Minor Histocompatibility Antigens Are Expressed in Syncytiotrophoblast and Trophoblast Debris

Implications for Maternal Alloreactivity to the Fetus
encoded by diallelic autosomal genes, others are encoded on the Y chromosome, and at least one gene is absent by deletion in some individuals.6 These antigens are HLA restricted: when broken down by antigen-presenting cells, one or more of the resulting peptides possess the correct structural properties to permit its presentation by class I or class II MHC molecules. Consequently, they are recognized by alloreactive CD4⁺ or CD8⁺ T cells from individuals lacking and, therefore, untolerized to, these proteins. mHAgs were discovered as a cause of chronic graft rejection, graft-versus-host disease, and the graft-versus-leukemia effect in HLA-matched donor-recipient pairs.7–9 In addition to eliciting immune responses after transplantation, mHAgs can also induce immune responses in the physiological context of pregnancy, in both mice and women. In murine pregnancy, CD4⁺ and CD8⁺ T cells are stimulated and tolerized by paternally inherited natural mHAgs and transgenically expressed model minor antigens present in the seminal fluid and fetus.10–12 These fetal antigen-specific T cells can be isolated from blood in multiparous mice and possess functional activity.13 The expansion and persistence of fetal antigen-specific cohorts of T cells in women long after pregnancy have also been recognized.14–16 The exposure of maternal T and B cells to fetal mHAgs and the ensuing response are presumably tolerogenic in normal pregnancy but may have important clinical consequences. For example, multiparity has a negative impact on organ and hematopoietic stem cell transplantation, effectively increasing the risk of transplant rejection and graft-versus-host disease.17–19

Although the antigen-specific response of maternal lymphocytes to paternally inherited antigens during pregnancy is well documented, the route of exposure of these antigens remains uncertain. One possible route of exposure is fetal microchimerism, in which fetal and/or placental cells actively traffic across the placenta and lodge within maternal organs, where they remain long-lived.20–23 A second likely source of fetal antigen is the placenta. The human placenta, which possesses a hemochorial arrangement, is bathed by maternal blood during the latter two thirds of pregnancy. The syncytiotrophoblast forms the outer layer of the placental villi and is the major interface between fetal tissues and maternal blood. An abundance of placental material is shed into the maternal circulation, and this physiological property of the placenta is thought to have important consequences on maternal physiological and pathological responses in pregnancy.24,25 The terminally differentiated syncytiotrophoblast is continuously renewed by the fusion of underlying cytotrophoblast precursors, whereas aged or damaged portions of the syncytiotrophoblast layer are extruded into the maternal blood as large multinucleated structures called syncytial knots.25 Other trophoblast debris shed into the maternal circulation includes mononuclear cytotrophoblasts and subcellular microparticles and nanoparticles.26–28 Gram quantities of trophoblastic elements may be deported from the placenta each day in normal pregnancy,29–32 and the quantity of deported material may substantially increase during preeclamptic pregnancy.33,34 This trophoblast debris is rapidly cleared from the maternal circulation without apparently generating an inflammatory immune response in normal pregnancy.31 Researchers25,35–37 have proposed that the shedding of trophoblast debris from the placenta provides an important avenue for immunological exposure of the mother to paternally derived fetal antigens, serving to establish maternal immune tolerance to the fetus. Whether such tolerance of the maternal adaptive immune system is possible is unclear, however, because the syncytiotrophoblast and villous cytotrophoblasts lack polymorphic HLA. The objective of this study, therefore, was to investigate whether mHAgs are present in the human placenta and in trophoblast debris shed from the placenta. We chose to analyze the expression of six minor antigens in the placenta, whose properties and mRNA expression pattern are summarized in Table 1: the autosomal antigens [human mHAg 1 (HMHA1), KIAA0020, and B-cell lymphoma 2–related protein A1 (BCL2A1)]; and the Y chromosome–encoded antigens [lysine demethylase 5D (KDM5D), ribosomal protein S4, Y linked (RPS4Y1), and DEAD box polypeptide 3, Y linked (DDX3Y)]. The HMHA1 and KDM5D antigens elicit a maternal response in pregnancy,13,15,16 and, like KDM5D, RPS4Y1 and DDX3Y were of interest as male antigens. BCL2A1 expression was explored because it is a regulator of apoptosis, a property that may be of significance given the putative role of apoptosis in trophoblast shedding.28,30 Finally, KIAA0020 is of interest as a potentially novel inducer of the maternal immune system. By using molecular, immunohistochemical (IHC), and proteomic approaches, we provide the first evidence that the human placenta is a rich source of these and other fetally derived mHAgs.

