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Chemokine Scavenger D6 Is Expressed by Trophoblasts and Aids the Survival of Mouse Embryos Transferred into Allogeneic Recipients

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Proinflammatory CC chemokines are thought to drive recruitment of maternal leukocytes into gestational tissues and regulate extra-villous trophoblast migration. The atypical chemokine receptor D6 binds many of these chemokines and is highly expressed by the human placenta. D6 is thought to act as a chemokine scavenger because, when ectopically expressed in cell lines in vitro, it efficiently internalizes proinflammatory CC chemokines and targets them for destruction in the absence of detectable chemokine-induced signaling. Moreover, D6 suppresses inflammation in many mouse tissues, and notably, D6-deficient fetuses in D6-deficient female mice show increased susceptibility to inflammation-driven resorption. In this paper, we report strong anti-D6 immunoreactivity, with specific intracellular distribution patterns, in trophoblast-derived cells in human placenta, decidua, and gestational membranes throughout pregnancy and in trophoblast disease states of hydatidiform mole and choriocarcinoma. We show, for the first time, that endogenous D6 in a human choriocarcinoma-derived cell line can mediate progressive chemokine scavenging and that the D6 ligand CCL2 can specifically associate with human syncytiotrophoblasts in term placenta in situ. Moreover, despite strong chemokine production by gestational tissues, levels of D6-binding chemokines in maternal plasma decrease during pregnancy, even in women with pre-eclampsia, a disease associated with increased maternal inflammation. In mice, D6 is not required for syngeneic or semiallogeneic fetal survival in unchallenged mice, but interestingly, it does suppress fetal resorption after embryo transfer into fully allogeneic recipients. These data support the view that trophoblast D6 scavenges maternal chemokines at the fetomaternal interface and that, in some circumstances, this can help to ensure fetal survival. The Journal of Immunology, 2010, 184: 3202–3212.

After embryo implantation in humans, the trophoblast cells of the chorioamniotic membrane completely surround the embryo in the form of proliferating cytotrophoblasts and a syncytial layer. During the first trimester, a subset of trophoblast cells, the extravillous trophoblasts, assumes a more invasive phenotype, moving through the outer syncytiotrophoblast to invade the decidua where they surround and invade the maternal spiral arteries. As a result, the trophoblast shell begins to break open, allowing maternal blood to enter the primitive intervillous space facilitating nutrient and gaseous exchange to the developing villous placenta and fetus. Further placental development dramati-
cally increases the surface area of the fetomaternal interface by the extensive folding and branching of trophoblast-covered fetal tissue, which remains bathed in maternal blood. The parts of the early chorion that do not develop into the placenta undergo atrophy to form the chorionic plate, which is sandwiched between the amniotic (which develops from the blastocyst inner cell mass) and the maternal derived decidua capsularis to form the gestational membranes that encase the developing fetus. Notably, at numerous distinct anatomical locations, trophoblast-derived cells directly contact uterine tissue or circulating maternal blood.

Implantation is associated with a robust maternal inflammatory response that is maintained throughout pregnancy and characterized by maternal leukocyte recruitment into the decidua (1, 2). This is thought to be a critical component of a successful pregnancy, but given the semiallogeneic or, during surrogacy, fully allogeneic nature of fetal tissues, mechanisms must also be in place to protect trophoblasts from maternal leukocyte attack. To achieve this balance, it is thought that maternal leukocytes and invading trophoblasts engage in complex reciprocal regulation (2), and a number of proteins have been characterized that may enable trophoblasts to control maternal decidua inflammation (3). This balance may be compromised in diseases of pregnancy, such as pre-eclampsia, which is frequently associated with overexuberant maternal inflammation (4). However, much is still unknown, partly because of marked differences in pregnancy and placentation between humans and experimental species, such as mice (5). In particular, trophoblasts invade much more deeply into the uterine wall in humans compared with other species, facilitating the diversion of large quantities of maternal blood to the placenta, bringing critical supplies of nutrients and oxygen. This puts greater immune pressure on...
fetomaternial interactions and has been proposed to underpin the susceptibility of humans to pre-eclampsia, in which an aberrant immune/inflammatory environment in the placental bed is thought to suppress trophoblast invasion, thereby compromising utero-placental blood flow.

Chemokines are small secreted proteins that, via heptahelical G protein-coupled receptors, direct the movement of leukocytes into, and within, tissues (6, 7). The chemokine family is divided into four subgroups (CC, CXC, CX3C, and XC) according to variations in a characteristic cysteine motif. The CC family contains a large subset of proinflammatory chemokines that are rapidly and transiently induced in response to damage or infection and that drive inflammation by regulating leukocyte recruitment through the chemokine receptors CCR1–5. Many proinflammatory chemokines are expressed in the decidua during pregnancy where they are believed to direct the recruitment of maternal leukocytes (8–13). Moreover, invasive trophoblasts can express chemokine receptors and have been reported to migrate toward chemokines in culture (14–17). To ensure a successful pregnancy, it would seem critical that the abundance and localization of proinflammatory chemokines is carefully controlled so that trophoblasts, leukocytes, and other responsive cells are appropriately recruited and positioned in gestational tissues.

We and others have proposed that specialized chemokine scavengers regulate chemokine bioavailability to control leukocyte trafficking (18–21). One such scavenger is D6, a heptahelial membrane molecule that is structurally related to CCR1–5 and that bind to at least 12 proinflammatory CC chemokines (CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, and CCL22) (19, 22–24). Unusually, when D6 is ectopically expressed in cell lines, it is unable to couple to the signal transduction pathways used by other chemokine receptors and cannot mediate cell migration. However, D6 can progressively remove large quantities of extracellular chemokines through its ability to undergo rapid, constitutive, ligand-independent trafficking to and from the cell surface (23, 25–29). Consistent with this scavenging function, D6-deficient mice often show exaggerated inflammatory responses associated with elevated abundance of bioavailable chemokines (30–35).

In humans, D6 protein is found on lymphatic endothelial cells and leukocytes (36, 37), but compared with other solid tissues, it is the placenta that shows by far the highest expression of D6 mRNA (22). Recent work has reported expression of D6 protein by trophoblasts in first trimester human placenta and found that D6, ectopically expressed in a trophoblast cell line, mediates chemokine scavenging (32). Moreover, D6-deficient mice carrying D6-deficient fetuses show increased fetal loss early in pregnancy in response to bacterial endotoxin or anti-phospholipid Abs from patients with anti-phospholipid syndrome (32, 38). In this report, we 1) provide a detailed immunohistochemical analysis of D6 expression in the human placenta, 2) reveal the function of endogenous D6 in a choriocarcinoma-derived cell line, 3) demonstrate that the D6 ligand CCL2 associates with syncytiotrophoblasts in situ, 4) quantify plasma chemokine levels during human pregnancy, 5) show that D6 mRNA is expressed in the mouse placenta, and 6) define the impact of placental D6 deficiency on fetal survival after embryo transfer in mice. These data indicate that trophoblasts use D6 to neutralize maternal chemokines at multiple locations in gestational tissue and, for the first time, reveal that fetal D6 can, in some contexts, help ensure a successful pregnancy outcome.

