Paternal MHC expression on mouse trophoblast affects uterine vascularization and fetal growth

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The mammalian fetus represents a semiallograft within the maternal uterus yet is not rejected. This situation is particularly pronounced in species with a hemochorial type of placenta, such as humans and rodents, where maternal tissues and blood are in direct contact with fetal trophoblast and thus potentially with paternal antigens. The main polymorphic antigens responsible for graft rejection are MHC antigens. In humans the trophoblast cells invading into the decidua have a unique pattern of MHC class I expression characterized by both classical (HLA-C) and nonclassical (HLA-G and HLA-E) molecules. Whether such an unusual MHC repertoire on the surface of trophoblast is a conserved feature between species with hemochorial placentation has not been resolved. Here we demonstrate, using a range of methods, that C57BL/6 mouse trophoblast predominantly expresses only one MHC class I antigen, H2-K, at the cell surface of giant cells but lacks expression of nonclassical MHC molecules. Antigen disparity between paternal MHCs affects trophoblast-induced transformation of the uterine vasculature and, consequently, placental and fetal growth. Maternal uterine blood vessels were more dilated, allowing for increased blood supply, in certain combinations of maternal and paternal MHC haplotypes, and these allogetic fetuses and placentas were heavier at term compared with syngeneic controls. Thus, maternal-fetal immune interactions are instrumental to optimize reproductive success. This cross-talk has important implications for human disorders of pregnancy, such as pre eclampsia and fetal growth restriction.

Intrauterine development in viviparous mammals is an immunological paradox because the conceptus expresses paternal MHC transplantation antigens, yet it is not rejected (1, 2). This situation is particularly evident in hemochorial placentation (found in humans and mice), where fetal trophoblast cells penetrate deeply into the maternal decidua and come into direct contact with maternal blood and tissues (3). During this invasive process, trophoblast cells transform the uterine arteries into large canals that funnel maternal blood toward the implantation site (4–6). Spiral artery remodeling is initiated early after implantation and is critical for the establishment of a fully functional placental nutrient and gas exchange unit throughout further development.

How precisely the conceptus evades immune rejection in this context remains elusive (7). An important mechanism may be provided by the distinctive MHC antigen expression profile on the surface of trophoblast. In humans, invasive extravillous trophoblast cells lack the highly polymorphic classical MHC class I (HLA-A and -B) and class II antigens but instead express a unique combination of classical HLA-C and nonclassical HLA-G and HLA-E molecules (8–10). Because nonclassical MHC class I molecules are invariant and HLA-G is expressed in the thymus, these antigens do not elicit prominent maternal immune responses (11, 12). Only HLA-C shows any appreciable polymorphism, and therefore the fetus will differ from the mother depending on the HLA-C allele contributed by the father. HLA-C is the dominant ligand for killer Ig-like receptors (KIR) expressed on natural killer (NK) cells (13). Indeed, uterine NK (uNK) cells are the most likely maternal cell type to interact with trophoblast antigens in the decidua basalis because of their sheer abundance: they constitute 70% of all maternal leukocytes in the placental bed (14). uNK cells differ from peripheral NK cells in that they have a reduced lytic activity and a distinct surface receptor repertoire (14–17). uNK cells support neovascularization of the decidua by producing proangiogenic and endothelial mitogenic stimulants and contribute to arterial transformation by initiating the loss of the arterial media (5, 14, 18, 19). In uNK cell-deficient mice the arteries do not undergo the dilation that is normally observed during pregnancy, and the resulting decrease in maternal blood supply may be associated with small placental size and embryonic lethality (20, 21). Thus, normal progression of pregnancy seems to be influenced by the functional activity of uNK cells, which in turn may be regulated by the paternal MHC expression on trophoblast through direct or indirect interactions.

The importance of trophoblast–uNK cell interactions has been reinforced by the finding that certain combinations of maternal KIRs and fetal HLA-C seem unfavorable to trophoblast cell invasion and are associated with pregnancy disorders (22, 23). These disorders include preeclampsia and fetal growth restriction and are all due to defective placentaion and inadequate arterial transformation. Thus, the immunological interplay between MHC expressed by fetal trophoblast and maternal uNK cells is critical for normal remodeling of decidual arteries.