Materials and Methods
Tissue Procurement

All tissues were obtained in accordance with protocols approved by the University of Kansas Medical Center Institutional Review Board (Kansas City, KS) and the Auckland Regional Ethics Committee (Auckland, New Zealand). First- and second-trimester placentas (8 to 12 and 13 to 21 weeks gestational age, respectively) were obtained from elective terminations of normal pregnancies from a Kansas City-area private office (Overland Park, KS), the University of Chicago Department of Obstetrics and Gynecology (Chicago, IL), the Epsom Day Unit, Greenlane Clinical Center (Auckland, New Zealand) and the Auckland Medical Aid Clinic (Auckland, New Zealand). Term placentas (≥37 weeks’ gestational age) were obtained from the University of Kansas Hospital (Kansas City, KS) from uncomplicated pregnancies after elective cesarean sections in the absence of labor. Amnionchoron membrane and placental sections were dissected from term placentas, and cord blood was collected after placentental delivery.
IHC Data

Samples of first-trimester ($n = 5$ to $8$ per antibody), second-trimester ($n = 2$ to $3$ per antibody), and term ($n = 8$ to $10$ per antibody) placentas, the latter with an attached basal plate (BP), and extraplacental membranes ($n = 6$ to $8$) were fixed in $4\%$ paraformaldehyde overnight, dehydrated through a graded series of ethanol, and embedded in paraffin. Sections ($10 \mu m$ thick) were placed onto slides, rehydrated, and stained as previously described, with modifications dependent on each antibody, as described later. For all antibodies, tissues were subjected to hot citrate antigen retrieval using Reveal buffer (BioCare Medical, Walnut Creek, CA). Nonspecific antibody binding was blocked in $10\%$ goat serum (Sigma-Aldrich, St. Louis, MO). The rabbit polyclonal primary antibodies targeting HMHA1 (12 $\mu g/mL$), KIAA0020 (2 $\mu g/mL$), and RPS4Y1 (12 $\mu g/mL$), all obtained from Sigma-Aldrich, were added to tissue sections, which were then incubated overnight at $4^\circ C$. For slides incubated with anti-KIAA0020 antibody, tissue sections were permeabilized in $0.5\%$ Tween 20 (Sigma-Aldrich) in PBS and subjected to enzymatic digestion using trypsin (Invitrogen) and DNase (Sigma-Aldrich). Dispersed cells were layered over fetal bovine serum and centrifuged at $1000 \times g$, and after washing, the pellet was resuspended in HBSS, layered over a $5\%$ to $70\%$ stepwise Percoll gradient (Sigma-Aldrich), and centrifuged at $1200 \times g$. The band corresponding to trophoblast cells was aspirated, and the cells were subjected to a second stage of purification using immunomagnetic beads (Miltenyi Biotec, Boston, MA) coated with anti-class I MHC (W6/32), as previously described. Cell purity was assessed using anti-cytokeratin 7 by IHC or flow cytometry and was determined to be $>90\%$.

RNA Isolation and RT-PCR

RNA was extracted from whole placenta ($n = 3$ female and 5 male), purified cytrophoblast cells ($n = 2$ female and 3 male), and cord blood mononuclear cells ($n = 2$ female and 2 male) using TRI Reagent (Applied Biosystems, Foster City, CA). RNA concentrations were quantified using a BioPhotometer (Eppendorf, Hauppauge, NY), and 1 $\mu g$ of RNA was reverse transcribed using Superscript III Reverse Transcriptase and random primers (Invitrogen) in a reaction volume of $20 \mu L$. Parallel reactions containing RNA but no reverse transcriptase were included to verify the absence of genomic DNA in samples in the subsequent PCR steps (Fermentas, Glen Burnie, MD). Conventional RT-PCR was performed for DDX3Y, RPS4Y1, and glyceraldehyde-3-phosphate dehydrogenase, with primers designed using PrimerBLAST (National Center for Biotechnology Information, Bethesda,