Materials and Methods

Immunodetection of D6 and cytokertatin-7 in tissue sections

Sections (5 μm) of paraffin-embedded tissue were prepared from an archived gestational series of placental samples obtained from patients after informed consent under the auspices of Glasgow Royal Infirmary ethical approval or from elective first trimester terminations (6–12 wk) under ethical approval from the Cambridge Local Research Ethics Committee. Sections were deparaffinized in xylene (Fisher Scientific, Loughborough, U.K.), rehydrated through decreasing concentrations of ethanol, and incubated for 30 min in 0.5% H2O2, and Ags were unmasked by boiling for ~10 min in a microwave or pressure cooker in 1 mM EDTA (pH 8) (for D6 [36]) or 10 mM citrate buffer (pH 6) (for cytokertatin-7 or for D6 [when using anti-D6 Abs from Sigma-Aldrich, St. Louis, MO]). After being blocked with serum, sections were incubated at 4˚C overnight or room temperature for 1 h in anti-human cytokertatin-7 (Dako UK, Ely, U.K. or Sigma-Aldrich), anti-human D6 Abs (Ref. 36 or Sigma-Aldrich), or isotype control Ab. After being washed, sections were incubated in an appropriate secondary Ab (Vector Laboratories, Burlingame, CA) at room temperature for 30 min. Bound Ab was detected using an avidin–biotin peroxidase detection system (Vector Laboratories, Burlingame, CA), according to the manufacturer’s instructions, and localized using diaminobenzidine or NovaRED substrate (Vector Laboratories). Sections were washed in water, counterstained with hematoxylin (Surgipath Europe, Peterborough, U.K.), dehydrated through increased concentrations of ethanol, cleared in xylene, and mounted in DPX mountant (VWR International, Lutterworth, U.K.). Images were captured using an Axiosview light microscope (Carl Zeiss, Welwyn Garden City, U.K.) and assembled using ThumbsPlus Software (Cerious Software, Charlotte, NC). All of the images show immunostaining that was present in sections stained with anti-D6 or anti-cytokertatin-7 Abs but absent from those exposed to appropriate isotype control Abs.

BeWo cell culture and molecular analysis

The human choriocarcinoma cell line BeWo (European Collection of Cell Cultures, Porton Down, Salisbury, U.K.) was grown in BeWo growth medium. DMEM/F12 medium (Gibco, Paisley, U.K.) supplemented with 10% FCS, 5 mM glutamine, and penicillin/streptomycin in 5% CO2 at 37˚C. HEK293 cells were grown under the same conditions in Dulbecco’s minimal essential medium with the same supplements. All of the tissue culture supplies were from Invitrogen (Paisley, U.K.). Detection of D6 by immunofluorescence, flow cytometry, and Western blot analysis was performed as described previously (25, 27, 39). The D6-GFP expression vector (25) was transfected into BeWo cells using Lipofectamine (Invitrogen), according to the manufacturer’s instructions, and stable transfecants were selected in 0.4 mg/ml G418. Chemokines were purchased from PeproTech (Rocky Hill, NJ) or Almac Sciences [bionitlated CCL3 (bioCCL3; Gladsmuir, U.K.) (25, 27)] or came from existing laboratory stocks [PM2 variant of mouse CCL3 (40)]. Radiolabeled (125I) mouse CCL3 (PM2 variant) was prepared as described (22, 41). BeWo cells were incubated in 5 or 10 μM [125I]CCL3 at 4 or 37˚C in BeWo growth medium (plus 20 μM HEPEPS [pH 7.4]) in the presence or absence of 100 nM competitor chemokine for up to 1 h. In some experiments, cells were loaded at 4˚C for 1 h in [125I]CCL3, shifted to 37˚C for 15 min, washed, and reincubated at 37˚C for up to 2 h. Medium was harvested and subjected to TCA precipitation as described previously (25). Radioactivity in the TCA precipitable and TCA nonprecipitable fractions and in cell pellets was determined using a Beckman Coulter (Fullerton, CA) γ 5500B counter. In each experiment, triplicate datasets were generated, and mean ± SD was calculated.

Detection of bioCCL3

To visualize internalized bioCCL3, BeWo cells were plated on glass eight-well slides, incubated with 10 nM bioCCL3 for 1 h (with or without a 10-fold molar excess of unlabeled competitor), washed, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% in C3-streptavidin (Pavida-Slirch), then washed, fixed, and mounted. Confocal images were captured using a Leica Microsystems (Deerfield, IL) SP-2 confocal microscope with 40× or 63× oil immersion objective lenses and digital zoom, with fluorochromes excited with lasers at 488 nm (GFP) or 543 nm (Cy3). To assess scavenging, 5 × 105 BeWo cells were incubated at 37˚C in 1 ml medium containing various concentrations of bioCCL3, samples of medium were removed at various times after biocellulose addition, and the remaining bioCCL3 was measured by Western blot analysis as described elsewhere (27), detecting with HRP-coupled streptavidin (Pavida-Slirch).

Small interfering RNA

Small interfering (siRNA) oligonucleotides from Qiagen (Valencia, CA) directed against the D6 coding sequence (SI1, AAGGCTGCCTCTCTGTGCAATG; SI2, AAGATGTTGAGCACCCTTAT) and the 3’ untranslated region of the mature mRNA (SI3, AAGCCCTTGGCTCAAGCAATT) were used previously to knock down human D6 (42). Scrambled oligonucleotides were also prepared. Oligonucleotides were transfected as a mixture at a concentration of 100 pM into subconfluent BeWo cultures
using lipofectamine (Invitrogen). Transfection was optimized using a fluorescein-coupled version of SI2, and conditions were selected that provided the highest transfection efficiency (∼90%) (data not shown). D6 protein knockdown was assessed by Western blot analysis as above. Mock transfections, to which siRNAs were not added, were also carried out as controls. In some experiments, 5 nM bioCCL3 was added to 1 ml medium bathing 5 × 10^5 transfected BeWo cells, and its depletion from the medium was assessed by Western blot analysis.