How these maternal–fetal immunological interactions dictate the outcome of pregnancy remains unknown. The mouse can provide a particularly useful tool for the detailed investigation of this mechanism, both because strains exist that are homozygous for specific MHC haplotypes and because it is possible to derive and maintain trophoblast stem (TS) cells in vitro that can differentiate into all trophoblast cell types (24). However, a key question is whether mouse trophoblast expresses MHC class I antigens that could functionally interact with uNK cell receptors. Several studies have suggested the presence of H2 molecules, which represent the main group of MHC class I antigens in the decidua artery remodeling | uterine natural killer cells


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mouse, either at the RNA level or by antisera binding (25–29). What is still unknown is which H2 loci are expressed as proteins, whether they are displayed at the cell surface, and on which trophoblast subsets they are found. Of particular importance is the nature of MHC expression on trophoblast giant cells as they form the boundaries of the implantation site, invade into the mesometrial decidua, and contribute to arterial remodeling. To gain better insights into the immunological competence of mouse trophoblast, we here characterized systematically its MHC antigenic profile and investigated the impact of an antigenic dissimilarity between mother and fetus on trophoblast invasion, uterine vascular remodeling, and fetal and placental growth rates. Our data provide insight into the importance of immunological interactions at the maternal–fetal interface for reproductive success and demonstrate how the immunological paradox has been redirected to the benefit of the fetus during the evolution of viviparity.

Results

MHC Class I mRNA Expression in Mouse Trophoblast. Expression of the two classical MHC class I molecules H2-K and H2-D on mouse trophoblast was first assessed by haplotype-specific RT-PCR on gestational day (E) 8.5 ectoplacental cone samples dissected from crosses between the C57BL/6 (B6; b haplotype) and BALB/c (d haplotype) strains. In all crosses, we detected expression of both H2-K and H2-D genes (Fig. 1A). The intercross-derived samples served to prove expression of the paternal MHC allele. Thus, the H2-Kb and H2-Dd products in the BALB/c × B6 cross (mother is always named first) stemmed from the b allele of the B6 father, and likewise the H2-Kd and H2-Dd bands were derived from the paternal d allele in the B6 × BALB/c cross. Haplotype specificity of the PCRs was proved by the absence of a product with the strain-specific haplotype-specific primers in the inbred crosses (Fig. 1A). Representative PCR products were sequenced to confirm their identity. Trophoblast-specific expression of H2-K and H2-D was also recapitulated in TS cells derived from the B6 strain (Fig. S1).

Although both antigens were detected, quantitative RT-PCR (qRT-PCR) revealed that H2-Kb was the predominant MHC molecule expressed by B6 mouse trophoblast, with 18-fold higher transcript levels in TS cells than H2-Dd (Fig. 1B). To determine whether expression levels differed between stem cells and differentiated trophoblast cell types, TS cells were cultured in the absence of FGF4 and feeder cell-conditioned medium for 6–8 d, which induces down-regulation of stem cell-expressed genes such as Cdx2 and differentiation mainly into trophoblast giant cells (24, 30). Intriguingly, H2-Kb and H2-Dd mRNA expression was strikingly higher in giant cells than in undifferentiated TS cells (15-fold for H2-Kb and fourfold for H2-Dd; Fig. 1B). Despite this overall increase, H2-Kb was still the predominant MHC class I antigen in giant cells (i.e., the trophoblast cell type that comes into direct contact with maternal tissues).

