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Atypical Chemokine Receptor ACKR2 Mediates Chemokine Scavenging by Primary Human Trophoblasts and Can Regulate Fetal Growth, Placental Structure, and Neonatal Mortality in Mice

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Inflammatory chemokines produced in the placenta can direct the migration of placental leukocytes using chemokine receptors that decorate the surface of these cells. Fetal trophoblasts can also express receptors for inflammatory chemokines, and they are one of the few cell types that express atypical chemokine receptor 2 (ACKR2), previously known as D6. ACKR2 binds many inflammatory CC chemokines but cannot stimulate cell migration or activate signaling pathways used by conventional chemokine receptors. Existing evidence suggests that ACKR2 is a specialized chemokine scavenger, but its function in primary human trophoblasts has not been explored. In mice, ACKR2 is thought to be dispensable for the reproductive success of unchallenged females that have conceived naturally, but it can suppress inflammation-induced abortion and aid the survival of implanted allogeneic embryos. In this article, we demonstrate that cultured primary human trophoblasts express ACKR2 far more strongly than genes encoding conventional receptors for inflammatory CC chemokines. Moreover, these cells are capable of the rapid internalization and efficient scavenging of extracellular chemokine, and this is mediated by ACKR2. We also report that in unchallenged DBA/1j mice, Ackr2 deficiency increases the incidence of stillbirth and neonatal death, leads to structural defects in the placenta, and can decrease fetal weight. Loss of Ackr2 specifically from fetal cells makes a key contribution to the placental defects. Thus, primary human trophoblasts use ACKR2 to scavenge chemokines, and ACKR2 deficiency can cause abnormal placental structure and reduced neonatal survival. The Journal of Immunology, 2014, 193: 5218–5228.
other cells. Numerous chemokines are expressed by maternal and fetal cells at specific anatomical locations in the human placenta (15–17), and placental leukocytes carry chemokine receptors and respond to chemokines (17–24). Cytotrophoblasts are also reported to express functional chemokine receptors, including CCR1 and CCR2 (16). It has been proposed therefore that chemokines attract immune cells into the placenta and contribute to the invasion and positioning of trophoblast cells (25).

Chemokines also bind atypical chemokine receptors (ACKRs). ACKRs structurally resemble conventional chemokine receptors but cannot couple to signal transduction pathways used by these receptors or directly stimulate cell migration. However, they are key components of chemokine networks in vivo, using a variety of mechanisms to regulate chemokine and chemokine receptor activity. For example, ACKRs can act as chemokine scavengers, internalizing chemokines to control their bioavailability, abundance, and distribution (26). There are four ACKRs: DARC, D6, CXCR7, and CCX-CKR. They have recently been renamed ACKR1 to ACKR4, respectively, to reflect their common functions and distinguish them from conventional chemokine receptors (27). ACKR2 is highly promiscuous and binds inflammatory CC chemokines that signal through conventional chemokine receptors CCR1 to CCR5 (26). Adult human tissues express low levels of ACKR2 on lymphatic endothelial cells (28) and some leukocytes (29, 30), but the placenta is the richest source of ACKR2 because of its expression by trophoblasts (31–33). Exogenous human ACKR2 in transfected immortalized cell lines cannot couple to signal transduction pathways used by conventional chemokine receptors, or stimulate cell migration, but it can efficiently internalize its chemokine ligands and target them for degradation (32, 34, 35). Consequently, human ACKR2 is thought to function as a chemokine scavenger. ACKR2-mediated scavenging has not been examined in primary human trophoblasts, but it could contribute to pregnancy-associated decreases in circulating levels of CC chemokines (33), and may regulate interstitial chemokines to control leukocyte positioning and trophoblast behavior. In mice, ACKR2 can suppress inflammation in some nonreproductive tissues (26, 36), and expression of Ackr2 by fetal cells in the mouse placenta enhances survival of embryos transferred into allogeneic wild-type (WT) recipients (33). Also, inbred Ackr2-deficient females that carry syngeneic Ackr2-deficient fetuses show increased susceptibility to abortion induced by LPS or anti-phospholipid Abs (32). However, no reproductive or placental abnormalities have been reported in unchallenged inbred Ackr2-deficient mice that carry naturally conceived embryos.

In this study, by studying primary human trophoblasts, we extend current understanding of ACKR2 function at the fetomaternal interface. We show that ACKR2 is the dominant inflammatory chemokine receptor gene expressed by these cells, and reveal that they use ACKR2 to mediate efficient internalization and progressive scavenging of chemokine. We also report unexpected reproductive and placental defects arising from Ackr2 deficiency in inbred DBA/1j mice that demonstrate that ACKR2 can serve indispensable roles during normal reproductive processes in unchallenged animals.