**Chemokine/trophoblast interactions in situ**

In situ interactions of chemokines with placental cells were studied using fragments of normal term human placenta (n = 3) within 3 h of delivery and following informed patient consent. The assay was performed essentially as described elsewhere (36, 43). Briefly, ∼1 cm^3, macroscopically intact, pieces of placenta were dissected and diced with a sharp scalpel into ∼1 mm^3 cubes. These were incubated for 30 min at room temperature with 1–10 μg D-1CCL8 or D-1CCL2 (200 μg/mg NEN Du Pont, Boston, MA), washed three times, fixed in 4% paraformaldehyde, and pro-cessed for histological examination. Five-micrometer-thick sections were cut, covered with K.2 autoradiographic emulsion (Ilford Imaging, Mobberley, U.K.), incubated for 2 wk in sealed black boxes, developed using D-19 developer (Kodak-Pathe, Paris, France), fixed (Unifix; Kodak-Pathe), washed three times, fixed in 4% paraformaldehyde, and pro-cessed for histological examination. Five-micrometer-thick sections were covered with K.2 autoradiographic emulsion (Ilford Imaging, Mobberley, U.K.), incubated for 2 wk in sealed black boxes, developed using D-19 developer (Kodak-Pathe, Paris, France), fixed (Unifix; Kodak-Pathe), contrasted with hemalaun, and examined using light microscopy.

**Detection of chemokines in human plasma**

All of the patient samples were collected from women attending the Princess Royal Maternity Unit at Glasgow Royal Infirmary for antenatal care. Healthy nonpregnant female volunteers were recruited through the University of Glasgow and the Glasgow Royal Infirmary. Ethical approval was received from the North of England Hospital Universities National Health Service Trust Research Ethics Committee, and all of the women gave informed consent. Blood samples from pregnant women were collected in the first trimester of pregnancy and 4 mo postpartum. For longitudinal comparison of plasma chemokines, pre-eclampsia and control pregnancy samples were collected for the Glasgow Outcome, APCR and Lipid Pregnancy Study (44). Plasma samples were available from both first and third trimester in women who subsequently developed pre-eclampsia (n = 34) and from age- and parity-matched controls (n = 34) who suffered no adverse pregnancy outcome. Pre-eclampsia was defined according to criteria defined by the International Society for the Study of Hypertension in Pregnancy. Blood was collected using citrate as an anticoagulant, and the plasma was harvested by low-speed centrifugation. Chemokine concentrations were determined using Luminex technology with Human Ab Bead Kits for CCL2, CCL3, CCL4, and CCL11 from Invitrogen on a Bio-PLEX array reader (Bio-Rad, Hercules, CA), according to the manu-facturer’s instructions. CXC10.10 was measured by ELISA (R&D Systems, Minneapolis, MN).

**Mouse studies**

All of the animal work was performed in accordance with U.K. Home Office regulations under the auspices of appropriate project and personal licenses after University of Glasgow ethical review and approval. For Northern blot analyses, wild-type (WT) C57Bl6/129 female mice were mated with WT C57Bl6/129 males. The appearance of a vaginal plug upon inspection each morning was considered 0.5 d postconception (dpc), and pregnant females were euthanized 10–19 d later. Embryos were separated from their placentas, both were homogenized, and RNA was isolated using TRIzol (Invitrogen), according to the manufacturer’s instructions. Due to their small size, placentas and embryos harvested at 10.5 dpc were not separated but were homogenized together, and RNA was isolated. RNA was also pro-cessed from 10-wk-old female WT C57Bl6/129 lung and liver as controls. Heat-denatured RNA (10 μg) was electrophoresed, blotted, and hybridized to [32P]-labeled mouse D6 cDNA as described previously (24).

For embryo transfer experiments, 0.5 dpc embryos were harvested from superovulated female D6 heterozygote mice (D6+/−) that had been fertili-zed by male D6−/− mice, both parents being on a C57Bl6/129 background. Embryos (10–20 per recipient) were transferred into the oviducts of pseudo-pregnant C57Bl6/129 or DBA/2 female mice (0.5 d postcoitum). These recipients were sacrificed 10–15 d later. Resorption plaques were counted; fetuses were dissected from their amniotic sacs, washed in PBS, briefly dried on filter paper, and weighed; and placentas were removed, weighed, fixed in formalin or snap-frozen in liquid nitrogen, processed for histology, and sectioned. Grossly malformed fetuses were counted, and any fetal weight >60% of the average fetal weight was scored as stunted. DNA was prepared from fragments of all of the embryos by lysing overnight at 55°C in 100 μl lysis buffer (100 mM Tris-HCl [pH 8.5], 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 100 μg/ml proteinase K), heating to 96°C for 5 min, adding 500 μl sterile deionized water, and centrifuging in a microcentrifuge at 13,000 rpm for 5 min. Genotyping was carried out on these DNA preparations using a triplex PCR reaction with the following primers: 5′−AGTTAACGGCTTCCGGGACAG−3′ (for WT allele only), 5′−ACCGGCTCACCCTTAATGCG−3′ (for targeted allele only), and 5′−CA-GAGCTGAGATGGTCCCAA−3′ (for both alleles, added at double the concentration of the other two primers). PCR conditions were 35 cycles of 94°C 30 s, 58°C 1 min, 72°C 1 min. Expected product sizes were 260 bp for WT allele and 550 bp for targeted allele. Products were electrophoresed on a 1% agarose gel containing ethidium bromide. Genotypes were confirmed by PCR using primers specific to the WT or the deleted D6 allele only. Placental sections (5 μm) were stained with H&E or immunostained with Abs against CD3 (T cells), myeloperoxidase (neutrophils), or CD68 (mac-rophages) or stained with periodic acid Schiff (decidual NK cells) and in-spected by light microscopy.

**Statistical analysis**

For human plasma chemokine data, normality testing was done using the Ryan-Joiner test, and data were transformed to log10 or square-root values to achieve a normal distribution where necessary. Differences among nonpregnant, pregnant, and postpartum women were tested using ANOVA and post hoc t tests. Differences between trimesters in pre-eclampsia and control pregnancies were tested using a paired t test. Differences between third trimester pre-eclampsia and healthy pregnancies were tested using an unpaired t test. All of the statistical analyses were performed using MiniTab (version 15.0, Coventry, U.K.). Student t tests, in GraphPad (San Diego, CA) Prism software, were used to analyze data from BeWo experiments, and the χ2 test was employed to analyze data from embryo transfer experiments.

