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An Alternative Role of C1q in Cell Migration and Tissue Remodeling: Contribution to Trophoblast Invasion and Placental Development

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Fetal trophoblast cells invading the decidua in the early phase of pregnancy establish complex interaction with the maternal extracellular matrix. We discovered that C1q was widely distributed in human decidual stroma in the absence of C4 and C3 and was actively synthesized by migrating extravillous trophoblasts. The cells expressed the messages for the three chains of C1q and secreted this complement component that interacted with the proteins of the decidual extracellular matrix. Solid phase-bound C1q promoted trophoblast adhesion and migration, and cell binding to C1q resulted in activation of ERK1/2 MAPKs. Ab inhibition experiments showed that the receptors for the globular head of C1q/p33 and $\alpha_4\beta_1$ integrin were both involved in this process and were colocalized on the cell surface following binding of C1q to trophoblasts. We also found that C1q$^{-/-}$ mice manifested increased frequency of fetal resorption, reduced fetal weight, and smaller litter sizes compared with wild-type mice. C1q deficiency was associated with impaired labyrinth development and decidual vessel remodeling. Collectively, these data suggest that C1q plays an important role in promoting trophoblast invasion of decidua and that defective local production of C1q may be involved in pregnancy disorders, such as pre-eclampsia, characterized by poor trophoblast invasion. *The Journal of Immunology*, 2010, 185: 4420–4429.

T he placenta is a new organ formed in pregnancy with the contribution of both the fetus and the mother and is absolutely essential to promote a peaceful feto–maternal interaction with continuous exchange of nutrients and protective fac-

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Abbreviations used in this paper: AU, arbitrary unit; C, conceptus side; COLL, collagen type; CTB, cytotrophoblast; D, decidual side; DCCM, decidual cell-conditioned medium; DEC, decidual endothelial cell; DSC, decidual stromal cell; EC, endothelial cell; ECM, extracellular matrix; EMBL, European Molecular Biology Laboratory; EMILIN1, elastin microfibril interfacer 1; EVT, extravillous trophoblast; FN, fibronectin; gC1q, globular head of C1q; gC1qR, receptor for the globular head of C1q/p33; IP, immunoprecipitate; NBD, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-y; PBDM, peripheral blood-derived macrophage; ROI, region of interest; RQ-PCR, real-time quantitative PCR; SA, spiral artery; vWF, von Willebrand factor; WT, wild-type.

tors. A characteristic feature of this interaction during placentation is the invasion of maternal decidua by fetal cytotrophoblasts (CTBs) that depart from the anchoring villi and migrate as extravillous trophoblasts (EVTs) into the maternal decidua, reaching the inner third of the myometrium (1). Some of the invading trophoblast cells penetrate the spiral arteries and migrate upward against blood flow, forming intravascular islands and mosaic vessels as a result of partial replacement of the endothelium (2, 3). Trophoblast cells share with tumor cells the ability to invade tissues, with the major difference that trophoblast invasion is a physiological event strictly restricted to pregnancy and spatially limited to the decidual microenvironment (4–7). Impairment of this process results in pathological conditions associated with pregnancy including intrauterine growth restriction and pre-eclampsia (8, 9).

CTBs migrating from chorionic villi into the decidua undergo a process of differentiation into the EVT cell lineage characterized by a progressive change in the surface expression of adhesion molecules that allow these cells to leave the chorionic villi and bind to the extracellular matrix (ECM) of decidua (10–13). The epithelial-like receptors E-cadherin and $\alpha_6\beta_4$ integrin expressed on villous trophoblasts are replaced by the adhesion molecules vascular endothelial cadherin, VCAM-1, and PECAM-1, characteristic of endothelial cells (ECs). In addition, EVTs express a variety of integrins that promote their binding to ECM of decidua (14). ECM proteins establish a special interaction with EVTs and, by virtue of their characteristic distribution, favor the invasion of decidua and the characteristic perivascular localization (15). Fibronectin (FN) is one of the most abundant ECM proteins distributed preferentially in the distal portion of the CTB column and in areas of decidua invaded by trophoblasts (16). This protein acts as a bridging ligand for $\alpha_5\beta_1$ integrin expressed on EVTs and mediates their adhesion and

migration at sites of anchoring villi and in the decidual tissue (17). Decidual stroma is also rich in laminins that surround the decidual cells as a distinct pericellular layer (18, 19) and interact mainly with $\alpha_1\beta_1$ and $\alpha_6\beta_1$ on EVTs (20, 21).

