Placental expression of the nonclassical MHC class I molecule Mamu-AG at implantation in the rhesus monkey

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During human implantation trophoblasts mediate attachment of the embryo to the uterine epithelium and invade and reorganize vessels of the maternal endometrium to initiate blood flow to the intervillous space. Expression of the nonclassical MHC class I molecule HLA-G by invading trophoblasts may play a central role in their protection from recognition by the maternal immune system; however, the ontogeny of trophoblast HLA-G expression during the earliest stages of implantation is difficult to evaluate in human pregnancy. We previously identified a novel nonclassical MHC class I molecule, Mamu-AG, which is expressed in the rhesus monkey placenta and shares many unique characteristics of HLA-G. Immunocytochemical analysis with a Mamu-AG-specific mAb and locus-specific *in situ* hybridization of rhesus implantation sites 7–12 days after embryo attachment (days 14–19 of pregnancy) demonstrated that Mamu-AG molecules are expressed predominantly in cytotrophoblasts invading the maternal vessels and endometrium, whereas syncytiotrophoblasts covering trophoblastic lacunae or newly formed chorionic villi remained largely Mamu-AG-negative. By day 36 of pregnancy, Mamu-AG glycoprotein also was expressed in villous syncytiotrophoblasts, and accumulation of Mamu-AG glycoprotein was noted at the border between maternal decidua and fetal trophoblasts. The ontogeny of a nonclassical MHC class I molecule at the implantation site supports the hypothesis that its expression is important for the establishment of maternal-fetal immune tolerance.

Implantation establishes the connection between the mammalian embryo and the mother. The unique maternal-fetal interface at the hemochorial placenta, where the fetal placenta is in direct contact with maternal blood, raises the question of how the semiallogeneic fetus escapes recognition by maternal immune cells. MHC class I molecules play a central role in immune recognition and alloreactivity by presenting peptides to cytotoxic T lymphocytes, and by interacting with MHC class I receptors on natural killer (NK) cells, T and B lymphocytes, and myelomonocytic cells (1–4). In the developing human embryo, trophoblasts directly contact maternal tissues and could be targets for maternal immune cells. Extravillous interstitial and endovascular trophoblasts that invade the uterus and uterine blood vessels in early pregnancy express the nonclassical MHC class I molecule HLA-G and classical HLA-C (5–8). The mRNA for another nonclassical MHC class I molecule, HLA-E, also is expressed in the placenta (9). The recent demonstration with *in vitro* experiments that HLA-G can activate the MHC class I inhibitory receptors ILT2, ILT4, and KIR2DL4 expressed on cells of lymphoid and myelomonocytic origin (4, 10–13) is consistent with the hypothesis that HLA-G plays an important role in establishing maternal-fetal tolerance. However, this remains to be demonstrated in an *in vivo* setting.

We recently have demonstrated the expression of a novel nonclassical MHC class I locus *Mamu (Macaca mulatta)-AG* in the placenta of the rhesus monkey, an Old World primate with an inactivated *G* locus (14, 15). Although *Mamu-AG* is evolutionarily related to the MHC class I *A* locus, it shares unique molecular and biochemical characteristics of human HLA-G, including limited polymorphism, a shortened cytoplasmic domain, an unusual pattern of alternative splicing, and synthesis in trophoblasts as multiple glycoprotein isoforms (14). Like *HLA-G*, *Mamu-AG* mRNA is expressed at high levels in the placenta and amniotic membranes, with low to undetectable levels of expression in others tissues (16). To define the ontogeny of MHC class I protein expression in primate implantation, we developed Mamu-AG-specific mAbs and evaluated Mamu-AG expression in early pregnancy when trophoblasts are actively invading maternal vessels and the endometrium. We demonstrate that expression of this nonclassical MHC class I molecule is initiated in rhesus trophoblasts during early trophoblast invasion and the establishment of the fetal-maternal border at implantation. The results strongly support the hypothesis that nonclassical MHC class I molecules in the primate placenta play a crucial role in the establishment of maternal-fetal immune tolerance.

Materials and Methods

Animals and Tissue Preparation. Female rhesus monkeys (*Macaca mulatta*) were from the colony maintained at the Wisconsin Regional Primate Research Center. Implantation sites from days 14–19 of pregnancy (day 0 = ovulatory luteinizing hormone peak) were collected as described by Enders and King (17). Day 14, 15 or 19 implantation sites were fixed for 4 h in 2% or 4% paraformaldehyde and were frozen or embedded in paraffin, respectively (18). Rhesus monkey placentas from day 36 were obtained by cesarean section as described (19). All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under the approval of the University of Wisconsin Graduate School Animal Care and Use Committee.