<table>
<thead>
<tr>
<th>Minor antigen</th>
<th>Gene</th>
<th>Chromosome</th>
<th>HLA restriction</th>
<th>Function</th>
<th>Tissue expression (mRNA)</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-1</td>
<td>HMHA1</td>
<td>19p13.3</td>
<td>HLA-A&lt;0201, HLA-A&lt;0206, HLA-B&lt;60, and HLA-B&lt;40012</td>
<td>GTPase activator (putative)</td>
<td>Hematopoietic cells</td>
<td>38–40</td>
</tr>
<tr>
<td>HA-8</td>
<td>KIAA0020</td>
<td>9p24.2</td>
<td>HLA-A&lt;0201</td>
<td>RNA-binding protein (putative)</td>
<td>Broad</td>
<td>41</td>
</tr>
<tr>
<td>ACC-1 and ACC-2</td>
<td>BCL2A1</td>
<td>15q24.3</td>
<td>HLA-A&lt;2402 and HLA-B&lt;4403</td>
<td>Negative regulator of apoptosis</td>
<td>Hematopoietic cells</td>
<td>42</td>
</tr>
<tr>
<td>SLC1A5</td>
<td>SLC1A5</td>
<td>19q13.3</td>
<td>HLA-B&lt;4002</td>
<td>Amino acid transporter</td>
<td>Some carcinomas, especially colorectal</td>
<td>43 and 44</td>
</tr>
<tr>
<td>LB-SSR1-1S</td>
<td>SSR1</td>
<td>6p24.3</td>
<td>HLA-A&lt;0201</td>
<td>Signal sequence receptor in endoplasmic reticulum</td>
<td>Hematopoietic cells, small intestine</td>
<td>44 and 45</td>
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<tr>
<td>A2/HY and B7/HY</td>
<td>KDM5D</td>
<td>Yq11</td>
<td>HLA-A&lt;0201 and HLA-B&lt;2705</td>
<td>Protein-containing zinc finger domains</td>
<td>Broad; high in hematopoietic cells</td>
<td>44 and 46</td>
</tr>
<tr>
<td>B52/HY and DBR3/HY</td>
<td>RPS4Y1</td>
<td>Yp11.3</td>
<td>HLA-B&lt;5201 and DBR3&lt;0301</td>
<td>RNA-binding protein</td>
<td>Broad</td>
<td>47 and 48</td>
</tr>
<tr>
<td>DQ5/HY and DBR1/HY</td>
<td>DDX3Y</td>
<td>Yq11a</td>
<td>DQB1&lt;05 and DBR1&lt;1501</td>
<td>RNA helicase, involved in spermatogenesis</td>
<td>Broad; high in testis</td>
<td>49</td>
</tr>
</tbody>
</table>

KIAA0020, pumilio domain-containing protein; KIAA0020, solute carrier 1A5; SSR1, signal sequence receptor 1.
MD) and synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences were as follows: DDX3Y forward, 5'-TGCTGTCGATACGCTGGTGG-3'; DDX3Y reverse, 5'-TCTCGAGACCAAAACTGCT-3'; RPS4Y1 forward, 5'-AGATTCTTCTCCGTCGAGA-3'; RPS4Y1 reverse, 5'-CATAGACGCGCGGAATGT-3'; glyceraldehyde-3-phosphate dehydrogenase forward, 5'-ACCACACTCCATGCCATCAC-3'; and glyceraldehyde-3-phosphate dehydrogenase reverse, 5'-TCCACTCCTTGCGTGA-3'. The RT reaction sample, 2 μL, was subjected to PCR (Invitrogen) using one times PCR buffer, 0.2 mmol/L deoxyribonucleotide triphosphate, 1.5 mmol/L MgCl₂, 1.5 U Taq polymerase, and 5 mmol/L of each primer in a volume of 20 μL. The PCR was performed with the following protocol: 94°C for 3 minutes; 35 cycles of 94°C for 45 seconds, 63°C for 30 seconds, and 72°C for 90 seconds, and 72°C for 5 minutes. TaqMan Gene Expression Assay kits for real-time RT-PCR were obtained from Applied Biosystems (Foster City, CA) (HMHA1, Hs00299628_m1; KIAA0020, Hs00972109_m1; BCL2A1, Hs00187845_m1; KDM5D, Hs01104415_m1; DDX3Y reverse, 5'-TCTCGAGACCAAAACTGCT-3'; and β-actin, Hs99999903) and run according to the manufacturer’s protocol. All RT-PCR samples were analyzed in triplicate, and a mean was generated from the three C_T values for each sample. For each individual placenta, purified trophoblast or cord blood sample, the mean for each mHAg was subtracted from the mean for β-actin to provide an mHAg C_T value that had been normalized to β-actin to account for discrepancies in RNA concentration or quality. These means were then pooled to generate an average C_T value for each antigen in each tissue type.