**Results**

**Distribution of D6 protein in gestational tissues during pregnancy**

Anti-D6 immunoactivity has been reported in villous trophoblasts from first trimester human placenta (32). To explore this further, we immunostained sections of human placentas (first trimester and term) and gestational membranes (from term and preterm births) with a mAb that we had previously raised against human D6 (Fig. 1A–D) (36) [in situ hybridization and in situ chemokine binding has pre-viously confirmed that this Ab provides reliable immunohistochemi-cal detection of D6-expressing cells (36)]. In first trimester samples (n = 5), D6 immunoactivity was consistently observed on cyto-trophoblasts and the syncytiotrophoblast layer of chorionic villi (Fig. 1A). In term chorionic villi (n = 8), when the cytotrophoblast layer has completely converted into a syncytium, it alone was strongly stained (Fig. 1B). Notably, in all of the sections examined from first trimester and term placentas, D6 staining was concentrated toward the apical surface of chorionic villi trophoblasts (i.e., the side contacting ma-ternal blood). No consistent differences in intensity of anti-D6 immunoactivity were apparent between samples of different gestational ages. In all of the samples of gestational membranes ex-mined (from preterm [32–35 wk, n = 3] and term births [38–41 wk, n = 7]), D6 immunoactivity was absent from the amnion (Fig. 1C), but large numbers of cells in the chorion laeve were stained (Fig. 1D). At high magnification, this staining appeared to be primarily intracellular, clustered around the hematolin-stained nucleus (Fig. 1D, inset). Staining of adjacent sections with Ab against the trophoblast marker cytokeratin-7 confirmed that, as expected from their location, D6-expressing cells in the chorion laeve were trophoblast-derived (data not shown). We also noted occasional weak D6 immuno-staining of large cells, probably invading extravillous trophoblasts, in decidual tissue that was often attached to gestational membranes or placental samples (data not shown). To confirm these data, we stained sections of placenta of varying gestational ages with commercially available anti-human D6 Ab developed as part of the HUPO Human Antibody Initiative (www.proteinatlas.org; gene name, CCBP2). This gave very similar immunostaining patterns to those of our home-made Abs, including strong immunoactivity with large decidual cells present in term (n = 4) and first trimester (n = 3)
samples (Fig. 1E1, 1F1). These cells had the appearance of extravillous trophoblasts, and indeed, in adjacent sections, cells with a similar morphology stained with Abs against cytokeratin-7 (E2, F2) and visualized with diaminobenzidine (A, C–H) or NovaRED substrate (B) are shown. Hematoxylin-stained nuclei are blue. A, Chorionic villi, first trimester placenta (12 wk gestation). c, cytotrophoblast; s, syncytiotrophoblast. B, Chorionic villi, term placenta (39 wk gestation). C, Amnion, with amniotic epithelium (AE) and amniotic connective tissue (ACT) indicated. D, ACT, chorion laeve (indicated by the double-headed arrow), and decidual layers of gestational membranes. Inset, A higher magnification image of the boxed region indicated. E and F, Decidua in adjacent term (E) and first trimester (F) placental sections stained with Abs against D6 (E1, F1) or cytokeratin-7 (E2, F2). G, Hydatidiform mole. H, Choriocarcinoma. G2 and H2 show close-up images of the regions boxed in G1 and H1, respectively. Original magnification ×400 (A, B), ×200 (C–F), ×100 (G1, H1). Details of the number of sections assessed are contained in the Results.

BeWo cells express D6 and can degrade CCL3

Insight into the molecular function of D6 has come primarily from experiments in which it has been ectopically expressed to high levels in heterologous cell lines (23, 25–29, 32). To our knowledge, the function of endogenous D6 has not been explored. We therefore chose to explore the function of D6 in the human choriocarcinoma cell line BeWo, which is widely used to model trophoblast function. Consistent with previous work (32), RT-PCR (data not shown) and Western blot analysis (Fig. 2A) confirmed expression of D6 by this cell line and at a much lower level than that in D6-transfected HEK293 cells. When samples were electrophoresed a sufficient distance into the gel, D6 protein in BeWo cells was consistently detectable as a doublet on Western blots (as in Fig. 2A), likely corresponding to glycosylated and non-glycosylated forms of the protein that we have previously reported in transfected cells, including HEK293 (25, 27, 39). In D6-transfected HEK293 cells, >95% of D6 protein is found in vesicles inside cells (25, 27, 39). Similarly, using anti-D6 Ab, it was apparent from flow cytometry that D6 was barely detectable on the surfaces of BeWo cells (Fig. 2B) but was abundant in vesicles inside these cells, as revealed by immunofluorescent staining of
subconfluent BeWo cells (Fig. 2C). Likewise, a D6-GFP fusion protein (25) stably expressed in BeWo cells was predominantly localized to intracellular vesicles (Fig. 2D). Consistent with this subcellular distribution, BeWo cells showed only minimal compatable binding of radiolabeled CCL3 in binding assays performed at 4°C (Fig. 3A, 3B). Importantly, this could be blocked by the addition of unlabeled D6 ligands (CCL2, CCL3, CCL4, CCL5, and CCL22), but not by non-D6-binding CC and CXC chemokine ligands (CCL19, CCL20, CXCL8, and CXCL12) (Fig. 3A, 3B), strongly suggesting that CCL3 uptake was mediated by D6, the only known CCL3 receptor capable of also binding CCL2 and CCL22. In addition, consistent with data from D6 transfectants, BeWo cells did not migrate through porous filters toward a range of concentrations (0.1–100 nM) of CCL2 or CCL3 or produce detectable calcium ion fluxes in response to these chemokines (data not shown).

Next, we examined the fate of internalized [125I]CCL3 (Fig. 3C). BeWo cells were loaded with [125I]CCL3 at 4°C, briefly incubated at 37°C to drive internalization, washed, and then incubated at 37°C for up to 2 h. The amount of radioactivity in the cell pellet and the media was determined after subjecting the media to TCA precipitation to distinguish intact (TCA precipitable) from degraded (TCA non-precipitable) protein. As in D6-transfected HEK293 cells, [125I]CCL3 internalized into BeWo cells was degraded and released into the medium. Notably, minimal intact [125I]CCL3 was released from

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the cells. This was confirmed by SDS-PAGE, which failed to detect the release of any intact internalized \(^{[125I]}\)CCL3 from BeWo cells (data not shown). These data showed that, as in D6-transfected cells (23, 25–28, 32), D6 in BeWo cells is able to internalize and degrade CCL3. These assays rely on short-term uptake of chemokine, yet one key feature of D6 in transfected cells is its ability to continuously scavenge chemokines (25, 27). To investigate this in BeWo cells, we assessed the removal, over time, of bioCCL3 added at a high concentration to the medium bathing cultures of BeWo cells (Fig. 3D). After 4 h, there was a clear reduction in the amount of bioCCL3 in BeWo cultures, and by 24 h nearly all of the chemokine had been removed. Thus, despite low levels of surface D6, BeWo cells showed effective progressive scavenging of bioCCL3.

**D6 is required for chemokine scavenging by BeWo cells**

To further demonstrate that chemokine scavenging by BeWo cells was D6-mediated, we developed a siRNA approach. To optimize the assay, D6 protein levels were measured by Western blot analysis for up to 96 h after siRNA transfection (Fig. 4A). By comparison to anti-actin blots (data not shown), it was possible to use densitometry to quantify the extent of D6 knockdown, revealing that after 72 h D6 protein levels had been reduced by \(\sim 90\%\). No D6 knockdown was observed in mock-transfected cells or when scrambled siRNA sequences were introduced, and cell viability was not affected by transfection (data not shown). Next, 70 h after siRNA or mock transfection of BeWo cells, bioCCL3 was added to the medium. Four hours later, cells and medium were harvested and assayed for D6 expression and bioCCL3, respectively. As before, effective knockdown of D6 was achieved. Importantly, this correlated with a marked reduction in the amount of bioCCL3 removed from the medium (Fig. 4B). Collectively, these data reveal, for the first time, that endogenous D6 behaves in a manner similar to D6 introduced by transfection into HEK293 and other cell lines (23, 25–28, 32).