Generation of Mamu-AG-Specific mAbs. Because of the similarity among the allelic products of *Mamu-AG* and *Mamu-A* loci (14, 20), we used a subtractive immunization technique (21, 22) to bias an immune response toward Mamu-AG-specific epitopes. Female BALB/c mice at 6–8 weeks of age received 10^7 *Mamu-A*04*-transfected 721.221 cells i.p. in 0.5 ml of PBS, followed by injection of cyclophosphamide (100 mg/kg) 10 min, 24 h, and 48 h after antigen exposure. This was repeated at 2-week intervals for a total of four treatments. Three and 5 weeks after the last treatment, mice were immunized i.p. with 10^7 *Mamu-AG*02012* transfectants. Efficiency of the tolerizing treatment and antibody titer were routinely checked by flow cytometry

Abbreviations: NK, natural killer; PBL, peripheral blood leukocyte.

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using *Mamu-A*04* and *Mamu-AG*02012* transfectants. A fusion of spleen cells with NS1 myeloma cells was completed 3 days after the last immunization. Hybridomas positive for Mamu-AG transfectant reactivity in cell ELISA (23) were screened by flow cytometry to select Mamu-AG-specific clones and cloned twice by limiting dilution. Ig isotype was determined by using a mouse mAb isotyping kit (Sigma).

Immunohistochemistry and *in Situ* Hybridization. Paraffin sections were stained with Abs and visualized by using the Vectastain ABC alkaline phosphatase kit (Vector Laboratories) and Vector Red substrate kit I (Vector Laboratories) (18). For Mamu-AG immunolocalization, frozen sections were prepared and air-dried briefly, incubated with 0.5% hydrogen peroxide in 50 mM Tris (pH 7.4) to quench endogenous peroxidase activity, and stained with mAbs after preincubation with 20% normal horse serum. Bound antibody was detected by using biotinylated horse anti-mouse Ig and Vectastain ABS peroxidase complex (Vector Laboratories). Peroxidase activity was revealed with NovaRed substrate (Vector Laboratories), yielding a brick-red precipitate. Tissue sections subsequently were counterstained with methyl green. *In situ* hybridization was performed by using a *Mamu-AG*-specific 141-nt mRNA probe as described (18).

Antibodies, Cell Lines, and Rhesus Monkey MHC Class I Transfectants. The mAbs used in this study were anti-human MHC class I mAb W6/32 (American Type Culture Collection), anti-human cytokeratin mAb (CAM 5.2; Becton Dickinson), anti-human neural cell adhesion molecule (CD56) (Sigma), and irrelevant monoclonal IgG1 or IgG2a mouse mAbs (Sigma). Rabbit anti-human β1 glycoprotein (Sp1) polyclonal antibody was from Zymed. Rhesus monkey cytotrophoblasts were isolated by trypsin-DNase dispersion and gradient centrifugation from placentas obtained by cesarean section and cultured as described (19). Human 721.221 B-lymphoblastoid cell lines stably transfected with rhesus MHC class I molecules were generated as described (15).

Results

mAb 25D3 Specifically Recognizes Mamu-AG Glycoproteins. Subtractive immunization is based on the observation that the induction of tolerance to one set of antigens by cyclophosphamide treatment, followed by immunization with a second set of antigens, can significantly improve the frequency of obtaining of antibodies that distinguish between the two sets of antigens (21, 22). By tolerizing mice toward *Mamu-A*04* transfectants followed by immunization with *Mamu-AG*02012* transfectants, we were able to obtain clones that selectively react with Mamu-AG*02012, but not with Mamu-A*04 glycoproteins. Screening by flow cytometry using 721.221 cells transfected with *Mamu-AG*02012*, *Mamu-A*01*, *Mamu-A*03*, *Mamu-A*04*, *Mamu-A*11*, *Mamu-B*01*, *Mamu-B*03*, *Mamu-B*04*, *Mamu-B*17*, as well as trophoblast cells and peripheral blood lymphocytes (PBLs) demonstrated that mAb 25D3 recognizes *Mamu-AG* transfectants and trophoblasts but does not recognize other transfectants or PBLs (Fig. 1 and data not shown). One-dimensional isoelectricfocusing analysis (Fig. 2) showed that mAb 25D3 precipitates Mamu-AG glycoproteins from trophoblasts and *Mamu-AG* transfectants. However, in contrast to the pan-MHC class I W6/32 mAb, no protein was precipitated from PBLs, *Mamu-B*01* (Fig. 1) or *Mamu-A*04* (not shown) transfectant lysates with 25D3. Because the VMAPRTLLL leader peptide found in Mamu-AG (14) might bind and bring HLA-E to the cell surface (24) it was important to exclude cross-reaction of 25D3 with HLA-E or Mamu-E. 25D3 mAb does not cross-react with a panel of other rhesus MHC class I transfectants that could induce surface HLA-E expression in 721.221 cells, therefore it is unlikely that this mAb recognizes HLA-E (e.g., *Mamu-A*04* transfectants in Fig. 1). In addition, although the MHC class I E-locus is highly expressed in rhesus lymphocytes (25), 25D3 does not react with MHC class I molecules from rhesus monkey PBL as detected by flow cytometry or one-dimensional isoelectricfocusing (Figs. 1 and 2).