**Protein Extraction**

For analysis of protein content of shed trophoblast debris, an established in vitro model of trophoblast shedding was used, together with a proteomic approach. Trophoblast debris was harvested from 12 first-trimester placentas (8 to 12 weeks’ gestational age). Placental tissue was washed with PBS (pH 7.4), dissected into approximately 400 mg of wet-weight explants, and transferred into Netwell inserts (Corning, Lowell, MA) placed in a 12-well culture plate (Becton Dickinson, Franklin Lakes, NJ). The explants were cultured in 3 mL of Dulbecco’s modified Eagle’s medium/F12 medium (Invitrogen) containing 5% fetal bovine serum and 1% penicillin-streptomycin for 12 hours at 37°C, in a humidified atmosphere containing 5% CO₂ and 95% ambient oxygen. Trophoblast debris shed from the explants was collected from the bottom of the culture plate, and red blood cells were depleted by hypotonic lysis, while contaminating leukocytes were removed using anti-CD45-coated magnetic beads, as previously described. The trophoblast debris was subjected to two independent protein extraction methods to enrich the plasma membrane components in the extracts: Ludox CL cationic colloidal silica (Sigma-Aldrich) and direct trypsinization. The colloidal silica extraction protocol followed the method of Robinson et al., with minor modifications. For direct trypsinization, briefly, the trophoblast debris was suspended in 30 μL of pre-warmed 50 mmol/L NH₄HCO₃ containing 1 μL of benzonase nuclease (Merck, Darmstadt, Germany). Sequencing-grade trypsin (Promega Corporation, Madison, WI), 1 μL, was added and incubated at 37°C for 80 minutes, with 1 μL of trypsin added at 20-minute intervals. The sample was centrifuged at 17,000 × g at 4°C for 15 minutes, and the supernatant was collected.

To characterize proteins from the syncytiotrophoblast, protein extraction was performed directly on six placentas after extensive washing in PBS. Syncytiotrophoblast proteins were isolated from the surface of placental explants using extraction methods to enrich the plasma membrane component in the extract. Two independent methods were used to extract proteins: Ludox CL cationic colloidal silica (previously described) and Triton X-114 detergent (Sigma-Aldrich). The Triton X-114 extraction protocol followed the method of Dickerson et al., with minor modifications. To confirm enrichment of plasma membrane components in the trophoblast debris, the relative proportion of proteins found primarily in the plasma membrane was compared between a non-plasma membrane enrichment technique (radioimmuno-precipitation assay lysis) and the two plasma membrane enrichment techniques, Ludox CL cationic colloidal silica and direct trypsinization. Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA) and showed a relative enrichment of 12% (O.J.H. and L.W.C., unpublished data).

**MS Data**

Liquid chromatography/tandem mass spectrometry (MS/MS) was used to sequence tryptic peptides in the extracts from which protein identities were determined. Samples were electrophoresed on a 4% to 15% SDS polyacrylamide gel, and either the entire lane was excised as a whole or the lane was excised in sections for MS analysis. The excised protein lanes were subjected to in-gel digestion with trypsin using a robotic workstation for automated protein digestion (DigestPro Msi; Intavis AG, Cologne, Germany), following the method of Shevchenko et al., The MS measurements were made on the LTQ-Orbitrap hybrid MS (Thermo Scientific, Lafayette, CO). Extracted MS/MS spectra were automatically assigned to the best-matching peptide sequence using the SEQUEST algorithm and the Sequest Browser software package (ThermoFinnigan, Lafayette, CO). The false-discovery rate for the data was calculated by running a decoy search against the reversed Human Reference Sequence database (National Center for Biotechnology Information) using the same search parameters as the main analysis. The reversed database was built from the same copy of the Human Reference Sequence database that has been used for the main searches using the Decoy Database Tool (Trans-Proteomic Pipeline; Seattle Proteome Center, Institute for Systems Biology, Seattle, WA).
Results