Elastin microfibril interfacer 1 (EMILIN1) is a connective tissue glycoprotein associated with elastic fibers (22) previously identified as another candidate ECM molecule involved in EVT invasion. A characteristic feature of this protein is to display a gradient distribution in decidua with a progressive increase in its expression toward remodeled spiral arteries that favors EVT migration from the anchoring columns to the perivascular areas (23). EMILIN1 shares a structural homology with the globular head of C1q (gC1q), the first component of the complement system (24), raising the possibility that C1q may contribute to trophoblast invasion of decidua. The main function of this complement component is to act as a recognition molecule of the complement system and trigger the activation of the classical pathway. This results in the release of biologically active products primarily involved in the removal of immune complexes and infectious and other harmful agents through the activation of various cellular responses, including enhancement of phagocytosis (25), stimulation of neutrophil, eosinophil, and mast cell chemotaxis (26), and clearance of apoptotic cells (27). However, C1q has also been shown to exhibit activities apparently unrelated to its defensive functions, such as promotion of EC adhesion and spreading (28) and regulation of fibroblast migration and apoptosis (26). Over the last few years, we have been analyzing human placenta for the presence and distribution of complement components with the aim to define their role in embryo implantation. Recently, we have shown that C1q is expressed on the surface of decidual ECs (DECs) and acts as a molecular bridge that favors adhesion of endovascular trophoblasts and endothelial replacement (29). Data are now presented suggesting that invasive trophoblast cells synthesize and secrete C1q that binds to ECM proteins and is used by EVTs to invade the decidua.

Materials and Methods
Abs and reagents

Goat antiserum anti-human C1q, mAb anti-human C1q, and purified human C1q were purchased from Quidel (San Diego, CA). Two mAbs anti-gC1q receptor (gC1qR; clones 74.5.2 and 60.11) recognizing distinct domains of the molecule were previously reported (30); rabbit IgG anti-human cC1qR/calreticulin and anti-gC1qR/p33 were previously reported (31). The neutralizing mAb M89D3 to CD31 was kindly provided by M. R. Zocchi (San Raffaele Hospital, Milan, Italy) (32, 33); rabbit IgG and mAb P1H4 to human β_1 integrin, mAb P5D2 to α_4 integrin, mAb CGA7 to human smooth muscle α-actin, and mAb 349 anti-paxillin were from Chemicon (Milan, Italy). Rabbit IgG to human C1q and to cow cytokeratin wide spectrum, mAb W6/32 anti-HLA, mAb OV-TL 12/30 anti-cytokeratin 7, mAb F8/86 mouse anti-human von Willebrand factor (vWF), mAb SS2/36 anti-human CD10, mAb SN6h anti-human CD105, and mAb WM-47 mouse anti-human CD13 were purchased from DakoCytomation (Milan, Italy). Rabbit antiserum to ERK MAPK was from Santa Cruz Biotechnology (Santa Cruz, CA), and mAb anti–p-ERK1/2 MAPK from Cell Signaling Technology (Danvers, MA); mAb MEM-G9 anti-human HLA-G was from Immunological Science (Friesoythe, Germany); and Cy3-conjugated F(ab')$_2$ goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). The IgG were purified from the antisera by affinity chromatography on protein G-Sepharose columns (GE Healthcare, Milan, Italy) and, when required, biotin-labeled as previously reported (34).

Alkaline phosphatase-conjugated goat anti-mouse IgG, alkaline phosphatase-conjugated goat anti-rabbit IgG, mouse IgG1 isotype-matched Abs, mAb V9 anti-human vimentin, and mAb UCHM-1 anti-human CD14 were from Sigma-Aldrich (Milan, Italy), and mAb 5D3 to human cytokeratin 8/18 was from Novocastra (Milan, Italy). Monoclonal 2D1 anti-human CD45 was from BD Biosciences (Milan, Italy).