**FIGURE 4.** siRNA-mediated D6 knockdown confirms that scavenging of bioCCL3 by BeWo cells is D6-dependent. A, BeWo cells were transfected with D6 siRNA and lysed 48, 72, and 96 h later. Control wells were mock-transfected and lysed 72 h later. Cell lysates were electrophoresed, blotted, and probed with anti-D6 Abs detected with HRP-coupled antismouse IgG Abs. B, A total of \(5 \times 10^5\) BeWo cells growing in 1 ml medium were transfected with D6 siRNA or mock-transfected, and bioCCL3 was added to a concentration of 5 nM 70 h later. A further 4 h later, media was collected, and the cells were lysed. BioCCL3 in the medium was detected by Western blot analysis using HRP-coupled streptavidin (upper panel). D6 or actin in the cell lysates (lower panels) were detected using anti-D6 or anti-actin Abs, respectively (detected by HRP-coupled anti-mouse IgG Ab). Two repeat experiments generated similar results.

**FIGURE 5.** CCL2 associates with the syncytiotrophoblast layer in situ. Pieces of fresh term placentas were incubated in 1–10 ng radiolabeled chemokine (CCL2 or CXCL8) for 30 min, washed, fixed, and sectioned (5 \(\mu\)m). Deposited radioactivity was detected using autoradiographic emulsion, after which sections were developed, fixed, counterstained with hemalaun (blue), and examined by light microscopy. Images of placentas incubated with \(^{[125I]}\)CCL2 (A) or \(^{[125I]}\)CXCL8 (B) are shown and are representative of data obtained from three placentas. Original magnification \(\times 400\).

Syncytiotrophoblasts interact with \(^{[125I]}\)CCL2 but not \(^{[125I]}\)CXCL8

The data above support the idea (32) that D6 can scavenge chemokines at the fetomaternal interface. To provide further evidence of this, we attempted to study the chemokine scavenging capabilities of primary trophoblasts freshly isolated from term placentas. These cells were able to internalize small but detectable amounts of D6 ligand, but we noted a substantial loss of D6 expression during the isolation and culture of these cells that precluded their use as a reliable model to examine D6 function (data not shown). As an alternative approach to examine interactions between chemokines and primary placental cells, we used techniques developed in our previous work (36, 43) that allow in situ detection of chemokine receptor activity. In this approach, small fragments of tissue are incubated briefly (e.g., 30 min) with radiolabeled chemokines, washed extensively, fixed and sectioned, and exposed to autoradiographic emulsion for several weeks to localize cell-associated radioactivity. We incubated pieces of fresh term placentas with either \(^{[125I]}\)CCL2 (a D6 ligand) or \(^{[125I]}\)CXCL8, which cannot bind to D6. Consistent with the strong anti-D6 immunoreactivity of trophoblasts in situ, the syncytiotrophoblast layer lining the large majority of villi in placentas incubated with \(^{[125I]}\)CCL2 became intensely and specifically radiolabeled during the short incubation period (Fig. 5A). In contrast, when \(^{[125I]}\)CXCL8 was used, little, if any, radioactivity was found associated with these or other placental cells (Fig. 5B). Thus, like BeWo cells, primary syncytiotrophoblasts are capable of interacting with D6-binding chemokines.

**Reduced plasma levels of D6-binding chemokines during pregnancy**

Strong expression of proinflammatory chemokines by inflamed tissues or cancers typically leads to elevated circulating plasma chemokine levels. Many D6-binding chemokines are abundantly expressed throughout pregnancy by maternal decidua and the enlarging placenta (10, 11), and we have confirmed high expression of CCL2 and CCL3 by delivered term human placentas by quantitative PCR (data not shown). Maternal plasma chemokine levels might therefore be expected to be elevated in early pregnancy and increase toward term as the placenta enlarges. However, maternal blood continuously flows over D6-expressing chorionic villi, the surface area of which increases during pregnancy. To explore the outcome of these counteracting processes of production and scavenging, we assessed the levels of four inflammatory chemokines known to bind to D6 (CCL2, CCL3, CCL4, and CCL11) and one non-D6 ligand...
Table I. Plasma concentrations of proinflammatory chemokines, CRP, and proinflammatory cytokines in pregnant and nonpregnant women

<table>
<thead>
<tr>
<th></th>
<th>Nonpregnant (n = 33)</th>
<th>Trimester 1 (n = 19)</th>
<th>Postpartum (n = 15)</th>
<th>p ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation (wk)</td>
<td>—</td>
<td>12.4 (1.1)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Postpartum (wk)</td>
<td>—</td>
<td>—</td>
<td>18.3 (2.4)</td>
<td>—</td>
</tr>
<tr>
<td>CCL2 (pg/ml)*</td>
<td>319.2 (184.4)</td>
<td>184.7 (35.3)*</td>
<td>228.6 (98.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>CCL3 (pg/ml)*</td>
<td>126.8 (45.1)</td>
<td>94.2 (17.5)*</td>
<td>106.7 (38.8)</td>
<td>0.002</td>
</tr>
<tr>
<td>CCL4 (pg/ml)*</td>
<td>94.5 (94.8)</td>
<td>57.5 (21.7)</td>
<td>66.1 (47.4)</td>
<td>0.085</td>
</tr>
<tr>
<td>CCL11 (pg/ml)*</td>
<td>75.4 (30.9)</td>
<td>32.3 (9.0)**</td>
<td>61.0 (25.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CXCL10 (pg/ml)*</td>
<td>70.7 (28.2)</td>
<td>67.4 (20.6)</td>
<td>93.9 (68.5)</td>
<td>0.33</td>
</tr>
<tr>
<td>CRP (mg/ml)*</td>
<td>—</td>
<td>6.38 (6.02)**</td>
<td>1.58 (1.81)</td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>—</td>
<td>2.18 (4.39)</td>
<td>0.87 (0.35)</td>
<td></td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>—</td>
<td>1.56 (1.19)</td>
<td>1.57 (0.56)</td>
<td></td>
</tr>
</tbody>
</table>

*Statistical analyses carried out on log-transformed data, using ANOVA and post hoc t test.