RNA for mHAgS in the Placenta and Cord Blood

To determine whether the placenta or fetal blood cells could serve as sources of mHAgS, whole placental lysates, purified cytotrophoblast cells, and fetal cord blood RNA were examined by real-time RT-PCR for the presence of four genes known to encode mHAg proteins. The expression of mRNA for HMHA1, KIAA0020, BCL2A1, and KDM5D was found in placental lysates, purified cytotrophoblast cells, and fetal cord blood (Figure 1A). No expression of the Y chromosome–encoded mHAg KDM5D was found in samples from pregnancies with a female fetus. Because of lack of availability of TaqMan real-time PCR gene expression assay kits that distinguish between the male and female isoforms of the DDX3Y (DDX3X) and RPS4Y1 (RPS4X) genes, we designed primers for conventional RT-PCR that would distinguish these transcripts. Like KDM5D, DDX3Y and RPS4Y1 were identified in whole placenta, cytotrophoblast cells, and cord blood from male, but not female, fetuses (Figure 1, B and C).

Analysis of mHAg Proteins in the Placenta

General Properties of mHAg Immunoreactivities in the Placenta

First-trimester, second-trimester, and term placental villi, as well as term extraplacental membranes, were examined by IHC using antibodies targeting the mHAg proteins HMHA1, KIAA0020, BCL2A1, and RPS4Y1. All four minor antigens could be observed in both villous and extravillous trophoblast (EVT) populations, with variation occurring within and between placental samples, across gestation, and according to antigen and cell type (Figure 2 and Figure 3). IHC for DDX3Y and KDM5D was attempted using commercially available antibodies, but failed to yield any positive staining under a variety of conditions, including positive control testis tissue.

HMHA1 and RPS4Y1 expression was cytoplasmic (Figure 2, A, B, G, and H), KIAA0020 and BCL2A1 were also usually seen in the cytoplasm, but this was frequently accompanied by nuclear expression (Figure 2, C–F, and Figure 3, C–F). The RPS4Y1 antibody stained trophoblast cells (all subpopulations) of both male and female placentas, because this antibody does not distinguish between the Y- and X-encoded isoforms of this protein. Expression observed using this antibody is, therefore, described as RPS4X/Y1 hereafter.

Villous Trophoblast Cells

In the syncytiotrophoblast, immunoreactivity for all four mHAgS examined was observed, although staining intensity for each antigen always varied, both within and between placentas. Immunoreactivity for BCL2A1 was generally stronger and more uniform in first- and second-trimester villous cytotrophoblasts and syncytiotrophoblasts than at term (Figure 2, E and F, and Figure 3E). However, in three of the eight term placentas examined, BCL2A1 was often concentrated or restricted to areas of aggregated syncytiotrophoblast nuclei, possibly representing syncytial knots before their shedding from the placenta.

KIAA0020 and RPS4X/Y1 proteins were observed in villous trophoblasts of all placentas throughout gestation. Immunoreactivity varied within and between placentas (Figure 2, C, D, G, and H, and Figure 3, C, D, and G).
KIAA0020 was always found in the cytoplasm, and some first-trimester samples exhibited nuclear staining as well. This protein was often concentrated at the microvillous surface and displayed stronger immunolocalization in the syncytiotrophoblast than in the cytotrophoblast.

HMHA1 immunoreactivity in the villous trophoblast was only observed consistently in first-trimester samples. One of five second-trimester samples, and none of the term samples, expressed this protein in the syncytiotrophoblast (Figure 2, A and B, and Figure 3A). Within each individual first-trimester sample, trophoblast expression of HMHA1 was variable. In the first- and second-trimester samples, syncytial immunoreactivity was always stronger than cytotrophoblast staining.