MHC Class I Protein on the Surface of Mouse Trophoblast Giant Cells. Next we determined whether transcript levels of these classical MHC class I antigens correlated with their presence on the surface of trophoblast cells. Three different methods (FACS, immunoprecipitation, and immunofluorescence staining) were used to study surface MHC expression. Compared with IgG control, the anti–H2-K monoclonal antibody unambiguously stained the surface of trophoblast cells (Fig. 2 and Figs. S2 and S3). By contrast, H2-D was only detectable at very low levels by FACS, with variable results presumably due to slightly varying

![Fig. 1. MHC class I expression in mouse trophoblast.](image-url)

![Fig. 2. H2-Kb and H2-Dd MHC class I antigens on the surface of TS cells.](image-url)
determination states of the TS cell cultures (Fig. 2A and Fig. S2). Locus and allele reactivity of the monoclonal antibodies used is well established (31), and we also confirmed specificity using splenic controls (Fig. S2). Immunoprecipitation of trophoblast surface proteins using a monoclonal antibody to β2-microglobulin (β2m), associated with all MHC class I molecules, confirmed that the MHC heavy chain on mouse trophoblast was the same molecule (45 kDa) as for classical H2 antigens of the RMA-S control cell line (Fig. 2B). Lower-molecular-weight bands suggestive of nonclassical MHC molecules were detected (32). In line with the RT-PCR data, we observed a clear increase in H2-Kb and β2m staining upon TS cell differentiation (Fig. S4). To resolve the cell type-specific expression pattern, we stained partially differentiated B6 TS cells, ectoplacental cone explants of B6 conceptuses, and E8.5 (BALB/c × B6) implantation sites for H2-Kb by immunofluorescence and assessed them by confocal microscopy. In all these contexts, clear and specific H2-Kb staining was detected both in the cytoplasm and on the cell membrane, as revealed by costaining with the trophoblast surface marker Cdh3 (Fig. 2C and Fig. S3). Importantly, the strongest H2-Kb staining was always confined to giant cells: those differentiated from TS cells in vitro (Fig. 2C), giant cells in ectoplacental cone explants (Fig. S3B), and those at the interface with the decidua in implantation sites in vivo (Fig. 2D).

In addition to the two classical MHC class I loci (K and D), mouse WT20 nonclassical class I genes have been isolated in the B6 genome that may be particularly important on trophoblast (32). Transcripts for many of these nonclassical class I genes have been detected in crude placental extracts (32). Classical and nonclassical H2 genes of B6 mice show significant homology, averaging 92% nucleotide sequence identity in the conserved α3 domain (32). To identify whether any nonclassical H2 genes are expressed, we performed RT-PCR with degenerate primers amplifying H2-K, H2-D, and each of the M, Q, and T families of nonclassical H2 genes encoded in B6 mice. This analysis revealed almost exclusively H2-K and H2-D molecules. Only few clones represented the nonclassical T22 transcript, but surface expression was not detected (Fig. S5). Thus, the predominant MHC class I molecule on mouse trophoblast is the classical H2-K and possibly some, albeit considerably less, H2-D on giant cells, but negligible nonclassical MHCs.

The cell surface localization of H2-K would allow for a functional interaction with maternal immune cells, in particular with the cognate Ly49 receptors on the surface of uNK cells (15). Indeed, we found that paternal H2-Kβ affected the maternal uNK cell repertoire, because more Ly49C-positive uNK cells were detected in the decidua of (d × b) allogeneic crosses compared with (d × d) syngeneic controls (Fig. S6). This result indicates that the maternal uNK cell receptor repertoire may adapt to the paternal H2-Kβ expressed by trophoblast.

**Physical Interactions Between Trophoblast and uNK Cells.** Surface expression of MHC antigens and their functional effect on uNK cells raised the question of whether trophoblast cells physically interact with uNK cells to influence their activity. Previous studies on E13.5 placentas suggested that uNK cells are separated from the farthest distal trophoblast cells by a considerable distance (5). Because the remodeling of uterine arteries is initiated much earlier during the phase of trophoblast giant cell invasion, we analyzed midsagittal sections of E8.5 conceptuses and found that maternal–fetal MHC combinations did not affect the depth of trophoblast invasion (Fig. S7A). By contrast, dramatic differences in the extent of decidual artery dilation were observed that depended on the direction of the cross. Staining the sections containing the most distally invading giant cells with an endothelial cell marker (von Willebrand factor) allowed us to measure the diameter of blood vessels in corresponding decidual regions in the four different crosses. Strikingly, decidual artery diameters of BALB/c × B6 conceptuses were approximately twice as large as those of the inbred strains as well as the reciprocal B6 × BALB/c cross (Fig. 4A and B). Thus, the B6 genotype of the father in the allogeneic BALB/c × B6 cross has a profound effect on uterine vascularization compared with the syngeneic BALB/c × BALB/c mating (i.e., same maternal genotype). The direction of the cross made a significant difference because the arterial diameters in the B6 × BALB/c intercross were much less affected than in the BALB/c × B6 cross. This indicated that the effect is specific to particular strain combinations and is therefore not simply the result of heterosis.