Materials and Methods

Isolation of primary human trophoblast cells

The study was approved by the West of Scotland Research Ethics Committee (reference no. 10/S1001/14). After receiving informed consent, term placentas were obtained from healthy women having elective caesarean sections at the Princess Royal Maternity Unit (Glasgow Royal Infirmary) or the Southern General Hospital, Glasgow, U.K. Trophoblast cells were isolated from placentas as previously described (37, 38). In brief, the umbilical cord and the edges of the placenta were removed, and the remaining tissue was cut into 5-cm³ cubes and washed twice in sterile PBS. Fragments containing large blood vessels, fibrous areas, and clotted blood were discarded. Remaining material was minced and incubated for 30 min on a shaker at 100 rpm at 37 °C in 150 ml Digestion Solution (HBSS, 0.25% Trypsin, 500 Kunitz Unit/ml DNase). Supernatant was collected and added to a one-fifth volume of heat-inactivated newborn calf serum (Life Technologies). Cells were collected by centrifugation at 500 × g for 10 min, and the cell pellet was resuspended in DMEM (Life Technologies). The cells were layered onto a discontinuous Percoll gradient (Sigma) with a range of densities (1.005 and 1.10 g/ml). Cells with densities between 1.05 and 1.06 g/ml were harvested for culture. Cells were cultured in Medium 199 (Life Technologies) supplemented with 10% newborn calf serum and 1% antibiotic/antimycotic solution (Life Technologies). Cells were maintained at 37°C/5% CO₂ in air for a maximum of 96 h with medium replacement every 24 h. Cells were harvested for analysis using Non-Enzymatic Cell Dissociation Solution (Sigma), collected by centrifugation at 500 × g for 5 min, and resuspended in media or buffer, as required. To quantify trophoblasts in the cultures, we incubated cell suspensions with human FcR blocking reagent (Miltenyi Biotech), fixed and permeabilized them with Cytofix fixation buffer (BD Biosciences) and Cytoperm permeabilization buffer (BD Biosciences), and added one of the following primary Abs: anti-cytokeratin 7 (clone 12/30; DAKO), anti-vimentin (clone V9; DAKO), or IgG1 isotype control Ab (Sigma). Primary Abs were detected using FITC-coupled rabbit anti-mouse Ig (Dako). Data were acquired using a FACScalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Cell lines

The culture of HEK293 cells, and the generation of ACKR2-expressing HEK293 cells (HEK-ACKR2), is described elsewhere (34).

RNA isolation and quantitative real-time PCR

Primary trophoblasts (4 × 10⁸) were collected into 1 ml TRIzol (Life Technologies) and total RNA extracted, according to the manufacturer’s instructions. A total of 5 μg RNA was treated with DNase I (DNA-free kit; Life Technologies). The High Capacity Reverse Transcription Kit (Life Technologies) was used, according to the manufacturer’s instructions, to synthesize cDNA. For each sample, a control reaction was prepared lacking reverse transcriptase, mRNAs encoding chemokine receptors were quantified using TaqMan technology on the ABI Prism 7900HT system (Applied Biosystems) using the following primer sets (Life Technologies): GAPDH (Hs02786624_g1), Ackr1 (Hs01011079_s1), Ackr2 (Hs00907876_s1), Ackr3 (Hs00664172_s1), Ackr4 (Hs00356608_g1), Ccr1 (Hs00928897_s1), Ccr2 (Hs0074702_1s), Ccr3 (Hs009999027_s1), Ccr4 (Hs00747615_s1), and Ccr5 (Hs00999949_g1). For each gene, reactions were also set up containing serial dilutions of human genomic DNA (100 ng, lambda) that contained defined numbers of gene copies (ranging from 30 to 30,000 copies). Each gene analyzed is present at two copies per diploid genome. For each experiment, at least two copies were used as a positive control, and a negative control containing no template was also performed. Each gene analyzed is present at two copies per diploid genome. All reactions contained 1.25 μl of 20× target assay, 12.5 μl of 2× MasterMix (Life Technologies), 10.25 μl diethylpyrocarbonate-treated water, and 1 μl template, and were incubated for 2 min at 50 °C, then 10 min at 95 °C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. SDS Version 2.3 software (Applied Biosystems) was used to determine threshold cycle (Ct). For each gene, the genome DNA reactions were used to generate standard curves that plotted Ct values against gene copy number. The Ct values from the PCRs that used cDNA as a template were converted into copy numbers using these standard curves, and expression of each chemokine receptor is presented as the number of copies of chemokine receptor per copy of GAPDH.

Chemokines

Alexa Fluor 647–coupled human CCL2 (CCL2AF647) and biotinylated human CCL2 (bioCCL2) were purchased from Almac Sciences. Unlabeled human chemokines were from Peprotech. Western blotting

Cells lysates were prepared according to manufacturer’s instructions using CellLytic MT Cell Lysis Reagent (Sigma) or streptavidin coupled to FCR (Invitrogen), respectively. Densitometry was performed using Quantity One software (Bio-Rad Laboratories).

Flow cytometric analysis of CCL2AF647 uptake

These experiments were performed essentially as previously described (30). In brief, 3 × 10⁷ cells were incubated in 25 nM CCL2AF647 (±250 nM...
unlabeled chemokine) at 37°C for 1 h. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Dead cells were excluded from the analysis using 7-aminoactinomycin D staining (BD Pharmingen).