**EVT Data**

The expression of all four mHAg proteins was frequently observed in EVTs. BCL2A1 expression was moderate in first-trimester (Figure 2E) and second-trimester (data not shown) trophoblastic columns, whereas at term, staining for BCL2A1 was weak or absent in the EVTs, basal plate, and chorion membrane (Figure 3F). KIAA0020, HMHA1, and RPS4Y1 antibodies consistently reacted with EVT cells throughout gestation; in the term chorion membrane, trophoblast cells proximal to the amnion exhibited stronger immunoreactivity for HMHA1 and RPS4Y1 than those distal to the amnion (Figure 3, B and H).

**Fetal Leukocytes**

Fetal leukocytes contained within the vasculature of the term placentas were generally negative for KIAA0020, BCL2A1, and RPS4X/Y1; however, a few weakly positive leukocytes were occasionally present in these samples. Fetal leukocytes in term placenta consistently and strongly expressed HMHA1 (data not shown).

**Hofbauer and Mesenchymal Cells**

Hofbauer cells, which are found within the villous mesenchyme and share lineage with macrophages, showed strong expression of HMHA1 throughout gestation (Figure 2, A and B, and Figure 3A). In the term placenta, stromal fibroblasts could also be seen to express HMHA1, BCL2A1, KIAA0020, and RPS4X/Y1 antibodies generally did not react with placental fibroblasts or Hofbauer cells, with the exception that most second-trimester placentas exhibited weak staining of both cell types.
Endothelial cells were consistently immunoreactive with the KIAA0020 antibody throughout gestation (Figure 2D and Figure 3C); with the exception of sporadic staining of these cells for RPS4X/Y1, endothelial cells generally lacked the other mHAg proteins investigated in this study.

**Maternal Tissues**

The expression of mHAgS in the decidua was determined by examining term basal plate and extraplacental membranes with their associated decidual tissue. HMHA1, RPS4X/Y1, and KIAA0020 were expressed by decidual cells, whereas BCL2A1 expression was weak or absent (Figure 3, B, D, F, H, and J). KIAA0020, BCL2A1, and RPS4X/Y1 were generally negative in maternal leukocytes associated with decidual tissue. Maternal leukocytes consistently expressed HMHA1 in both first- and third-trimester tissues. These cells could be observed both in peripheral blood located within the intervillous space and within decidual tissue associated with the chorion and basal plate.

**Proteomic Analysis of the Syncytiotrophoblast and Trophoblast Debris**

Proteomic analysis was performed on the syncytiotrophoblast and on the trophoblast debris shed from cultured first-trimester placental explants. A total of 1590 proteins were identified as being present in the syncytiotrophoblast, and 375 proteins were identified as being expressed in trophoblast debris, with a false-discovery rate of 0.0087. The identity of these proteins was confirmed by examining term basal plate and extraplacental membranes with their associated decidual tissue. HMHA1, RPS4X/Y1, and KIAA0020 were expressed by decidual cells, whereas BCL2A1 expression was weak or absent (Figure 3, B, D, F, H, and J). KIAA0020, BCL2A1, and RPS4X/Y1 were generally negative in maternal leukocytes associated with decidual tissue. Maternal leukocytes consistently expressed HMHA1 in both first- and third-trimester tissues. These cells could be observed both in peripheral blood located within the intervillous space and within decidual tissue associated with the chorion and basal plate.

**Discussion**

Multiparous women have previously been shown to possess expanded cohorts of T cells specific for fetal mHAgS. However, the source of the antigens that trigger this T-cell expansion has not been definitively identified. In this study, we identified the placenta, a fetal organ, as a probable source of foreign mHAgS to which the mother is exposed during pregnancy. By using RT-PCR, we demonstrated that mRNAs for the autosomal mHAgS (HMHA1, KIAA0020, and BCL2A1) and the Y chromosome–encoded mHAgS (KDM5D, DDX3Y, and RPS4Y1) are found in placental tissue and purified trophoblast cells. These results are consistent with those of Warren et al who, by tissue array, identified mRNA for another male mHAg, UTY, in human placental homogenate. IHC analysis revealed that four of the six mHAgS studied were expressed by several different placental cell types, including trophoblast subpopulations, mesenchymal cells, placental macrophages, and cord blood leukocytes. Importantly, all four mHAgS examined by IHC were expressed within the syncytiotrophoblast, which, for most of the pregnancy, is bathed directly in maternal blood. This then provides an open avenue for release of mHAg-containing shed material from the placenta into the blood and subsequent trafficking to maternal lymphoid organs, where an immune response might be generated. Indeed, we show that at least one antigen, DDX3Y, is present in trophoblast debris shed from first-trimester placenta.