The gC1q fragment was kindly provided by G. J. Arlaud (Institut de Biologie Structurale, Grenoble, France). FN was purchased from Calbiochem-Novabiochem (La Jolla, CA). Vitronectin was purified from human plasma as previously described (35). Human collagen type (COLL) II, III, IV, and V, human plasma fibrinogen, and high m.w. hyaluronan were obtained from Sigma-Aldrich; rat tail COLL I was purchased from BD Biosciences. Native laminin-1–nidogen complex from Engelbreth-Holm-Swarm mouse tumor was obtained as previously described (36). Chick COLL VI tetramers were purified as previously described (37). Tenascin and human perlecan were kindly provided by Luciano Zardi (Advanced Biotechnology Centre, Genoa, Italy) and Anders Malmström (Lund University Hospital, Lund, Sweden), respectively. The synthetic peptide DELPQLVTLPHNLHGPEILDVPST (CS-1), the cell-attachment site of FN, was synthesized and purified as previously described (38).

Human tissues

Placenta and decidual biopsy specimens were obtained from women undergoing elective termination of pregnancy at 8–12 wk gestation. This study was approved by the institutional board of the Maternal-Children's Hospital (Istituto Di Ricovero e Cura a Carattere Scientifico "Burlo Garofolo," Trieste, Italy), and informed consent was obtained from women providing the tissue specimens.

Immunohistochemical staining

Tissue samples were fixed in 10% buffered formalin and paraffin embedded. Four-micrometer–thick sections of placental tissue were stained with H&E and examined for the presence and distribution of invasive trophoblasts. For immunohistochemical analysis, the slides were microwaved three times in Tris-HCl/EDTA (pH 9) buffer (DakoCytomation) for 5 min, brought to room temperature, and washed in PBS. Postneutralization of the endogenous peroxidase with H_2O_2 for 10 min, the sections were first incubated with protein block (Novocastra) for 10 min. Sections were incubated with the appropriate primary Abs for 30 min at room temperature. Binding of anti-C1q IgG or anti-cow cytokeratin wide spectrum were detected using the Labeled StreptAvidin Biotin + HRP kit and diaminobenzidine as substrate (DakoCytomation) and that of mAb anti-cytokeratin 7 was revealed with the Alkaline Phosphatase Anti-Alkaline Phosphatase Complex (DakoCytomation) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/NBT or Fast Red (DakoCytomation) as chromogens. The sections were viewed under a Leica DM3000 optical microscope (Leica Microsystems, Mannheim, Germany), and captions were collected using a Leica DFC320 digital camera (Leica Microsystems).

Cell isolation and culture

CTBs were purified from placental specimens postremoval of decidual tissue and fetal membrane as previously described (2). Briefly, placental tissue was incubated with HBSS containing 0.25% trypsin and 0.2 mg/ml DNase (Roche, Milano, Italy) for 20 min at 37°C. Postfractionation through Percoll gradient, the leukocytes were totally removed by immunomagnetic beads coated with mAb to CD45 (Dynal, Invitrogen, Milan, Italy). CTBs collected by negative selection were seeded in a 25-cm^2 flask coated with 5 μg/cm^2 FN (Roche), cultured overnight in RPMI (Life Technologies, Invitrogen) supplemented with 10% FCS, and finally detached by trypsin-EDTA treatment. The cells obtained under these conditions contained 95% cytokeratin 7-positive CTBs and a few vimentin-positive decidual stromal cells (DSCs). The presence of contaminating leukocytes and ECs was excluded by RT-PCR assay for CD45 and FACS analysis with anti-vWF and anti-CD31 Abs, respectively.

HLA-G$^+$ and HLA$^-$ cells were purified by incubating freshly isolated CTBs (3×10^6) with Dynabeads M-450 (Dynal, Invitrogen) conjugated with mAb anti-human HLA-G at 4°C for 45 min with gentle rotation. The cells adsorbed to magnetic beads were collected with a magnetic collector, washed five times with Dulbecco's PBS containing 0.1% BSA (Sigma-Aldrich), and used as a source of HLA-G–positive CTBs. The unadsorbed cells were incubated with CD45 magnetic beads (Invitrogen) to remove contaminating leukocytes and subsequently with Dynabeads M-450 conjugated with mAb W6/32 that recognized a conserved epitope of human HLA-A, -B, and -C at 4°C for 45 min with gentle rotation. The negatively selected cells were 100% HLA negative.