**Chemokine scavenging assay**

Primary human trophoblasts were seeded into the wells of 6-well plates (1.5 × 10^6 cells per well) in 1.5 ml medium and cultured at 37°C, 5% CO2 in air overnight. To each well, 7.5 pmol bioCCL2 was added (±7.5 pmol CCL3,1 or CCL26). Wells without cells but containing media and 7.5 pmol bioCCL2 were also set up. A 60 μl sample of medium was collected immediately from each well (0 h samples) and additional 60 μl aliquots were taken every 24 h for 3 d thereafter.

**Animals**

Animal studies were performed under U.K. Home Office license and after approval from the University of Glasgow’s ethical review panel. Mice were maintained in specific pathogen-free conditions at the University of Glasgow’s Central Research Facility. WT and Ackr2-deficient DBA/1j mice were derived from C57Bl6 Ackr2-deficient mice (41) after backcrossing for 11 generations with WT DBA/1j mice (Taconic). To generate pregnant mice, we housed 8- to 12-wk-old female mice overnight with male mice. The presence of a vaginal plug the following morning, designated as embryonic day 1 (E1), indicated that copulation had occurred. Male mice were removed and females were monitored daily.

**Tissue collection and stereology**

Embryos and placentas were weighed, and tail tips were taken from offspring for genotyping when required. Placentas were hemisectioned in a random orientation perpendicular to the CP, and half were processed for stereological analysis. Blood was collected in Lithium Heparin BD Microtainer tubes (BD Biosciences), and plasma was collected and stored at −80°C, as previously described (42). Histological preparation of samples for stereology was performed as previously described (7). Placenta halves were stored in 4% paraformaldehyde (Sigma) in PIPES buffer (100 mM PIPES, 1.36 mM CaCl2, 0.75 mM polyvinyl-pyrrolidone, pH 7.2; all from Sigma) for 16 h at room temperature, transferred to PIPES buffer, dehydrated, and embedded in paraffin such that tissue sections would extend from chorial to basal plate. Eight-micrometer sections were cut using a microtome until the entire tissue had been sectioned. All sections were transferred to slides and labeled according to the order they were taken. Fifteen sections of each E14 placenta and eight sections of each E18 placenta were chosen for analysis using a systematic random sampling method (7, 43), stained with H&E (Sigma), and examined under 100× magnification using an Olympus BX50 light microscope. Images covering the entire section were captured using Image Pro 6.2 Plus software (Media Cybernetics) and overlaid to exclude areas of overlap. Within each individual image, five points were randomly marked with a plus sign (+) using the Image Pro 6.2 Plus software. The placental zone where the center of each grid point fell was identified and recorded as DB, JZ, LZ, or CP. For each placenta analyzed, the total number of grid points that fell on each zone was divided by the total number of grid points assigned during the analysis of that placenta and multiplied by 100 to calculate the percentage of the total placental volume occupied by each zone.

**Quantification of plasma chemokines in mice**

Chemokine concentration in plasma was determined using Singleplex Luminex assays (Life Technologies) and the Bio-Plex 200 System and Bio-Plex Manager Software 4.0 (Bio-Rad), according to the manufacturer’s instructions.

**Statistical analysis**

All statistical analyses were carried out using GraphPad Prism Version 5.0 software with p < 0.05 accepted as significant. The tests used are indicated in the table and figure legends.

**Results**

**ACKR2 is strongly expressed by cultured human trophoblasts**

First, we wished to examine the expression of genes encoding inflammatory CC chemokine receptors and ACKRs in primary human trophoblast cultures. To ensure that cultures established from term placentas contained substantial numbers of trophoblasts, they were examined by flow cytometry after intracellular immuno-
proach to demonstrate the expression of functionally competent ACKR2 by mouse innate-like B cells (30). The internalization of CCL2AF647 is required for cell labeling in this assay, because incubation at 4°C (which prevents chemokine internalization but not receptor binding) results in little, if any, accumulation of cell-associated fluorescence (Ref. 30 and data not shown). ACKR2 and CCR2 are the only chemokine receptors able to internalize CCL2. Because transcripts encoding ACKR2 are ∼50-fold more abundant in cultured trophoblasts than those encoding CCR2 (Fig. 1B), we anticipated that ACKR2 would be primarily responsible for any CCL2AF647 uptake by these cells. However, to specifically examine this, we also performed CCL2AF647 uptake assays in the presence of a 10-fold molar excess of various unlabeled chemokines (30). Chemokines such as CCL3L1, CCL5, CCL11, and CCL17, which are ligands for ACKR2, but not CCR2, will only reduce CCL2AF647 uptake mediated by ACKR2, whereas chemokines that bind both CCR2 and ACKR2 (such as CCL7, CCL8, and CCL13) will reduce CCL2AF647 uptake mediated by both these receptors. Nonspecific CCL2AF647 uptake mechanisms, such as pinocytosis, will be unaffected by any of the unlabeled chemokines. The extent to which an unlabeled chemokine will reduce receptor-mediated CCL2AF647 uptake will depend on the relative concentrations of the labeled and unlabeled chemokine, and the nature of their interactions with the receptors. A 10-fold molar excess of the unlabeled chemokine might be expected to cause a 10-fold reduction in CCL2AF647 uptake, if CCL2AF647 and the unlabeled chemokine have similar receptor interaction properties. However, if they differ in this regard, then more or less inhibition of CCL2AF647 uptake will be seen. This could conceivably be influenced by cell background, so by assessing the impact of unlabeled chemokines on CCL2AF647 uptake by HEK-ACKR2 and cultured trophoblasts, the behavior of endogenous ACKR2 in immortalized cell lines (HEK-ACKR2) and endogenous ACKR2 in primary cells (trophoblasts) can be compared.