Our findings of robust expression of fetal mHAgS in the placenta align with earlier work demonstrating that pregnancy results in the expansion of maternal minor antigen–specific CD8+ T cells. One antigen that elicits antigen-specific maternal immunoreactivity, HMHA1, was strongly expressed in the syncytiotrophoblast and EVTs, both of which are fetal cells to which the maternal immune system has direct contact. Protein expression studies of other Y chromosome–encoded antigens known to elicit maternal T-cell reactivity, such as KDM5D, are limited by antibody availability and specificity. Indeed, caution in interpretation of RPS4Y1 IHC studies is warranted because the peptide immunogen for the RPS4X/Y1 antibody derives from regions conserved between the male and female isoforms of this protein, thus rendering the antibody undiscerning between male and female placentas. Nonetheless, we have shown the presence of the male-specific isoform, RPS4Y1, by both RT-PCR and proteomic analyses in the trophoblast of male placentas.

### Table 2. Antigenic Minor Histocompatibility Proteins Detected by MS in Shed Trophoblast Debris and in the Syncytiotrophoblast

<table>
<thead>
<tr>
<th>mHAg</th>
<th>Protein name</th>
<th>Gene</th>
<th>Trophoblast debris</th>
<th>Syncytiotrophoblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1/HY and DQ5/HY</td>
<td>ATP-dependent RNA helicase DDX3Y</td>
<td>DDX3Y</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BS2/HY and DRB3/HY</td>
<td>40S ribosomal protein S4, Y isoform 1</td>
<td>RPS4Y1</td>
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<td>+</td>
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<tr>
<td>SLC1A5</td>
<td>Neutral amino acid transporter B(0) isoform 1</td>
<td>SLC1A5</td>
<td>–</td>
<td>+</td>
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<tr>
<td>LB-SSR1-1S</td>
<td>Translocon-associated protein subunit α</td>
<td>SSR1</td>
<td>–</td>
<td>+</td>
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<tr>
<td>HA-1</td>
<td>Minor histocompatibility protein HA-1</td>
<td>HMHA1</td>
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<td>HA-8</td>
<td>Pumilio domain-containing protein KIAA0020</td>
<td>KIAA0020</td>
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<td>ACC-1 and ACC-2</td>
<td>Bcl-2-related protein A1</td>
<td>BCL2A1</td>
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<td>–</td>
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<tr>
<td>A2/HY and B7/HY</td>
<td>Lysine-specific demethylase 5D</td>
<td>KDM5D</td>
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</tbody>
</table>

The protein name and gene symbol of each minor antigen are given.

N/A: antigen not found.
The finding that mHAgs are expressed in the syncytiotrophoblast and in trophoblast debris shed from the placenta provides the first direct evidence that trophoblast and deported trophoblast debris contain proteins that are responsible for triggering cell-mediated immune responses. A single mHAg was found in proteomic analysis of trophoblast debris shed from the placenta. It seems likely that other mHAgs are also present in the debris, because MS proteomic analysis is only able to detect proteins that are present in relatively high abundance; some of the mHAgs may be expressed at a level too low to detect by this method. Current efforts are focused on identifying these antigens in trophoblast debris using more sensitive methods.

Much trophoblast debris is thought to be shed into the maternal blood daily in a normal pregnancy. This enormous amount of trophoblast debris is rapidly cleared from the maternal blood by an unknown mechanism, but it is likely that maternal phagocytes, such as macrophages, are at least partially responsible. Phagocytosis of mHAg-containing trophoblast debris by maternal antigen-presenting cells would likely produce some form of maternal adaptive immune response. Additionally, the context in which the adaptive immune system detects antigen is critical in determining the nature of the ensuing response. Antigens produce tolerogenic immune responses when they are presented from apoptotic cells. Because trophoblast debris is thought to be shed from the placenta via apoptosis, it seems likely that DDX3Y and other mHAgs and/or HLAs in deported the recipient antigen-presenting cell. Phagocytosis of gen processing occurring either before or after transfer to necrotic cellular material by dendritic cells, with anti-cells, and occurs as a result of phagocytosis of apoptotic Cross presentation can result in tolerance or priming of T responses. A single mHAg was found in proteomic analysis of trophoblast debris shed from the placenta. It seems likely that other mHAgs are also present in the debris, because MS proteomic analysis is only able to detect proteins that are present in relatively high abundance; some of the mHAgs may be expressed at a level too low to detect by this method. Current efforts are focused on identifying these antigens in trophoblast debris using more sensitive methods.