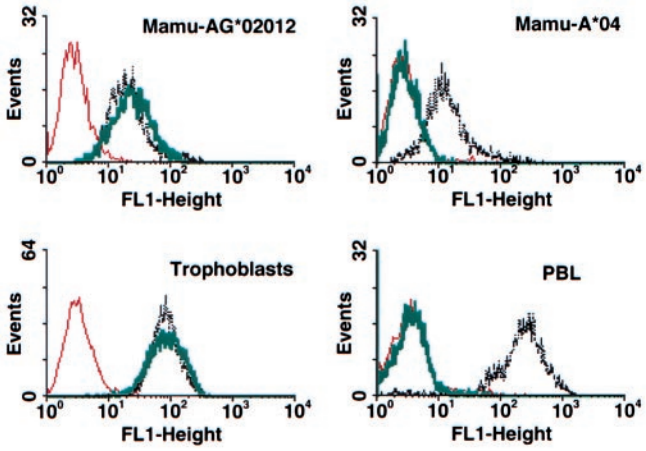

Fig. 1. Flow cytometric analysis with mAb 25D3. The thin red line depicts irrelevant mouse IgG1 + IgG2 (negative control), the dotted black line depicts W6/32 mAb, and the thick green line depicts mAb 25D3.

Mamu-AG mRNA and Glycoprotein Are Highly Expressed in Postimplantation Cytotrophoblasts. We conducted *in situ* hybridization and immunohistochemical analyses to localize *Mamu-AG* mRNA and glycoprotein in early postimplantation placental development. The earliest stage examined was 14 days of gestation (approximately 6–7 days after attachment of the blastocyst to the uterus). The morphological aspects of implantation in the rhesus monkey have been characterized in detail (17, 26, 27). Briefly, initial invasion in the macaque occurs 1–2 days after

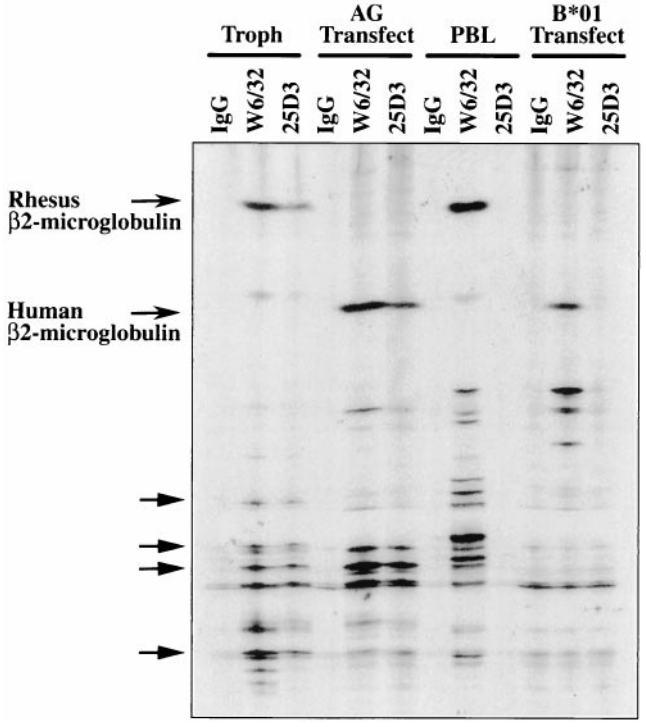

Fig. 2. One-dimensional isoelectricfocusing analysis of ^{35}S-methionine-labeled cellular lysates precipitated with control IgG, W6/32, or 25D3 mAbs. 25D3 precipitates Mamu-AG-specific bands (four lower arrows) from trophoblasts and *Mamu-AG* transfectants, but not from PBLs or *B*01* transfectants.