As expected, HEK-ACKR2 cells efficiently internalized CCL2AF647 (Fig. 2A and Supplemental Fig. 1). This was ACKR2 dependent, because the fluorescence intensity of HEK-unt cells incubated with CCL2AF647 was similar to that of HEK-ACKR2 cells incubated in the absence of CCL2AF647 (Supplemental Fig. 1). Strikingly, despite expressing far less ACKR2 protein than HEK-ACKR2 cells (Fig. 1C), we were unable to immunostain CCL2AF647-positive cells with anti-vimentin or anti–cytokeratin-7 Abs (because cell permeabilization resulted in the loss of cell-associated AF647 fluorescence), but CCL2AF647-labeled cells had the physical properties of trophoblasts (forward and side scatter characteristics), and the proportion of cells that failed to internalize any CCL2AF647 was consistently similar to the proportion of non-trophoblast cells in the culture (i.e., vimentin+, cytokeratin-7− cells (Fig. 1A and data not shown)). CCL2AF647 uptake by HEK-ACKR2 cells and trophoblasts was reduced by inclusion of a 10-fold molar excess of unlabeled CCL3L1, CCL5, CCL11, CCL17, and CCL22 (i.e., che-
mokines able to bind ACKR2, but not CCR2), but was unaffected by CCL26, a chemokine that does not bind to ACKR2 (Fig. 2A, 2B). CCL7, CCL8, and CCL13 were also able to substantially reduce CCL2AF647 uptake by HEK-ACKR2 cells (Fig. 2C). These chemokines, which can bind CCR2 and ACKR2, also reduced uptake by trophoblasts (Fig. 2D), but they were no more effective than those chemokines that lack the ability to bind CCR2 (Fig. 2B), suggesting that CCR2 plays little, if any, role in driving CCL2AF647 uptake by these cells. Thus, ACKR2 expressed by cultured primary human trophoblasts can mediate the efficient internalization of extracellular chemokine.

There were some marked differences between HEK-ACKR2 cells and trophoblasts in the ability of unlabeled chemokines to reduce CCL2AF647 uptake by these cells. With HEK-ACKR2 cells, the chemokines could be split into three groups: CCL7, CCL8, CCL13, and CCL22 were potent inhibitors of CCL2AF647 uptake; CCL3L1, CCL11, and CCL17 were less effective; and CCL5 resulted in only a small reduction in cell labeling. In contrast, these chemokines showed few differences in their ability to reduce CCL2AF647 uptake by trophoblasts, although CCL11, and particularly CCL8, had reduced activity. Thus, as with the post-translational modification of ACKR2 (Fig. 1C), it appears that interactions between chemokines and endogenous ACKR2 seen in trophoblasts are not fully recapitulated when ACKR2 is overexpressed in immortalized HEK-293 cells.

FIGURE 2. Efficient ACKR2-mediated internalization of CCL2 by primary human trophoblasts. Flow cytometry profiles of HEK-ACKR2 cells (A and C) or primary human trophoblasts (B and D) after incubation at 37°C with or without CCL2AF647 (≥ a 10-fold molar excess of the unlabeled chemokine indicated). Dead cells (7-aminoactinomycin D+) were excluded by pregating. In (A) and (B), the unlabeled chemokines used are ligands for ACKR2, but not CCR2, or are unable to bind to either ACKR2 or CCR2 (i.e., CCL26), whereas in (C) and (D), the unlabeled chemokines used are able to bind ACKR2 and CCR2. Data are representative of results generated using cells from three or more placentas.

Progressive chemokine scavenging by cultured human trophoblasts mediated by ACKR2

