maternal immune system is not restricted to NK cell inhibition (53) suggests a multifaceted role of nonclassical MHC in promoting pregnancy success at the maternal-fetal interface.

Villous cytotrophoblasts are polarized cells, which differentiate into the multinucleated and also highly polarized syncytiotrophoblast. Villous cytotrophoblast may arise from the non-polarized cytotrophoblasts of the column of anchoring villi which also give rise to the extravillous trophoblast population. Thus, the expression of HLA-E in human and Mamu-E in rhesus villous cytotrophoblasts probably reflect some basal level of membrane bound HLA-G and Mamu-AG expression, and the expression of soluble HLA-G (38) and Mamu-AG (31) molecules. Alternatively, hsp60 has been reported to donate peptide to HLA-E (16,54), and hsp60 expression has been reported in the human placenta (55–57). However, colocalization of HLA-E and hsp60 remains to be demonstrated. Finally, due to the low affinity interactions of HLA-E with β 2-microglobulin, it is likely that HLA-E competes for β 2-microglobulin with other HLA class I heavy chains (11) and in villous cytotrophoblasts there is a balance between availability of nonamer peptides and free β 2-microglobulin.

The role of HLA-G molecules in successful pregnancy is considered to include the modulation of cytokine production by responding cells (58,59). The receptors recognizing HLA-G are present in all populations of decidual immune cells (NK cells, macrophages/dendritic cells, T cells) (25,58). KIR2DL4, a putative receptor for HLA-G expressed more highly by decidual NK cells than by peripheral NK cells (25), was reported to induce proliferation and IFN- γ production, but not cytotoxicity in resting NK cells (60–62). Although INF- γ production is usually associated with a pro-inflammatory response and the activation of T cells, macrophages and NK cells, it has been shown that the presence of INF- γ is necessary for the normal murine pregnancy (decidual vascular remodeling, decidual integrity and decidual NK cell maturation) (63). HLA-E and HLA-G synergism is an attractive candidate mechanism for trophoblast regulation/modulation of decidual development. Analysis of the transcriptional regulation of the HLA-E gene showed that IFN- γ is a strong inducer of HLA-E (64), which contains a STAT1 binding site, unique amongst other HLA class I genes. Up-regulation of HLA-E in response to HLA-G induced INF- γ production by decidual leukocytes may be a way to promote trophoblast tolerance via activated immune cells and to maintain a necessary level of supporting cytokines, such as IFN- γ .

It remains to be determined what cellular interactions are relevant to Mamu-E expression in villous cytotrophoblasts. These cells are not in direct contact with maternal immune cells with specific receptors to Mamu-E, but it is possible Mamu-E expression contributes to a normal intravillous environment, or to signal cellular stress or bacterial and viral presence to villous fetal immune cells. HLA-E is reported to bind peptides derived from viruses, including human cytomegalovirus, Epstein-Barr virus and influenza virus (17,21,65), and intracellular bacteria (*Listeria monocytogenes*, *Mycobacterium tuberculosis*) (66). Nonhuman primates represent an experimental system in which functional investigations of placental infection can be designed. Although the MHC-E locus is remarkably conserved during evolution and recognition of MHC-E by the CD94/NKG2 receptor family appears to have an ancient origin, cross-species tetramer staining experiments demonstrated that the interaction surfaces in CD94/NKG2 and MHC-E have diverged between primates and rodents (67). Thus, nonhuman primates represent an exceptional model for the study of the biology of early human pregnancy. Characterizing the expression pattern of Mamu-E and its functions in the rhesus monkey is a necessary step for the complete study of the function of MHC-E in placentation and immunoregulation at the maternal-fetal interface.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.