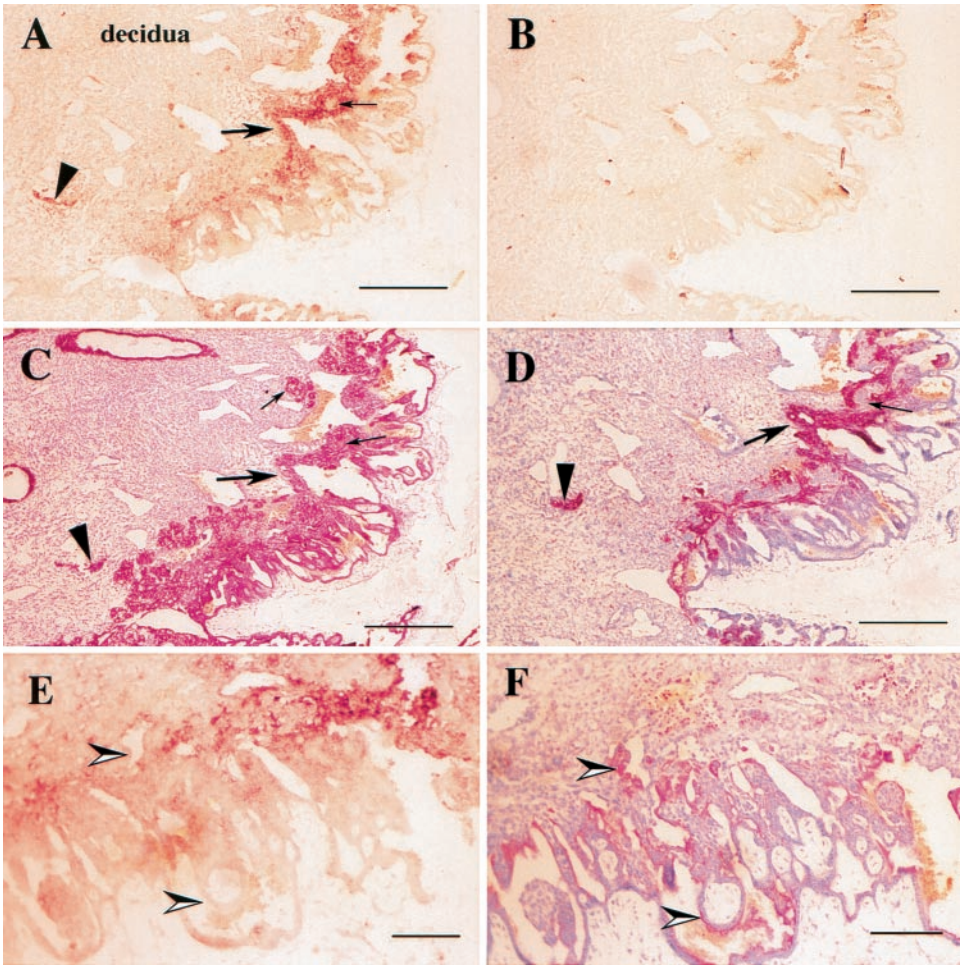

Fig. 3. Day 15 rhesus monkey implantation site. *In situ* hybridization for *Mamu-AG* mRNA. Serial paraffin sections of a day 15 implantation site were hybridized with *Mamu-AG* antisense (*A* and *E*) or sense probe (*B*), or stained with anticytokeratin mAb (*C*), anti-CD56 mAb (*D*), or anti-Sp1 Ab (*F*). Positive *in situ* hybridization signal or immunostaining appears red; cell nuclei are counterstained blue with hematoxylin. Location of the uterine decidua is noted in *A* in this and subsequent figures. (Scale bars: *A–D*, 500 μm; *E* and *F*, 100 μm.)