Previous work using transfected cell lines has shown that ACKR2 can, over time, progressively remove substantial quantities of extracellular chemokine (34, 35). This relies on its ability to rapidly traffic to and from the cell surface, and to avoid the downregulation and desensitization experienced by conventional chemokine receptors when they bind chemokine. To examine
chemokine scavenging by trophoblasts, we added bioCCL2 to trophoblast cultures, and its removal from the medium was assessed by Western blotting aliquots of medium taken over the course of a 3-d period (Fig. 3). To determine whether ACKR2 was responsible for any observed bioCCL2 scavenging, we included incubations in which a 10-fold excess of unlabeled CCL3L1 or CCL26 was included in addition to the bioCCL2. As in the CCL2 AF647 uptake experiments, CCL3L1, but not CCL26, would be expected to compete with bioCCL2 for access to ACKR2, and thereby slow any scavenging specifically mediated by this receptor. To verify that any loss of bioCCL2 was not due to degradation in the medium, control wells lacking trophoblasts, but containing media and bioCCL2, were also set up. Visual inspection of the Western blots indicated that, when trophoblasts were present, bioCCL2 was progressively removed from the culture medium, and that this could be slowed by unlabeled CCL3L1, but not CCL26 (Fig. 3A, 3B). This was confirmed by using densitometry to quantify the intensity of the bands in Western blots of samples of medium taken from three replicate wells (Fig. 3C, 3D). These data could also be used to estimate the rate of bioCCL2 scavenging. A total of 7.5 pmol bioCCL2 was added to 1.5 × 10⁶ cells at the start of the assay, and typically 75–98% of this bioCCL2 had been scavenged after 72 h of culture. On average, therefore, each cell in the culture was capable of scavenging ~3–4 × 10⁶ molecules of bioCCL2 every hour. Thus, like ACKR2-transfected cells (32, 34, 35), primary human trophoblasts can use ACKR2 to progressively scavenge inflammatory chemokines.

ACKR2 suppresses neonatal mortality in DBA/1j mice

In addition to the experiments on primary human trophoblasts, we were keen to further define indispensable functions for ACKR2 in vivo. In mice, ACKR2 protects fetuses from inflammation-driven abortion (32) and aids the survival of embryos transferred into pseudopregnant allogeneic recipients (33), but it is not thought to be involved in regulating the reproductive success of unchallenged pregnant mice. Indeed, during standard breeding regimens, Ackr2 deficiency did not result in any change in the number of weaned healthy offspring generated from mice with a C57Bl6/FVB/N mixed C57Bl6/FVB/N or mixed C57Bl6/129 background. However, as part of ongoing studies to explore Ackr2 function in mouse models of autoimmunity, we backcrossed Ackr2 deficiency onto DBA/1j mice (to enhance their susceptibility to collagen-induced arthritis) and noticed that Ackr2-deficient DBA/1j pairs consistently generated fewer weaned offspring than WT DBA/1j pairs housed under the same conditions. We therefore collected data on litter size and neonatal mortality during the course of generating mice for the autoimmunity study (Table I). Pregnant mice were monitored twice daily as they approached term, and their offspring were counted every day for the first 3 d after birth, and then every 1–2 d thereafter. Dead offspring (or their remains) found when the litter was first discovered were scored as stillborn, whereas neonatal death was defined as animals that did not survive to weaning (18–21 d after birth), although most deaths that we observed (>85%) occurred in the first few days of life. WT DBA/1j mice are reported by commercial breeders to generate relatively small numbers of offspring per pregnancy (mean 4.5 per litter; Harlan Laboratories, Indianapolis, IN, http://www.harlan.com), and it was apparent from our data that rates of stillbirth and neonatal mortality are high in this strain (Table I), and greater than we typically observe with WT C57Bl6 mice (data not shown). Moreover, although Ackr2-deficient DBA/1j mice gave birth to equivalent numbers of offspring as WT DBA/1j females, it was clear that the absence of Ackr2 substantially increased the likelihood of stillbirth and neonatal death. This resulted in significantly fewer offspring being successfully weaned from each litter (Table I). WT and Ackr2-deficient offspring that were not stillborn appeared grossly normal, and it was not possible to predict which would die before weaning. Also, there was no significance difference in mean weight or growth rate between WT and Ackr2-deficient DBA/1j offspring from day 5 after birth onward; no abnormalities were apparent in the growth or survival of Ackr2-deficient mice after weaning; and there was no difference in the male/female ratio of successfully weaned mice between strains (data not shown). Complete or partial consumption of dead offspring by the parents precluded detailed autopsies on dead neonates, and the causes of neonatal death remain uncertain. Nonetheless, the data clearly demonstrate that in an inbred mouse strain with relatively poor reproductive success, the presence of Ackr2 reduces the incidence of stillbirth and reduces neonatal mortality.

Pregnant Ackr2-deficient DBA/1j mice have increased levels of plasma CCL2

Neonatal death can be caused by compromised placental function and, from E12 onward, Ackr2 is strongly expressed in the mouse...
placenta while being virtually undetectable in the embryo (33). Thus, we were interested in whether the increased neonatal death in Ackr2-deficient mice was associated with defects during pregnancy. Before embarking on a detailed analysis of the placenta, we first examined plasma from a group of pregnant WT and Ackr2-deficient mice (E14–E18) in an effort to generate evidence of molecular changes arising from Ackr2 deficiency. ACKR2 can decrease the plasma level of some ACKR2-binding chemokines, including CCL2, shortly after LPS administration to pregnant C57Bl6 mice (32), so we wondered whether a similar molecular signature might exist in unchallenged pregnant Ackr2-deficient DBA/1j animals. This analysis revealed that the levels of the ACKR2 ligands CCL3 and CCL5, and the non-ACKR2 ligand CXCL1, were not different between pregnant WT and Ackr2-deficient mice, but interestingly, that circulating levels of CCL2 were significantly higher in Ackr2-deficient mice (Fig. 4). Thus, even in the absence of challenge, pregnant Ackr2-deficient DBA/1j mice show evidence of aberrant chemokine regulation.