DSCs were isolated as described by Carlino et al. (39). Briefly, decidual tissue was digested with 0.5% trypsin (Sigma-Aldrich), 50 μg/ml DNase1 (Boehringer Mannheim, Mannheim, Germany) in HBSS overnight at 4°C and then treated with collagenase type I (3 mg/ml; Worthington Biochemical, Milan, Italy) for 30 min at 37°C. After Ficoll (Roche) density gradient centrifugation, the decidual cells were cultured for 24 h in RPMI supplemented with 10% FCS, and the decidual cell-conditioned medium (DCCM) was collected, stored at −80°C, and used as source of chemotactic stimuli in the migration assays. Nonadherent cells were removed by extensive washing, and adherent cells were used at the third passage of culture.

Under these conditions, the cells were negative for CD14, CD45, cytokeratin 8-18, vWF, or CD31 and positive for α-actin, vimentin, CD13, CD10, and CD105.

Peripheral blood-derived macrophages (PBDMs) were obtained from Ficoll-purified PBMCs cultured for 7 d in RPMI supplemented with 10% FCS as previously described (40).

RT- and real-time quantitative PCR

Cells and tissues were harvested in Omnizol (Euroclone, Milan, Italy) according to the supplier's instructions. Total RNA was extracted and reverse transcribed as previously described (29). RT-PCR was performed in a Techne TC-312 thermal cycler (Boneworld Scientific, Milan, Italy) using Taq DNA polymerase (AccuTaq, Sigma-Aldrich).

Real-time quantitative PCR (RQ-PCR) was carried out on a Rotor-Gene 6000 (Corbett, Explera, Ancona, Italy) using iQ SYBR Green Supermix (Bio-Rad, Milan, Italy). Table I lists the primers used for PCR following Kaul and Loos (41). Amplification conditions, sequences, and concentrations of the primers are similar to those of RT-PCR. The melting curve was recorded between 55˚C and 99˚C with a hold every 2 s. Purified *C1qC* and *β-actin* PCR products synthesized from pooled deciduas were used to establish standard curves, and the data were analyzed using Rotor-Gene Software (Corbett, Explera). The relative amount of *C1qC* in each sample was normalized with β-actin and expressed as arbitrary units (AUs) considering 1 AU obtained in PBDMs used as positive control (42).

Immunoenzymatic assays

The level of C1q secreted by CTBs in the culture supernatant and the presence of gC1qR in immunoprecipitated samples were measured by a sandwich ELISA as previously described (43).

ECM proteins (25 μg/ml) bound to wells of 96-well ELISA plates (Corning Costar, Acton, MA) in 0.1 mM sodium bicarbonate buffer (pH 9.6) by overnight incubation at 4˚C were tested for their interaction with C1q. Hyaluronan and laminin-5 were used at the concentration of 50 μg/ml and 11.5 μg/ml, respectively. After washing the wells with PBS containing $CaCl_2$ and $MgCl_2$ (0.7 mM) and 0.05% Tween-20 and blocking the residual-free sites with PBS plus 1% BSA for 1 h at 37˚C, C1q was added at the concentration of 10 μg/ml in PBS plus 1% BSA containing 0.05% Tween-20 and 0.7 mM Ca^{2+} and Mg^{2+} and allowed to bind to ECMs for 90 min at 37˚C. Bound C1q was revealed using mAb anti-C1q (1 μg/ml) followed by alkaline phosphatase-conjugated goat anti-mouse IgG.

CTB adhesion and migration assays

Adhesion and migration of CTBs were measured as previously reported (19). Briefly, CTBs (10^5 cells/100 μl) were labeled with the fluorescent dye *FAST Dil* (Molecular Probes, Invitrogen) and added to C1q-coated wells of a 96-well plate (Iwaki, Bibby Scientific Italia, Milan, Italy) for 30 min at 37˚C in an air/CO_2 incubator. The number of adherent cells were counted with Infinite200 (absorbance 544 nm, emission 590 nm) (TECAN Italia, Milan, Italy) and referred to a calibration curve established with an increasing number of labeled cells.