embryo attachment (days 9 and 10) by intrusion of embryonic syncytial trophoblasts between uterine epithelial cells (26, 27). By days 11–15, lacunae filled with maternal blood are formed and cytotrophoblasts penetrate the endometrium, passing around clusters of epithelial plaque cells and ultimately tapping maternal blood vessels. *In situ* hybridization of a *Mamu-AG*-specific probe (18) with sections from a day 15 implantation site yielded a strong signal in trophoblasts invading the endometrial stroma and maternal vessels (Fig. 3 *A* and *E*). The mAb to cytokeratin (Fig. 3*C*) identified cytotrophoblasts (large arrow) and endovascular trophoblasts (arrowhead) and also stained epithelial plaques (small arrows) (compare with *Mamu-AG* mRNA localization in Fig. 3 *A* and *E*). As previously reported (28), anti-CD56 mAb (Fig. 3*D*) stained cytotrophoblasts at the fetal-maternal junction (large arrow) and endovascular cytotrophoblasts (arrowhead) but not epithelial plaque cells (small arrow), thus CD56 immunostaining matches closely *Mamu-AG* mRNA expression (compare Fig. 3 *A* with *D*). Syncytiotrophoblasts covering lacunae are identified with an Sp1-specific Ab (Fig. 3*F*, black and white arrowheads), but are negative for *Mamu-AG* mRNA (compare Fig. 3 *E* with *F*). No hybridization was detected with *Mamu-AG* sense probe (Fig. 3*B*).

The pattern of Mamu-AG glycoprotein localization with the 25D3 mAb (Fig. 4) was similar to that seen for *Mamu-AG* mRNA. Distinct staining was seen in cytotrophoblasts at the fetal-maternal junction (Fig. 4 *A* and *C*, large arrows), endovascular cytotrophoblasts within arterioles (Fig. 4*A*, arrowhead and Fig. 4*G*, large arrow) and venules (Fig. 4*A*, black and white arrowhead). Trophoblasts have been previously noted in superficial maternal venules at the rhesus implantation site (27). Mamu-AG was localized to knobs of cytotrophoblasts projecting into lacunae (Fig. 4*E*, large arrow), which will give rise to chorionic villi at later stages of placental development, whereas the syncytiotrophoblasts lining these lacunae (Fig. 4*E*, black and white arrowhead) did not express Mamu-AG. The closely matched pattern of immunostaining with 25D3 and CD56 mAbs (compare Fig. 4*A* with *B* and *C* and Fig. 4 *D* and *E* with *F*) suggests that Mamu-AG at the implantation site is expressed predominantly in extravillous cytotrophoblasts. Epithelial plaques (Fig. 4 *A* and *C*, small arrows) do not express CD56 or Mamu-AG. The smaller round cells within the endometrium are CD56-positive decidual NK cells (Fig. 4 *B*, *D*, and *H*). Most endovascular cytotrophoblasts within arterioles express Mamu-AG glycoprotein (Fig. 4*G*, large arrows) and are CD56-positive (Fig. 4*H*) and cytokeratin-positive (not shown). However, some cytotrophoblasts within vessels (Fig. 4*G*, black and white arrowhead) are Mamu-AG-negative.

Mamu-AG Expression Is Restricted to Extravillous Trophoblasts at the Early Villous Stage. At the end of the lacunar stage of implantation (day 15 of pregnancy) the proliferation of knobs of cytotropho-