**Paternal MHC Affects Decidual Vascularization, as Well as Placental and Fetal Growth.** We hypothesized that the strain-specific effect on decidual vessel dilation was due to maternal BALB/c uNK cells interacting with paternal H-2Kb on trophoblast. To test this hypothesis, we narrowed down the genetic variability between the parents by using the congenic BALB.B strain that carries the b haplotype at the H2 locus on a BALB/c genetic background (34). Because of the dramatic effect of a B6 father (carrying the b haplotype) crossed to a BALB/c female on decidual vessels, we concentrated on the same combination of haplotypes in the
are key adjustments in early pregnancy to ensure a sufficient blood and nutrient supply for normal fetal growth and development. To determine whether the allogeneic MHC could indeed have a long-lasting effect on developmental outcome, we assessed the weights of placentas and fetuses at E18.5 of BALB/c × BALB.B, as well as from BALB/c × BALB.B F1 in which syn- and allogeneic conceptuses develop within the same mother. To achieve the most powerful statistical analysis, we pooled all weight data of BALB/c × BALB/c syngeneic controls from two different sets of matings, from BALB/c × BALB.B, as well as from BALB/c × BALB.B F1 in which syn- and allogeneic conceptuses develop within the same mother. To determine whether the allogeneic MHC could indeed have a long-lasting effect on developmental outcome, we assessed the weights of placentas and fetuses at E18.5 of BALB/c × BALB.B, as well as from BALB/c × BALB.B F1 in which syn- and allogeneic conceptuses develop within the same mother. To achieve the most powerful statistical analysis, we pooled all weight data of BALB/c × BALB/c syngeneic and BALB/c × BALB.B allogeneic fetuses and placentas from both experimental sets. BALB/c × BALB.B fetuses allogeneic at the H2 locus were larger than syngeneic controls (P = 0.006), and this effect was not influenced by litter size (P = 0.228) (Fig. 5C). Allogeneic BALB/c × BALB.B placentas were also larger, but this effect was dependent on litter size: the bigger the litter, the smaller the difference (P = 0.01) (Fig. 5D). These data imply that allogeneic placentas may be more efficient in supporting fetal growth in larger litters, in which maternal resources are more restricted. The increased fetal/placental weight ratios of BALB/c × BALB.B conceptuses in larger litters do indeed demonstrate an augmented placental efficiency (Fig. 5E), suggesting a growth-enhancing effect of the increased maternal blood supply through larger spiral arteries on these allogeneic conceptuses.

**Discussion**

Our ignorance about which MHC molecules are expressed on the surface of murine trophoblast has hindered research in the field of reproductive immunology. Using a variety of approaches, we here show that mouse trophoblast is immunologically competent and expresses the classical MHC class I molecule H2-K on the cell surface. Taking advantage of intercrosses between mouse strains with defined H2 haplotypes, we further demonstrate presence of the paternal H2-K allele on trophoblast giant cells. These results correlate with previous reports of the detection of MHC transcripts in preimplantation embryos and trophoblast samples from E7.5 onward (27, 28) and presence of paternal H2-K antigens on early trophoblast and on the sinusoidal face of mouse labyrinthine trophoblast cells in the late-gestation placenta (29, 30, 31). In contrast to the typical expression of H2-K and H2-D on the surface of virtually all nucleated cells, however, we find that mouse trophoblast expresses only one predominant classical polymorphic MHC molecule, H2-K. This situation resembles that of human trophoblast, which expresses only HLA-A and HLA-B antigens. An unusual trophoblast MHC class I repertoire is thus conserved between both species and may be essential for trophoblast function at the maternal–fetal interface.