Ackr2-deficient DBA/1j embryos show stunted growth at E14

Next, we set up timed matings and harvested fetuses and placentas at E14 and E18 to examine whether placental or fetal growth was compromised in Ackr2-deficient DBA/1j mice. This study was motivated by recent evidence that the expression of the fetal receptor Ackr2 can significantly affect placental mass (33). Indeed, it performs this function with considerable efficiency. Interestingly, however, although Ackr2-deficient fetuses were morphologically normal at E14, they were found to weigh significantly less than their WT counterparts (Fig. 5A). By E18, fetal weight had increased ~5-fold, and there was no longer any difference between WT and Ackr2-deficient fetuses (Fig. 5B). Thus, Ackr2 deficiency is associated with stunted fetal growth at E14 in DBA/1j mice.

Fetal Ackr2 deficiency reduces the size of the placental LZ in DBA/1j mice

Changes in the structure of the placenta could account for the reduced growth of Ackr2-deficient DBA/1j embryos. Thus, we undertook a detailed stereological analysis of E14 and E18 WT and Ackr2-deficient DBA/1j placentas (Fig. 6). By using randomized, unbiased sampling at several stages of the process, this approach allows placental volumes to be objectively extrapolated from the systematic plotting of areas in a series of two-dimensional images of placental sections (7). At E14, there were no differences between WT and Ackr2-deficient placentas in the volume occupied by the DB or the CP, but strikingly, compared with WT placentas, the JZ was ∼25% larger in Ackr2-deficient placentas, whereas the LZ was nearly 50% smaller (Fig. 6A, 6B). By E18, when there was no difference in weight between WT and Ackr2-deficient fetuses, there was also no difference in zone volumes between WT and Ackr2-deficient placentas (Fig. 6C).

To determine whether the structural defects in Ackr2-deficient E14 placentas were due to a defect in the fetal component of the placenta, Ackr2<sup>−/−</sup> females were bred with Ackr2-deficient males so that Ackr2-deficient placentas could be compared with those expressing Ackr2 (<i>i.e.</i>, Ackr2<sup>+/−</sup> placentas) in the context of the same Ackr2-expressing (<i>i.e.</i>, Ackr2<sup>+/+</sup>) mother. Fetuses and placentas were harvested on E14. Although no significant differences in fetal weight, placenta weight, or FPI were detectable between Ackr2<sup>+/−</sup> and Ackr2-deficient placentas/fetuses (data not shown), when stereology was performed, a significantly smaller proportion of the placentas supporting Ackr2-deficient fetuses was occupied by the LZ compared with those supporting Ackr2<sup>+/−</sup> siblings (Fig. 7). The JZ fraction of the Ackr2-deficient placenta tended to be larger than the equivalent area of the Ackr2<sup>+/−</sup> placenta, but this was not statistically significant, and there was a small, but statistically significant, increase in tissue scored as CP (Fig. 7). Thus, the loss of expression of Ackr2 by fetal cells results in a reduction in the size of the LZ in the E14 placenta.

Discussion

Current theories regarding human ACKR2 function at the fetomaternal interface rely, in large part, on data from studies that have examined its behavior in immortalized cell lines. Our experiments are the first to have explored the molecular function of ACKR2 in primary human trophoblasts, and importantly, they firmly indicate that ACKR2 functions as a chemokine scavenger in these cells. Indeed, it performs this function with considerable efficiency. For example, despite containing considerably less ACKR2 protein than HEK-ACKR2 cells, in short-term CCL2<sup>AF647</sup> uptake experiments,
trophoblasts accumulate nearly as much fluorescence as these highly expressing ACKR2-transfected cells. Moreover, cultured trophoblasts are able to mediate the continuous scavenging of extracellular chemokine through ACKR2, and thus in these cells, as in cell lines, ACKR2 appears to avoid the desensitization and downregulation processes that limit the activity of conventional chemokine receptors. With trophoblasts able to scavenge $3-4 \times 10^7$ molecules of chemokine per hour in culture, and estimated to cover $10 \text{ m}^2$ of the human placental surface at term, ACKR2 has enormous potential to regulate chemokines in the placenta and circulating blood of pregnant women.

It was notable that the m.w. of ACKR2 protein was more uniform in trophoblasts than it was in HEK-ACKR2 cells, and this may be important for optimal chemokine scavenging activity by trophoblasts. The molecular heterogeneity of ACKR2 in HEK-ACKR2 cells likely arises from variations in posttranslational modification. ACKR2 is subject to a variety of posttranslational modifications, including sulfation, glycosylation, and phosphorylation, and interfering with these processes enhances the electrophoretic mobility of ACKR2 (39, 40). More over, these modifications are known to be functionally significant. Constitutive phosphorylation, for example, enhances ACKR2 stability, whereas sulfation is important for ligand binding (40, 44). Variation in posttranslational modification could be responsible for the differences we have observed between HEK-ACKR2 cells and trophoblasts in the way that unlabeled chemokines reduce their ability to internalize CCL2AF647. The ligand specificity of ACKR2 appears not to differ between the two cell types, but some chemokines, such as CCL8 and CCL22, were far better at reducing CCL2AF647 uptake by HEK-ACKR2 cells than they were at blocking its internalization into trophoblasts. These differences indicate that the ligand recognition properties of ACKR2 are affected by cellular background, and serve to emphasize the importance of examining ACKR function in primary cells.