(CXCL10) in plasma samples taken from pregnant women during the first trimester (n = 19) and from women ~4 mo postpartum (n = 15). Samples from an unrelated nonpregnant control group (n = 33) were also assessed. Notably, levels of CCL11 were significantly lower in first trimester samples compared with those of samples taken after birth, and CCL2, CCL3, and CCL11 were significantly less abundant in the plasma of pregnant women compared with that of nonpregnant controls (Table I). Conversely, but as anticipated, the general marker of inflammation, C-reactive protein (CRP), was significantly elevated in the first trimester samples compared with those taken postpartum, whereas CXCL10 and the inflammatory cytokines IL-6 and TNF-α were not significantly different.

To examine whether circulating chemokine levels were further reduced with increasing gestation and placental mass, we undertook an analysis of a second cohort of women (n = 34) from whom paired plasma samples had been taken in the first and third trimesters of their pregnancy. Samples were also analyzed from women who had developed pre-eclampsia during their pregnancies (n = 34). As before, plasma levels of CCL2, CCL3, CCL4, and CCL11 were low in first trimester samples from all of the women, but interestingly, they were further reduced in the third trimester (Table II). This was also true in the pre-eclamptic group, and despite a trend toward slightly higher levels of plasma chemokines in mothers with pre-eclampsia (compared with those of mothers with normal pregnancies), this failed to achieve statistical significance for any of the chemokines analyzed. In contrast, CXCL10 levels did not change from first to third trimester in control pregnancies but were significantly increased over this period in mothers with pre-eclampsia such that, consistent with a previous study (45), they were significantly greater than those of controls by the third trimester. Thus, despite robust expression of proinflammatory chemokines by gestational tissues, D6-binding chemokines are less abundant in maternal plasma during pregnancy.

D6 is expressed in the mouse placenta

Although placentas in mice and humans are anatomically different, the availability of D6-deficient mice enables an examination of the dispensability of D6 during pregnancy. First, however, we wished to confirm that D6 is expressed in the mouse placenta. Effective anti-D6 Ab is currently unavailable, so we chose to perform Northern blot analyses. To do this, RNA was isolated from WT C57Bl6/129 placentas and embryos harvested between 12.5 and 19.5 dpc and a 10.5 dpc placenta/fetus mix. In addition, because we had previously detected D6 expression in adult mouse lung and weaker expression in liver (24), RNA was also isolated from these tissues from a nonpregnant female WT C57Bl6/129 for use as controls. The Northern blot analyses clearly showed that D6 mRNA was present in the placenta from 12.5 dpc onwards and in the 10.5 dpc placenta/fetus mix but was undetectable in the fetuses between 12.5 and 19.5 dpc (Fig. 6). Notably, in contrast to humans (22), expression of D6 in the mouse placenta was much lower than that in adult lung and at a level equivalent to that of adult liver. As expected from the trophoblast-specific expression of D6 seen in humans, placental D6 expression in mice came from fetal-derived tissues because placentas feeding D6-deficient embryos in D6−/− females showed no detectable expression of D6 by Northern blot analysis (data not shown).

Fetal D6 aids fetal survival after embryo transfer in mice

From standard breeding programs, it was clear that D6 was not required for the successful production of syngeneic offspring because, on two inbred genetic backgrounds (C57Bl6 and FVB/N) and on the mixed C57Bl6/129 background, crosses between D6+/− mothers and D6−/− fathers yielded normal, healthy offspring. D6+/− mothers, on the other hand, consistently produced D6−/− offspring. These results were consistent with a requirement for D6 in placental trophoblasts for fetal survival after embryo transfer. First, however, we wished to confirm that D6 is expressed in the mouse placenta. Effective anti-D6 Ab is currently unavailable, so we chose to perform Northern blot analyses. To do this, RNA was isolated from WT C57Bl6/129 placentas and embryos harvested between 12.5 and 19.5 dpc and a 10.5 dpc placenta/fetus mix. In addition, because we had previously detected D6 expression in adult mouse lung and weaker expression in liver (24), RNA was also isolated from these tissues from a nonpregnant female WT C57Bl6/129 for use as controls. The Northern blot analyses clearly showed that D6 mRNA was present in the placenta from 12.5 dpc onwards and in the 10.5 dpc placenta/fetus mix but was undetectable in the fetus between 12.5 and 19.5 dpc (Fig. 6). Notably, in contrast to humans (22), expression of D6 in the mouse placenta was much lower than that in adult lung and at a level equivalent to that of adult liver. As expected from the trophoblast-specific expression of D6 seen in humans, placental D6 expression in mice came from fetal-derived tissues because placentas feeding D6-deficient embryos in D6−/− females showed no detectable expression of D6 by Northern blot analysis (data not shown).

Table II. Plasma concentrations of proinflammatory chemokines from first (T1) and third (T3) trimester in women with normal or pre-eclampsia–associated pregnancies

<table>
<thead>
<tr>
<th>Control (n = 34)</th>
<th>T1 versus T3</th>
<th>Pre-eclampsia (n = 34)</th>
<th>T1 versus T3</th>
<th>Pre-eclampsia (T3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age</td>
<td>10.5 (2.4)</td>
<td>31 (3.3)</td>
<td>10.7 (2.2)</td>
<td>29.5 (2.4)</td>
</tr>
<tr>
<td>CCL2 (pg/ml)*</td>
<td>143 (47)</td>
<td>105 (55)</td>
<td>148 (80)</td>
<td>115 (48)</td>
</tr>
<tr>
<td>CCL3 (pg/ml)*</td>
<td>153 (74)</td>
<td>87 (72)</td>
<td>145 (118)</td>
<td>100 (104)</td>
</tr>
<tr>
<td>CCL4 (pg/ml)*</td>
<td>22 (25)</td>
<td>17 (23)</td>
<td>30 (47)</td>
<td>26 (48)</td>
</tr>
<tr>
<td>CCL11 (pg/ml)*</td>
<td>31 (13)</td>
<td>14 (11)</td>
<td>26 (15)</td>
<td>16 (11)</td>
</tr>
<tr>
<td>CXCL10 (pg/ml)*</td>
<td>70 (42)</td>
<td>75 (27)</td>
<td>77 (53)</td>
<td>105 (39)</td>
</tr>
</tbody>
</table>

*Statistical analyses carried out on log-transformed data, using ANOVA and post hoc t tests.

**Statistical analyses carried out on square-root values, using paired t tests for tests of differences between control and pre-eclampsia third trimester.
mice consistently produced offspring with the expected Mendelian frequency of 1:2:1 (WT; D6+/-; D6-deficient). Moreover, crosses between D6+/- mice of different genetic backgrounds failed to reveal any indispensable role for D6 in the survival of semi-allogeneic pups. In addition, consistent with previous work (32), D6-deficient pairs on these backgrounds produced litters with similar frequency and size as WT pairs, and neonatal weight and health were not detectably compromised (data not shown).