Interestingly, however, unlike human trophoblast that characteristically expresses HLA-G and -E, nonclassical MHC class I antigens are not a prominent feature on B6 mouse trophoblast. Thus, we did not detect mRNA or/and surface expression of the nonclassical M, O, or T family genes, and immunoprecipitation using a monoclonal antibody to $\beta$-m only revealed heavy chains with a molecular weight of classical MHC molecules. This lack of nonclassical H2 molecules on murine trophoblast corresponds with the absence of their cognate receptors (CD94 and NKG2ACE) on the Dba+ uNK cells both in the B6 and BALB/c strains (15). Thus, nonclassical MHC class I molecules are not a prerequisite for trophoblast function in species with hemochorial placentation.

We have also determined the anatomical location of MHC antigens in murine trophoblast subsets. Early studies suggested the absence of MHC class I antigens on giant cells of the ectoplacental cone, but this view was revised by experiments using in situ hybridization and immunogold labeling techniques (25, 28, 35). In contrast to the typical co-expression of H2-K and H2-D on the surface of virtually all nucleated cells, however, we find that mouse trophoblast expresses only one predominant classical polymorphic MHC molecule, H2-K. This situation resembles that of human trophoblast that characteristically expresses HLA-G and -E, nonclassical MHC class I antigens are not a prominent feature on B6 mouse trophoblast. Thus, we did not detect mRNA or/and surface expression of the nonclassical M, O, or T family genes, and immunoprecipitation using a monoclonal antibody to $\beta$-m only revealed heavy chains with a molecular weight of classical MHC molecules. This lack of nonclassical H2 molecules on murine trophoblast corresponds with the absence of their cognate receptors (CD94 and NKG2ACE) on the Dba+ uNK cells both in the B6 and BALB/c strains (15). Thus, nonclassical MHC class I molecules are not a prerequisite for trophoblast function in species with hemochorial placentation.

We here used TS cells, ectoplacental cone explants, and implantation sites to unequivocally resolve the cell type-specific distribution of H2 expression. All of our approaches perfectly correlate in demonstrating that H2-K is more strongly expressed on giant cells than in undifferentiated trophoblast. We also show that giant cells frequently come into intimate contact with uNK cells both in the B6 and BALB/c strains (15). Thus, nonclassical MHC class I molecules are not a prerequisite for trophoblast function in species with hemochorial placentation.
frequency of Ly49C+ uNK cells was greater in the presence of paternal H2-K6, providing in vivo evidence of an adaptation of uNK cell receptors to paternal MHC. Investigations on the potential contribution of paternal H2 antigens to Ly49-mediated uNK cell education will shed light on the mechanisms of interaction between uNK cells and paternal MHC class I molecules. In addition to H2-K and Ly49 receptors, other types of interactions could occur between trophoblast and uNK cells. Indeed, we found that mouse trophoblast expresses transcripts for E-cadherin (Cdh1), the ligand for KLRG1, which is highly expressed on uNK cells (15), and Gas6 as well as Pros1 transcripts, which are implicated in the regulation of NK cell differentiation (Fig. S8) (38).

Because both trophoblast and uNK cells contribute to the transformation of decidual arteries, an intriguing functional consequence of their interaction may be to regulate the progression of this remodeling process. Indeed, we show here that the allelogeneic paternal H2-K expressed on fetal trophoblast enhances the dilation state of maternal blood vessels. This in turn correlates with a growth advantage in late gestation, with larger pups and more efficient placentas at term.

Overall our findings give great confidence that murine models will provide useful tools to study the importance of immunological cross-talk during placentation. The impact on fetal growth makes this immunological interaction also a key player in fetal programming for developmental origins of adult diseases (39).

Materials and Methods

Mice and TS Cell Lines. Mice of inbred strains BALB/cAnNcr and C57BL/6j (B6) and their reciprocal intercrosses were used. BALB.B mice were originally generated by backcrossing C57BL/10 mice (carrying the H2b haplotype) to the BALB/c strain for 13 generations and selecting for the H2b genotype (34). Cryopreserved BALB.B embryos were a kind gift of Dr. A Erlebacher (New York University School of Medicine).