Although ACKR2 appears to be the dominant receptor for inflammatory chemokines expressed by trophoblasts, other receptors, such as CCR1, are also expressed and could direct trophoblast migration. Interestingly, however, there is emerging evidence that ACKR2 can suppress the activity of coexpressed conventional chemokine receptors, including CCR1 (30, 45, 46), and this could limit the migratory responses of trophoblasts to extracellular chemokines. In specific contexts in vivo, reciprocal regulation of ACKR2 and conventional chemokine receptors in trophoblasts could conceivably switch off chemokine scavenging and switch on migratory potential. CCR1 and CCR4 are induced as cells differentiate to form invasive extravillous trophoblasts, and these receptors enable these cells to migrate toward their cognate chemokine ligands (47, 48). It will be of interest to examine whether there is a coincident loss of ACKR2 expression, because this could reinforce the ability of these cells to respond to chemotactic cues through these conventional chemokine receptors.

Our work has shown that, in addition to ACKR2, cultured trophoblasts express transcripts encoding ACKR3 and ACKR4. ACKR3, which binds the chemokines CXCL11 and CXCL12, has previously been reported to be expressed by trophoblasts (in culture and placental sections) (49, 50). Interestingly, CXCL12, by signaling through CXCR4, can regulate trophoblast invasion, differentiation, and survival in vitro (51–53), and control gene expression in decidual stromal cells (53), whereas both CXCL11 and CXCL12 induce migratory responses in leukocytes capable of homing to the fetomaternal interface (20, 22, 23). It seems possible that these responses could be regulated by ACKR3 through its ability to control extracellular chemokine abundance and modulate the activity of coexpressed CXCR4. The potential significance of ACKR4 expression by human trophoblasts is less clear. However, if functional ACKR4 protein is produced then by scavenging the chemokines CCL19 and CCL21, it is conceivable that it could contribute to the entrapment of dendritic cells in the pregnant uterus that has been reported to occur in mice (54).
CCL19 and CCL21 are ligands for the conventional chemokine receptor CCR7, which is critical for the intravasation of dendritic cells into lymphatic vessels as they migrate from peripheral tissues to lymph nodes (55). Clearly, the role of these ACKRs in trophoblast biology, placental function, and reproduction merits further investigation.

Previous work has shown that fetal ACKR2 aids the survival of C57Bl6/129 embryos transferred into fully allogeneic (DBA2), but not syngeneic, pseudopregnant WT recipients (33), and that the absence of ACKR2 from pregnant C57Bl6 mice and their fetuses enhances susceptibility to inflammation-induced abortion (32). The work presented in this article using DBA/1j mice reveals several additional reproductive phenotypes that can arise from loss of Ackr2. First, unlike unchallenged pregnant C57Bl6 mice (32), Ackr2 deficiency in pregnant DBA/1j mice is associated with increased levels of circulating CCL2. Second, although loss of Ackr2 from mother and fetus does not alter the size of the placenta, it does alter its structure at E14. This is characterized by

![Figure 6](image_url) **FIGURE 6.** Ackr2 deficiency is associated with structural changes in the placenta in DBA/1j mice. Placentas were collected from pregnant WT and Ackr2-deficient (knockout [KO]) DBA/1j mice (both \( n = 4 \)) at E14 (A and B) and E18 (C). From each mother, the two placentas closest to the mean placental weight were selected. Serial 8-\( \mu \)m sections were prepared from half of each placenta. Fifteen (E14) or eight (E18) sections from each placenta were randomly chosen, stained with H&E, and stereological analysis was performed to calculate the proportion of placental volume occupied by the DB, JZ, LZ, and CP within each pregnant animal. (A and C) Median and interquartile range within each group are indicated. Data were analyzed using the Mann–Whitney U test. *\( p < 0.05 \). (B) Representative images of an H&E-stained section from a WT and Ackr2-deficient (KO) E14 placenta. Original magnification \( \times 100 \). White dashed lines indicate the boundaries of each zone.