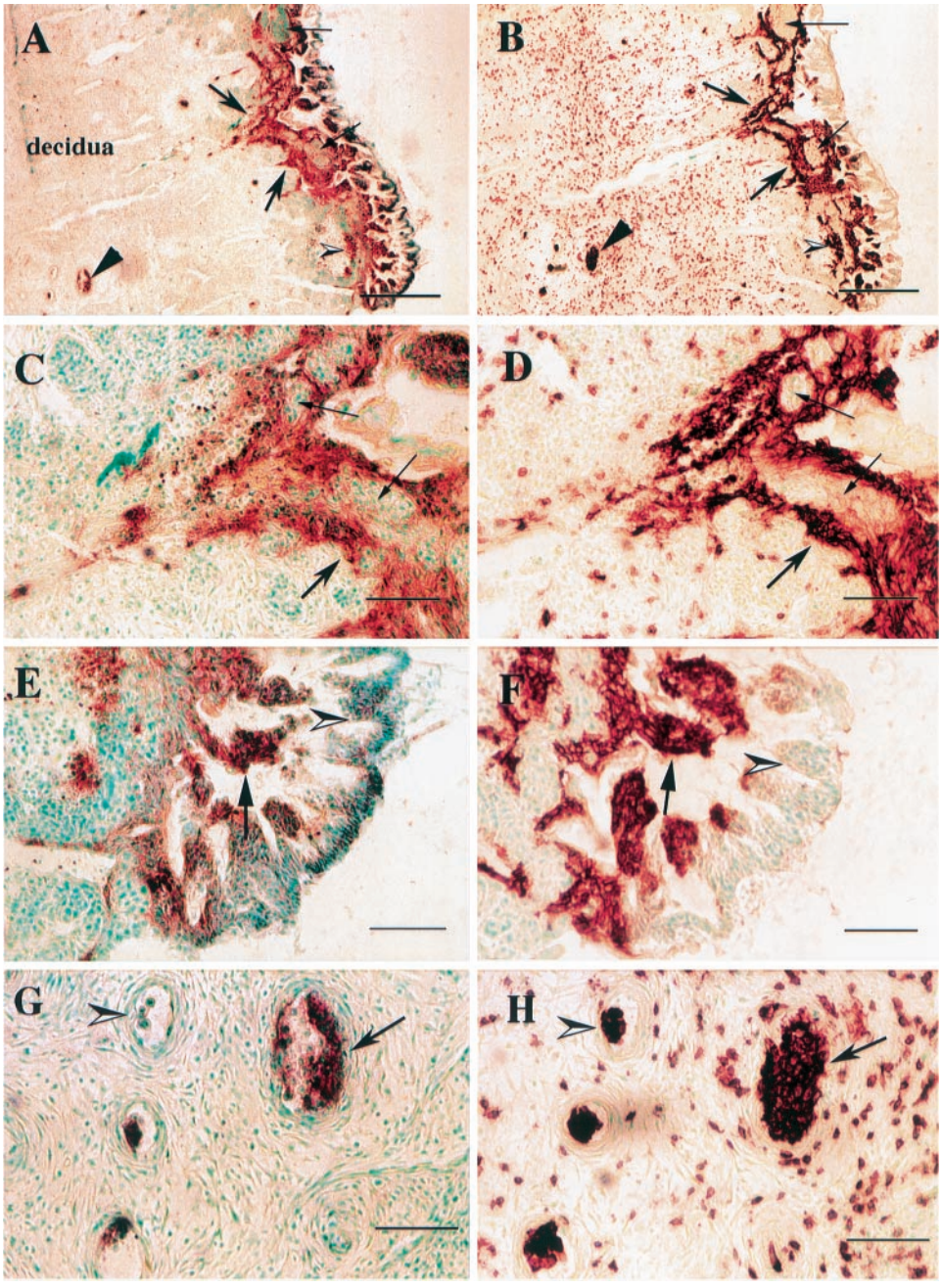

Fig. 4. Day 14 rhesus monkey implantation site. Immunocytochemical localization of Mamu-AG glycoprotein. Serial sections of the implantation site were immunostained with the 25D3 Mamu-AG-specific (*A*, *C*, *E*, and *G*), or CD56 (*B*, *D*, *F*, and *H*) mAbs. Cell nuclei are counterstained with methyl green. (Scale bars: *A*, *B*, *E*, and *F*, 500 μm; *C*, *D*, *G*, and *H*, 40 μm.)

blasts localized between the lacunae gives rise to primary villi, whereas cytotrophoblasts interdigitated into the endometrium begin to form the trophoblastic shell (Fig. 5*A*, arrow). At this stage of pregnancy stem villi emerging from the chorionic plate also can be easily distinguished (Fig. 5 *A* and *C*). Abundant *Mamu-AG* mRNA (Fig. 5*A*) and protein (Fig. 5*B*) can be seen in the trophoblastic shell (arrows), trophoblast columns (black and white arrowheads), and endovascular trophoblasts within a maternal vein (Fig. 5*B*, arrowhead). On the other hand, villous syncytiotrophoblasts (Fig. 5 *C* and *D*, arrows) and cytotrophoblasts (Fig. 5 *C* and *D*, arrowheads) do not express Mamu-AG glycoprotein (Fig. 5*D*), and only a very weak mRNA hybridization signal (Fig. 5*C*) is detected in villous syncytiotrophoblasts.

Mamu-AG Glycoprotein Is Highly Expressed in Villous Syncytiotrophoblasts and Accumulates at the Trophoblast-Decidua Junction in the Definitive Placenta. By day 36 of pregnancy in the rhesus monkey, the placenta has attained its definitive organization. The staining pattern with day 36 placental sections with the 25D3 mAb (Fig. 6) generally agrees with our previous report (18) with a *Mamu-AG*-specific mRNA probe: there is strong staining of villous syncytiotrophoblasts (small arrows), no staining of villous cytotrophoblasts (arrowheads), and weak staining of extravillous cytotrophoblasts (*). Surprisingly, despite a low level of mRNA expressed in the trophoblastic shell overall, Mamu-AG glycoprotein accumulation is readily apparent at the trophoblast-decidua junction (Fig. 6, large arrows): in essence, Mamu-AG glycoprotein immunostaining