RT-PCR and qRT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen); 2 μg was used for cDNA synthesis followed by qPCR. Each sample was analyzed in triplicate; each analyzed group contained 6–14 independent samples. Normalization was performed against Dynein. All primer pairs were designed to span introns (Table S1). PCR products were sequence-verified. For degenerate PCRs, four reactions were used, one each for the Q and M nonclassical genes would not be amplified: T13, T15, and T24 differ substantially in size from the H2 molecule immunoprecipitated by the anti-H2K antibody, and M1 and M10 are well established components of vomeronasal pheromone receptors.
Immunochemistry/Immunofluorescence. TS cells and ectoplacental cone explants were cultured on coverslips, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS or postfixed with acetone. Antibodies used were purified anti-mouse H-2K and H-2D (AF-688S, BD Biosciences, Pharmingen) at 1:100, and anti CD4 (MS-1741-50, NeoMarkers) at 1:200. H-2K\(^\text{a}\) staining on implantation sites was on frozen sections as above. For trophoblast-unknown localization studies, sagittal paraffin sections of 8.5 µm were stained with anti-pan-cytokeratin (20622, DAKO) at 1:100, followed by staining with FITC-conjugated DBA (L9142, Sigma). Counterstaining was performed with DAPI. Epifluorescent images were taken on an Olympus BX41 microscope and confocal images on a Zeiss 510 Meta microscope.

FACS Analysis. B6 TS cells were harvested using 2 mM EDTA and used in parallel to cell suspensions from spleens of B6 (positive control) and BALB/c (negative control) mice. Antibodies used were phycoerythrin (PE)-labeled H-2K, H-2D, and H-2L (Amersham Biosciences), PE-labeled H-2Z (clone KH95, BD Biosciences, Pharmingen), and isotype controls. Anti-β2-m antibody (clone S198, BD Biosciences) was detected with polyclonal PE-conjugated antibody to mouse IgG (Sigma-Aldrich). Cells were analyzed on a BD Biosciences LSRII flow cytometer. Analyses of data were performed using FlowJo (Tree Star) software.

Immunoprecipitation. TS cells were biotinylated with 0.2 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 20 min at 4 °C. Unconjugated reagent was quenched with 10 mM glycine, and cells were lysed in a fresh preparation of 20 mM Tris (pH 7.4), 140 mM NaCl, 1 mM EGTA, 1% Triton, 10% glycerol, 50 mM iodoacetamide, and protease inhibitor mixture (Roche). Lysates were precleared with protein G-Sepharose beads (Amersham Biosciences) before immunoprecipitation with anti-β2-m antibody or isotype control preclear protein G-Sepharose beads. Immunoprecipitated complexes were washed with lysis buffer containing 3 mM SDS before bond proteins were eluted with NuPAGE LDS Sample Buffer (Invitrogen), denatured by heating to 95 °C, resolved on reducing NuPAGE Bis-Tris 10% gels (Invitrogen), electrophoresed, transferred, and blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20. Blots were probed with the indicated antibodies (see figure legends) and processed for chemiluminescence (Amersham Biosciences).

Histology and in situ Hybridization. Whole uteri horns of pregnant females were dissected, the main uterine artery to minimize bleeding, fixed overnight in 4% paraformaldehyde, and processed for paraffin histology. Conceptuses were embedded in transverse orientation and serially sectioned at 7 µm. In situ hybridizations were performed with digoxigenin-labeled riboprobes according to a standard protocol. The Pri4a1 probe was a kind gift of Dr. M. Soares (University of Kansas Medical Center, Kansas City, KS). Where indicated, sections were subsequently stained with HRP-conjugated DBA lectin (Sigma). Counterstaining was performed with hematoxylin or nuclear fast red.

Vessel Diameter Measurements. To minimize variations in the level of dehydration, all conceptuses that were directly compared were processed in parallel to murine trophoblast cell lines. The inner diameter of the implantation sites was measured for each vessel using imageJ software. At least four independent conceptuses per cross were analyzed.

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