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cells traffic into the maternal circulation is unknown. The presence of detectable mHAgS within fetal leukocytes is suggestive that microchimerism can be a mechanism of maternal alloimmunization to these antigens, as has been suggested by others.\textsuperscript{16} Indeed, some mothers possessing T cells directed against their children’s mHAgS have possessed significant fetal microchimerism.\textsuperscript{14,21} Yet, another potential mechanism that could result in maternal exposure to fetal blood antigens is leakage into the maternal circulation during placental hemorrhage before or during parturition. Indeed, this occurs with immunization of rhesus antigen–negative women carrying rhesus-positive fetuses.

Maternal alloimmunization by fetal antigens during pregnancy is thought to have important clinical implications for organ and stem cell transplantation, subsequent pregnancies, and autoimmunity. Donor parity has been identified as a risk factor for acute and chronic graft-versus-host disease in patients undergoing hematopoietic stem cell transplantation,\textsuperscript{17,18} and recipients of parous female hematopoietic stem cell transplantation have both higher rates of graft-versus-host disease and graft-versus-leukemia effects.\textsuperscript{6,69–71} Researchers hypothesize that mHAg-specific T cells, which had resulted from exposure of fetal mHAgS to the maternal compartment during pregnancy, are provided by the female donor. The long-term impact of pregnancy-induced maternal sensitization is demonstrated by the findings that mHAg-specific T cells may persist up to 22 years after pregnancy.\textsuperscript{6,15,72}

Secondary recurrent miscarriage is defined as three or more consecutive miscarriages after a birth at 24 weeks’ gestation or later. Two independent research groups have found a significant association between the development of secondary recurrent miscarriage and prior birth of a male infant. This may be due, at least in part, to an aberrant maternal immune response to fetal Y chromosome–encoded antigens.\textsuperscript{73,74} In addition, maternal carriage of HY-restricting MHC class II alleles is associated with poor pregnancy outcomes in women with a prior live birth of a male infant and a diagnosis of secondary recurrent miscarriage.\textsuperscript{75} Our data indicate that maternal exposure to Y chromosome–encoded fetal antigens is likely to occur during pregnancy, at least in part as a result of shedding and deportation of trophoblast debris into the maternal blood. Therefore, aberrant responses to mHAgS may contribute to inappropriate adaptive maternal immune responses to the placenta/fetus.

Finally, our results have implications for the relationship between fetal microchimerism and maternal autoimmune disease, in that they further the possibility of differential antigen recognition by the maternal immune system and/or engrafted fetal cells. Nelson et al\textsuperscript{76b} and other researchers\textsuperscript{77–79} noticed an increase in the prevalence of male DNA of fetal origin within blood and skin lesions of women with systemic sclerosis. The relationship between microchimerism and systemic sclerosis has not been definitively elucidated, but possibilities include mutual recognition of minor and major histocompatibility antigens by maternal cells or engrafted fetal cells.\textsuperscript{80,81} Because placental deportation may be a mechanism of priming toward fetal antigen, the environmental context during pregnancy may determine future reactivity toward antigen on re-exposure in various states, including reactivity to microchimeric fetal cells, future pregnancy, and transplanted organs and cells.

In conclusion, we have demonstrated that paternally inherited fetal mHAgS are expressed in the fetal blood, the syncytiotrophoblast of the placenta, and trophoblast debris shed from the placenta. This finding provides support for the hypothesis that mHAg-specific T cells are generated during pregnancy as the result of both trophoblast deportation from the placenta into the maternal blood and fetal microchimerism. Importantly, these results refute the concept that the trophoblast is antigenically inert. Rather, the trophoblast is a source of fetal antigen that is likely to tolerize the maternal adaptive immune system in normal pregnancy, and may stimulate adverse responses by maternal immune cells in diseases of pregnancy and complications of transplantation.

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References