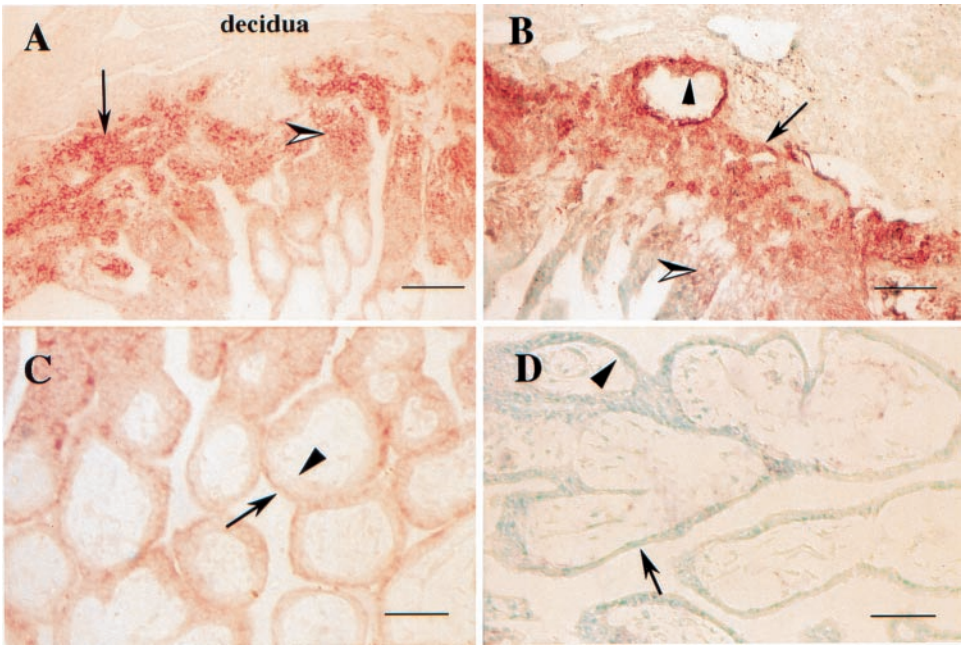

Fig. 5. Localization of Mamu-AG mRNA and glycoprotein in a day 19 rhesus monkey implantation site. Paraffin (*A* and *C*) or frozen (*B* and *D*) sections were hybridized with *Mamu-AG* antisense cRNA probe (*A* and *C*) or stained with 25D3 Mamu-AG-specific mAb (*B* and *D*). (Scale bars: *A* and *B*, 500 μm; *C* and *D*, 100 μm.)

demarcates maternal and fetal tissues both at the central zone of the placenta (Fig. 6*A*) as well as the periphery (Fig. 6*B*). The appearance at the central zone also suggests localization of Mamu-AG within some extravillous trophoblasts (Fig. 6*A*).

Discussion

The functions of nonclassical MHC class I molecules expressed in the human placenta remain incompletely defined. The restriction of HLA-G expression to trophoblasts invading the maternal decidua has led to the hypothesis that HLA-G may play an important role in protecting trophoblasts from killing by abundant uterine NK cells. This has been supported by *in vitro* studies of decidual NK cell activity against *HLA-G* transfectants (29, 30), and the recent identification of putative receptors for HLA-G on human immune cells (11, 12, 31–34). However, there is a paucity of *in vivo* evidence supporting the functional significance of trophoblast-immune cell interactions in human pregnancy. A more complete understanding of the *in vivo* roles of placental MHC class I molecules, and HLA-G in particular, would be significantly advanced by the use of appropriate animal models. There is close homology of implantation and placentation among the Old World primates (35). The rhesus monkey MHC contains orthologs of the human classical MHC class I loci *Mamu-A* and *-B,* and the nonclassical loci *Mamu-E, -F*, and *-G* (14, 20, 25, 36–38). The identification of a novel MHC class I locus expressed in the rhesus monkey placenta that shares unique molecular characteristics of *HLA-G* (14) and has a tissue distribution closely matching that seen for *HLA-G* (16) provides a unique opportunity for the study of nonclassical MHC class I molecules in the primate placenta.