![Figure 7](image_url) **FIGURE 7.** Fetal Ackr2 maintains placental LZ volume in DBA/1j mice. Ackr2 DBA/1j female mice heterozygous for the deleted Ackr2 allele (HET) were mated with Ackr2-deficient (knockout [KO]) DBA/1j male mice. E14 fetuses and placentas were harvested and weighed, the fetuses genotyped by PCR, and the placentas subjected to stereological analysis using 15 randomly selected 8-\( \mu \)m sections from each placenta (\( n = 8 \) for each group, derived from three litters). Data were analyzed using the Mann–Whitney U test. *\( p < 0.05 \).
find fetal demands. The placental defects in E14 because, in the same pregnant possible that early defects in the formation or enlargement of the late gestation to meet the nutrient needs of the fetus (12), and it is for the reduced weight of Ackr2+/- mouse (data not shown), but fur- failed to provide evidence that maternal interface (18, 61), the recruitment of Th17 cells into the preeclampsia (60), the regulation of Th2 responses at the feto- Ackr2+/- placenta or maternal tissues in confirmed that there are no changes in Ackr2+/- mice (data not shown). Local or systemic chemokine dysregulation could conceivably contribute to the placental phenotypes observed at E14 in Ackr2-deficient DBA/1j mice, by, for example, altering leukocyte recruitment into the placenta. Excessive or uncontrolled inflam- mation within the placenta can compromise its function and has been well documented to cause fetal loss in both animals and humans (56–59), and the CCL2/CCR2 axis has been implicated in pre eclampsia (60), the regulation of Th2 responses at the feto- maternal interface (18, 61), the recruitment of Th17 cells into the decidua (24), and the promotion of decidual stromal cell growth (62). Preliminary quantitative real-time PCR and histology have failed to provide evidence that Ackr2 deficiency modulates leukocyte abundance in the E14 placenta (data not shown), but further comparative analysis of immune cells in WT and Ackr2- deficiency placenta throughout gestation could provide insight into the cellular basis for the placental defects arising from Ackr2 loss.

The supply of nutrients through the placenta is a crucial factor in successful intrauterine growth and development (63). A smaller LZ would be expected to compromise the exchange of nutrients, gases, and waste between mother and fetus, and this could account for the reduced weight of Ackr2-deficient E14 fetuses. The structural defects in the Ackr2-deficient placenta at E14 are absent by E18. Placentas can adapt morphologically and functionally in late gestation to meet the nutrient needs of the fetus (12), and it is possible that early defects in the formation or enlargement of the LZ in Ackr2-deficient placentas are corrected in late gestation to meet fetal demands. The placental defects in E14 Ackr2-deficient mice are at least partially caused by loss of Ackr2 from fetal cells because, in the same pregnant Ackr2+/- mouse, the LZ occupies a smaller fraction of a placenta supporting Ackr2-deficient fetuses than one feeding an Ackr2+/- sibling. However, changes in these E14 Ackr2-deficient placentas are not as profound as those seen in E14 Ackr2-deficient placentas growing in Ackr2-deficient female mice, and there was no difference in the weight between E14 Ackr2-deficient and Ackr2+/- fetuses developing in the same Ackr2+/- mother. Thus, Ackr2 expressed by the mother, or by the placentas of siblings growing in the same mother, appears to lessen the impact of fetal Ackr2 deficiency on placental structure and fetal growth.

WT DBA/1j mice appear to be prone to neonatal death. The reason for this is unknown, but what is clear is that loss of Ackr2 substantially increases the likelihood of this occurring. We speculate that this is linked to the defects in placental structure and fetal weight that we have observed, and changes in placental function are linked to neonatal survival in humans and animals. However, it is possible that other defects caused by loss of chemokine scavenging in Ackr2-deficient neonates or mothers underpin the increased rate of neonatal death. For example, Ackr2-deficient DBA/1j neonates might develop exaggerated, and potentially life-threatening, responses to the microorganisms they encounter after birth. Indeed, adult Ackr2-deficient C57Bl6 mice have been shown to develop excessive inflammatory responses after intranasal infection with low doses of Mycobacterium tu- berculus, and these responses, rather than the infection itself, cause a substantial increase in mortality in this model (64). Thus, further investigations are required to understand the causes of neonatal death in WT and Ackr2-deficient DBA/1j mice. None- theless, our data demonstrate that Ackr2, influenced by genetic background, can make an indispensable contribution to the re- productive success of mice that have conceived naturally. To our knowledge, this is the first example of a reproductive defect in unchallenged mice arising from a genetic deficiency in a compo- nent of the inflammatory CC chemokine network.

In summary, our study has clearly demonstrated that primary human trophoblasts use ACKR2 to mediate efficient chemokine scavenging, and that Ackr2 can play indispensable roles in regu- lating placental structure, fetal weight, and neonatal mortality in mice. ACKR2 is a prominent player in the regulation of placental chemokine networks, and further investigation of its role in hu- man placentation is merited. Moreover, with evidence implicating ACKR2 as a key regulator of inflammatory disease (26), it will be of interest to extend these studies to consider its role during chorioamnionitis, intrauterine growth restriction, pre eclampsia, and other conditions that impact on reproductive health in humans.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Data

The atypical chemokine receptor ACKR2 mediates chemokine scavenging by primary human trophoblasts, and can regulate fetal growth, placental structure, and neonatal mortality in mice.


Supplementary Figure 1: Representative overlaid flow cytometry profiles of HEK-ACKR2 cells or HEK-unt cells after incubation at 37°C with or without CCL2^{AF647}. 5 or more repeat experiments gave similar results.