The migration assay was performed in transwell chambers using *FAST Dil*-labeled (Molecular Probes, Invitrogen) CTBs (2×10^5 cells) resuspended in RPMI with 0.1% BSA and added to the upper chamber. The cells were allowed to migrate through HTS FluoroBlokTM polycarbonate inserts with 8-μm pores (Becton Dickinson, Falcon, Milan, Italy) coated on the upper side with C1q, FN, or BSA, all used at the concentration of 40 μg/ml. For some experiments, the membranes were first coated with FN (20 μg/ml) and then incubated with C1q (20 μg/ml) for 30 min at room temperature to allow interaction of C1q with bound FN. DCCMs obtained by culturing decidual cells for 24 h and diluted 1:2 in RPMI with 0.1% BSA were added

to the lower chamber as a chemoattractant for CTBs. The number of cells transmigrated to the lower side of the insert after 4 h was evaluated as indicated above for the adherent cells.

Immunoprecipitation and immunoblot analysis

CTBs (2×10^6) were allowed to adhere to wells coated with BSA (10 μg/ml), FN (10 μg/ml), or C1q (10 μg/ml) for 30 min at 37˚C in RPMI without serum. At the end of incubation, both adherent and nonadherent cells were recovered and lysed in ice-cold lysis buffer containing 1% (v/v) Nonidet P-40 (Sigma-Aldrich), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% NaN_3, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 150 mM NaCl in 50 mM Tris (pH 7.5) for 30 min at 4˚C. Cell lysates were centrifuged at $15,000 \times g$ for 10 min at 4˚C, precleared with 100 μl 20% suspension of protein G-Sepharose beads (Sigma-Aldrich) for 1 h at 4˚C, and sequentially immunoprecipitated with normal mouse IgG (Sigma-Aldrich), mAb 60.11 anti-C1qR, and mAb 4B4 anti-$β_1$ integrin (Beckman Coulter, Fullerton, CA) previously bound to protein G-Sepharose beads. After washing with Tris buffer (50 mM, pH 7.4) containing 150 mM NaCl, 5 mM EDTA-Na, 0.25% Triton X-100 (Sigma-Aldrich), and 0.1% SDS, the immune complexes were resuspended in sample buffer with 2-ME, boiled for 3 min, resolved on 9% SDS-PAGE, and transferred to nitrocellulose membrane (Schleicher & Schuell, Dussel, Germany). The membrane was then sequentially immunoblotted with rabbit IgG against the $β_1$ integrin subunit and mAb against gC1qR (mAb 60.11). Immunoreactivity was revealed using an ECL kit (Amersham International, Amersham, U.K.).

Phosphorylation of ERK1/2 MAPKs in trophoblast cells

CTB cells were left to adhere to C1q (20 μg/ml), FN (20 μg/ml), or BSA-coated (20 μg/ml) wells for the indicated periods of time at 37˚C. At the end of the incubation time, ice-cold lysis buffer was added, and an equal amount of total cell lysates were resolved by 9% SDS-PAGE, transferred to nitrocellulose, and sequentially immunoblotted with anti-phosphorylated ERK1/2 mAb and anti-ERK antiserum. Immunoreactivity was revealed as described above. The amount of p-ERK1/2 MAPK was quantified by densitometric analysis using the ImageJ 1.37v program (National Institutes of Health, Bethesda, MD).

Confocal analysis

CTBs were plated at 37˚C on eight-chamber culture slides (BD Biosciences Discovery Labware, Milan, Italy) coated with C1q (10 μg/ml) or FN (10 μg/ml) and left to adhere for various lengths of time between 15 min and 2 h. Postfixation, the cells were permeabilized and stained with *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-y)-conjugated phallacidin (NBD-phallacidin) (Molecular Probes, Invitrogen) and mAb antipaxillin followed by Cy3-conjugated F(ab')₂ goat anti-mouse IgG (1:300) as previously described (19).

In other experiments, the C1q-bound CTBs were fixed with 3% paraformadehyde and incubated with Ultra V Block (Lab Vision, Bio Optica Milan, Milan, Italy) for 10 min at room temperature. The cells were then incubated with mAb P1H4 anti-human $β_1$ integrin (2 μg/ml) for 1 h at room temperature followed by Cy3-conjugated F(ab')₂ goat anti-mouse IgG. After extensive washing and blocking with mouse serum (1:100) in Ultra V Block (Lab Vision) for 10 min, the cells were incubated with biotin-labeled mAb anti-gC1qR 74.5.2 for a further 1 h at room temperature followed by FITC-conjugated streptavidin (1:100) (DakoCytomation).