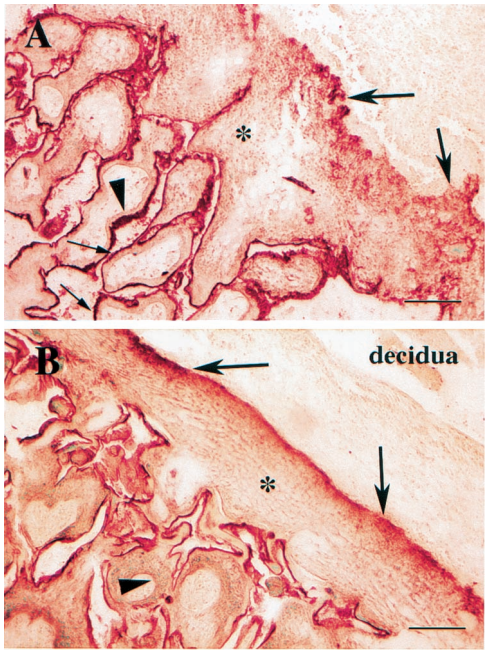

Fig. 6. Immunocytochemical localization of Mamu-AG glycoprotein in a day 36 rhesus monkey placenta. Frozen sections were stained with the 25D3 mAb. *A* is from the central placenta, *B* is from the placental periphery. Placental-decidual junction is noted by large arrows. (Scale bar = 500 μm.)

We examined the expression of Mamu-AG in the rhesus placenta during the first 2 weeks of pregnancy. HLA-G may (39) or may not (40) be expressed in the human preimplantation embryo; however, the immediate postimplantation stages of development are not available for evaluation with human pregnancy. The pattern of Mamu-AG expression predominantly in cytotrophoblasts invading the maternal vessels and endometrium strongly resembles the localization of HLA-G in the human placenta at 6–12 weeks of gestation, where HLA-G expression is restricted to cytotrophoblasts that have differentiated along the invasive pathway (5, 6, 8). Trophoblast invasion into the arterial wall, accompanied by displacement of the endothelial basement membrane and loss of smooth muscle cells (27, 41), limits the contractile capacity of endometrial arteries and permits a substantial and constant blood flow to the placenta and fetus. Endovascular trophoblasts are postulated to

initiate appropriate metalloproteinase, integrin, and extracellular matrix synthesis to accomplish these changes (28, 41). Trophoblast HLA-G expression (or Mamu-AG in the macaque) may protect from attack by decidual lymphocytes or PBLs and macrophages. The current studies demonstrate that nonclassical MHC class I expression is established in trophoblasts within a few days after implantation. Interestingly, some trophoblasts within maternal arterioles did not express Mamu-AG molecules. Perhaps these occasional trophoblasts would be eliminated by maternal NK cells without detriment to the pregnancy. However, significant loss of nonclassical MHC class I molecules may substantially affect invasive trophoblast survival and function, alter uteroplacental blood flow, and increase the risk for the development of preclampsia (42).

By the sixth week of gestation, *Mamu-AG* mRNA and protein in the definitive placenta primarily is localized to villous syncytiotrophoblasts (ref. 18 and the current studies). Intriguingly, despite a low level of *Mamu-AG* mRNA expression in the trophoblastic shell (18), there is distinct Mamu-AG immunostaining at the border between the maternal decidua and trophoblasts of the cytotrophoblastic shell. This accumulation of Mamu-AG glycoprotein at the fetal-maternal border was difficult to appreciate by previous immunostaining with the W6/32 mAb, because of the high level of MHC class I expression in the maternal decidua (18). It had been generally accepted that human villous syncytiotrophoblasts and cytotrophoblasts do not express MHC class I molecules (43–46). However, staining of human first-trimester villous syncytiotrophoblasts has been noted with some HLA-G-specific mAbs, which may recognize a soluble form of HLA-G (6, 8, 47). The pattern of Mamu-AG immunostaining at the maternal-fetal interface suggests the possibility of accumulated soluble Mamu-AG at the decidual border of the rhesus placenta.

In conclusion, the expression of nonclassical MHC class I molecules in the rhesus monkey placenta at implantation is strongly supportive of the hypothesis that nonclassical MHC class I molecules are required for appropriate trophoblast invasion and establishment of a unique immunologic microenvironment at the maternal-fetal interface. Understanding this microenvironment will have significance not only for pathologies of trophoblast invasion such as preeclampsia, but also for placental infection and maternal-fetal viral transmission. Although *Mamu-AG* and *HLA-G* evolved independently (14, 15), striking similarities between these two nonclassical MHC class I molecules in biochemical features and the pattern of expression in the developing placenta strongly suggests functional homology and a unique role at the fetal-maternal interface during the establishment of pregnancy in human and nonhuman primates.

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