D6-deficient fetuses in D6-deficient females are more susceptible than WT fetuses in WT females to resorption induced by LPS or anti-phospholipid Abs (32), suggesting that loss of D6 may be particularly significant at times of inflammatory or immunological stress. Notably, however, although it is likely that loss of placental D6 contributed to this phenotype, this was not formally proven, and because D6-deficient females show exaggerated inflammatory responses in a variety of models (30, 34, 35), it is possible that the absence of maternal D6 contributed to the increased resorption rate. To specifically explore placental D6 function, we chose to examine the impact of D6 deficiency on fetal survival after embryo transfer so that maternal and fetal genotype could be independently controlled. Moreover, by using donor embryos from D6+/- × D6+/- crosses, we could explore competition between fetuses of different genotypes and, by transferring into both syngeneic and allogeneic pseudo-pregnant females, also examine whether genetic background incompatibility between mother and fetus influences the significance of D6 deficiency. Thus, superovulated D6+/- female mice were mated with D6+/- males (both on the C57Bl6/129 background), and the resultant embryos were harvested at 0.5 dpc and transferred into pseudo-pregnant WT female mice that were either syngeneic (C57Bl6/129 [H2h]) or allogeneic (DBA/2 [H2d]) to the donors. Recipients were culled 15 d later, resorption plaques were counted, and fetuses and placentas were removed and examined (Fig. 7). Notably, resorption plaques were much more abundant in the DBA/2 recipients, and in contrast to fetuses retrieved from C57Bl6/129 recipients, many fetuses in the DBA/2 mice were grossly abnormal, appearing to be markedly stunted relative to their siblings or in the process of resorption (Fig. 7A). Healthy fetuses were genotyped by PCR (Fig. 7B), and their genotype ratio from syngeneic recipients did not differ significantly from the expected ratio of 1:2:1 ($\chi^2$ test, $p = 0.71$). However, among those retrieved from DBA/2 recipients, there was a marked reduction in the proportion of healthy fetuses that were D6-deficient, and the genotype ratio was significantly different from the expected 1:2:1 ratio ($\chi^2$ test, $p = 0.0009$) and from that observed in C57Bl6/129 recipients ($\chi^2$ test, $p < 0.0001$). Abnormal fetuses in DBA/2 recipients were also genotyped (Fig. 7C). Half were found to be D6-deficient, and the genotype ratio differed significantly from the expected 1:2:1 ratio ($\chi^2$ test, $p = 0.033$).

Next, we examined placentas supporting fetuses in DBA/2 recipients. D6 deficiency had no discernible impact on the weight or size of placentas supporting healthy embryos or on the gross structure of the placenta, as assessed by examining sections stained with H&E under light microscopy (data not shown). Moreover, there was no detectable impact of D6 deficiency on the abundance of T cells, macrophages, neutrophils, or decidual NK cells in these placentas (data not shown). In contrast, placentas supporting abnormal embryos (WT and D6-deficient) usually contained an obvious inflammatory cell infiltrate, consisting mainly of macrophages and T cells, and the placental structure was often disrupted. However, these parameters were highly variable between samples, and there were no consistent differences between placentas feeding WT or D6-deficient embryos (data not shown). Similar observations were also made when placentas were harvested 11 or 13 d after embryo transfer (data not shown).

Collectively, these data show that, although D6 is dispensable for the successful carriage and delivery of syngeneic and semi-allogeneic embryos in mice, it can, in the context of a fully allogeneic recipient, reduce the likelihood of fetal resorption.

Discussion

In this report, we have shown that 1) D6 is expressed by trophoblast-derived cells in the chorion laeve and chorionic villi of early and term normal human placentas and in hydatidiform mole and...
choriocarcinoma; 2) endogenous D6 in the human choriocarcinoma cell line BeWo scavenges extracellular proinflammatory chemokines; and 3) despite robust chemokine expression by decidua and placenta throughout pregnancy (10, 11), circulating plasma levels of D6-binding chemokines are lower in pregnant women than nonpregnant controls. D6 is also expressed in the mouse placenta, and although it provides no selective advantage to developing fetuses in unchallenged pregnant mice, it does aid fetal survival after embryo transfer into allogeneic, but not syngeneic, recipients. These data, along with previous work (32), support the concept that D6 on trophoblasts limits the availability of maternal chemokines and that this scavenging activity, in some contexts, is critical for successful pregnancy.

From our studies of BeWo cells, it is clear that endogenous D6 behaves like D6 ectopically expressed in cell lines. This is reassuring, because until now transfected cell lines with unnaturally high levels of D6 expression have been used to provide mechanistic insight into D6 function (23, 25–28, 32). As in transfecteds, D6 protein is found predominantly inside BeWo cells with only a small fraction available on the cell surface at any one time, yet it can progressively remove extracellular chemokines, without apparent desensitization. Presumably, like D6 in transfected cells, it achieves this by continuously trafficking to and from the cell surface, and indeed, like cells expressing ectopic D6 (25), BeWo cells can internalize surface-bound anti-D6 Abs in the absence of chemokines (data not shown). Notably, the distribution of D6 protein in BeWo cells growing in sparse culture appears to be recapitulated in situ by cells in the chorion. Moreover, in BeWo cells cultured to form polarized monolayers, D6-containing vesicles cluster near the apical surface (32). This has obvious parallels with the polarized distribution of D6 seen in cytotrophoblasts and syncytiotrophoblasts in first trimester and term placentas and in organized trophoblast layers in choriocarcinoma and hydatidiform mole. This striking distribution strongly suggests that villous D6 is directed against maternal blood chemokines, and indeed, when term placentas are incubated in solutions containing CCL2, this D6 ligand readily associates with the syncytiotrophoblast layer (Fig. 5).

Although immunohistochemistry does not provide sufficient resolution to precisely determine subcellular location, we anticipate that a significant proportion of D6 in polarized trophoblasts in situ will be found in vesicles underneath the apical cell surface from where it traffics to and from the cell surface. Immuno-electron microscopy studies of term placenta are underway to explore this in more detail. We also detect D6 expression in decidual extravillous trophoblasts, particularly when using the commercial anti-D6 Abs. This is consistent with the reported expression of D6 by anchoring and invading trophoblasts (32) and immunostaining published online (www.proteinatlas.org; gene name, CCBP2). Notably, D6 immunoreactivity is more uniformly distributed in these trophoblasts compared with those lining villi or within the chorion laeve, although the functional implications of this difference in subcellular distribution remain to be defined.

On all these cells, our data and previous work (32) point to a role for D6 in scavenging inflammatory CC chemokines. D6-binding chemokines are produced by the decidua and are likely responsible for recruiting and positioning maternal leukocytes (1). Indeed, decidual NK cells, which make up a large proportion of placental leukocytes and are known to engage in complex regulatory interactions with trophoblasts, respond to several proinflammatory chemokines that can be scavenged by D6, including CCL3 (9). Decidual chemokines may also control the localization of invasive cytotrophoblasts, which can migrate in vitro toward proinflammatory CC chemokines, including CCL3 (15). Bearing in mind this dual regulation of leukocytes and trophoblasts, D6-mediated chemokine scavenging during human pregnancy could have different outcomes depending on where scavenging occurs.

First, by scavenging chemokines in the uterine wall, D6 on invasive extravillous trophoblasts may control trophoblasts themselves by 1) directly modulating their responsiveness to chemokine-induced migration, and/or 2) serving as a feedback mechanism that regulates chemokine abundance to thereby limit the number of trophoblasts recruited into the uterine wall. Second, D6-mediated chemokine scavenging in the chorion laeve and decidua may regulate the number, position, or both of maternal leukocytes recruited into these tissues or, by keeping trophoblast surfaces free of proinflammatory chemokines, help to prevent these leukocytes from forming close physical interactions with trophoblasts. In this way, D6 may reduce the likelihood of maternal leukocytes sensing and responding to fetal non-self. Similarly, D6 on syncytiotrophoblasts may keep chorionic villi surfaces free of inflammatory chemokines to limit the arrest of maternal leukocytes passing through the intervillous space and prevent damage (46) to this critical interface between mother and baby.

Alongside these local consequences of D6-mediated scavenging, our data suggest that fetal D6 also has a systemic impact on the mother during pregnancy. Chorionic villi are continuously bathed in maternal blood and have an estimated surface area of ~10 m² in a term placenta (47). Their syncytiotrophoblast coating is therefore by far the most abundant source of D6 in human placentas, particularly late in pregnancy. CCL2 readily associates with these cells in situ, and it seems reasonable to assume that this makes a major contribution to reducing the levels of D6-binding chemokines in the plasma of pregnant women as it passes through the intervillous space. This reduction occurs in the face of robust chemokine expression by the placental/decidual unit (10, 11), and it is striking that in other situations when proinflammatory chemokines are persistently produced (e.g., in patients with chronic inflammatory disease or cancer) marked increases in plasma chemokine abundance are often observed and can be used to predict disease severity. It is also notable that CXCL10, which cannot bind to D6, is not significantly reduced during pregnancy. Hypothetically, because blood-borne chemokines can desensitize chemokine receptors on circulating leukocytes to suppress their extravasation, D6-mediated scavenging of plasma chemokines may help to ensure that circulating maternal leukocytes remain responsive to these chemokines produced by the decidua. Importantly, unlike pathological inflammatory responses, uterine inflammation during pregnancy is a physiological response that must be maintained for many months. Perhaps a placental sink for circulating chemokines helps to prevent chemokine receptor desensitization on maternal leukocytes to maintain efficient leukocyte recruitment to the decidua and other gestational tissues.

Pre-eclampsia is characterized by increased systemic maternal inflammation and elevated circulating levels of many inflammatory proteins (4). Consistent with this and in line with a previous report (45), CXCL10 is elevated in women with pre-eclampsia. However, these women still show reductions in circulating D6-binding chemokines during pregnancy, suggesting that the mechanisms responsible for this reduction, including D6-mediated scavenging, are maintained in pre-eclampsia. As in control placentas, immunohistochemistry has revealed that D6 is found exclusively on trophoblast-derived cells in pre-eclamptic placentas (n = 6; data not shown). However, we have found a small, but statistically significant, increase in the expression of the D6 gene (relative to 18S or cytokeratin-7) by quantitative PCR in term pre-eclamptic placentas compared with that in controls (1.8-fold, p = 0.024; control, n = 23; pre-eclampsia, n = 15) (data not shown). It is
tempting to speculate that this is a response to the increased systemic inflammation associated with this disease and that it enhances the chemokine scavenging capabilities of pre-eclamptic placentas to keep circulating chemokine levels low.

On the basis of the discussion above, we propose that, through the combined regulation of local and circulating chemokines, D6 makes an important contribution to human pregnancy. Testing this hypothesis in vivo is difficult in humans. Mouse pregnancy, despite its limitations, has provided unique insights into placental function, and notably the labyrinthine mouse placenta is similar in structure to the early stages of human placental development. The D6 gene is expressed in the mouse placenta but at levels that appear much lower than those in humans. Human placental D6 expression is at least an order of magnitude greater than that in the liver (the highest expressing tissue in nonpregnant humans) (22), but in mice, the liver and placenta show equivalent levels of expression, which are much lower than those in lung (the most abundant tissue source of D6 mRNA in this species). We are unable to explain why there is this clear interspecies difference in placental D6 expression, but perhaps anatomical differences, the extent of trophoblast invasion, or the multiparity of mouse pregnancy (which would result in a high overall D6 load in the pregnant uterus) reduce the need for high placental D6 expression in mice. Placental D6 comes from fetal tissues in mice, and it seems reasonable to assume that trophoblasts are the source, but we should stress that this has not yet been formally proven. Indeed, its presence on lymphatic endothelial cells in mice [which has been assumed based on data from human studies (36) and used to interpret phenotypes in D6-deficient mice] has, to our knowledge, not yet been formally demonstrated. This has been hampered by the lack of an effective anti-mouse D6 Ab, and despite trying numerous commercial products, we are still unable to detect D6 protein in mouse tissues that express abundant D6 mRNA.

Previous work has shown that mouse D6 is dispensable for the successful development and delivery of naturally conceived syngeneic pups (32). From our breeding programs (using parents heterozygous or homozygous for the deleted D6 allele on various backgrounds), we have confirmed that fetal D6 deficiency gives no survival advantage to syngeneic or semiallogeneic fetuses. However, we were unable to find any difference in the size or inflammatory infiltrate of placentas supporting healthy WT versus D6-deficient fetuses harvested 11–15 d after transfer, and the histological appearance and inflammatory infiltrate of placentas associated with abnormal fetuses was so variable (probably due to variation in the extent of fetal resorption) that detecting any influence of genotype was not possible. Clearly, more detailed studies are required before the underpinning cellular processes are defined.

In summary, our data reveal widespread expression of D6 by trophoblasts in human placenta and gestational membranes and demonstrate that endogenous D6 in trophoblast-derived cells can mediate proinflammatory chemokine scavenging. We propose that D6 is responsible for reducing the abundance of chemokines in the circulating plasma of pregnant women and for controlling chemokine distribution and bioavailability in the decidua and gestational membranes and at the surface of chorionic villi. We envisage that collectively these activities fine-tune chemokine-driven leukocyte and trophoblast responses. Our data, alongside previous work (32), emphasize the significance of chemokine networks in fetal health and encourage further investigations of the role of these networks in pre-eclampsia, recurrent spontaneous abortion, and other pregnancy-associated pathologies.

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Disclosures

The authors have no financial conflicts of interest.

References