Images were acquired with a Leica TCS SP2 confocal system (Leica Microsystems) using the Leica Confocal Software and a 63× fluorescence objective on a Leica DM IRE2 microscope (Leica Microsystems) or with a Nikon C1Si confocal system, using the Nikon EZ-C1 Confocal Software and a 63× fluorescence objective on a Nikon TE2000-U inverted microscope (Nikon, Melville, NY).

Table I. *Primers used for PCR analysis in accord to the method of Kaul and Loos (41)*

Sample	Primers	Sequence 5'→ 3'	Annealing Temperature (˚C)	Size of PCR Product (bp)	EMBL Database Accession No.
β-actin	Forward	CTACAATGAGCTGCGTGTGG	59	528	M10278
	Reverse	AAGGAAGGCTGGAAGAGTGC			
C1qA	Forward	ATGGTGACCGAGGACTTGTG	62	276	BC071986
	Reverse	GTCCTTGATGTTTCCTGGGC			
C1qB	Forward	ACCCCAGGGATAAAAGGAGAG	60	216	BC008983
	Reverse	GGCAGAGAAGGCGATTTTCTG			
C1qC	Forward	AAGGATGGGTACGACGGACTG	65	213	NM_172369
	Reverse	TTTCTGCTTGTATCTGCCCTC			

EMBL, European Molecular Biology Laboratory.

Pregnancy studies in mice

Eight- to 10-wk-old virgin female C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) and C1q-deficient mice (provided by Dr. Marina Botto, Imperial College, London, U.K.) were mated with 8–14-wk-old C57BL/6 or C1q-deficient mice, respectively. A group of pregnant females was killed at day 15, and the frequency of fetal resorption was calculated as previously described (44). Fetal weights were also determined. Some mice were studied until delivery, and the litter sizes were recorded at birth. Procedures that involved mice were approved by the Institutional Animal Care and Use Committee of the Hospital for Special Surgery and were conducted in strict accordance with guidelines for the care and use of laboratory research animals promulgated by the National Institutes of Health.

Immunohistochemical studies were performed on implantation sites from 6–8-wk-old pregnant mice kept at the Animal House of Istituto Clinico Humanitas (Rozzano, Italy) and collected at days 9.5, 10.5, and 12.5 of pregnancy. The experimental procedures were performed in accordance with the institutional guidelines and in compliance with the European (86/609/EEC) and Italian (D.L.116/92) laws.

Statistical analysis

The results are expressed as the mean ± SD. Statistical significance was determined using the Student t test to compare two groups of data. Values of $p \leq 0.05$ were considered to be statistically significant.

Results

C1q is localized in areas of invading trophoblasts

Immunohistochemical analysis of paraffin-embedded first-trimester placenta with rabbit IgG to human C1q showed a wide distribution of this complement component in the decidual stroma in the vessel wall. A strong signal was also seen in the Hofbauer cells (Fig. 1B, arrow) and in limited surface areas of the villi, whereas the overall staining of syncytiotrophoblasts and the underlying CTB layer of the villi was fainter (Fig. 1B). This finding was confirmed using goat IgG anti-C1q. Double staining of tissue sections for C1q and cytokeratin 7 to identify trophoblast cells documented the presence of C1q on trophoblasts localized in the trophoblast shell (Fig. 1C) and at the level of the CTB columns on their way to invade the decidua (Fig. 1D). Staining for C1q was particularly evident in the EVT spread in the decidua (Fig. 1E) and surrounding the spiral arteries (Fig. 1F), and a closer analysis of these cells revealed marked cytoplasmic C1q localization. We failed to detect C4 and C3 in the decidual tissue at sites of C1q deposition (Supplemental Fig. 1).

CTB cells express C1q at mRNA and protein level

The finding of intracellular staining for C1q in EVTs led us to investigate these cells for their ability to synthesize and secrete this complement component. Initially, we performed RT-PCR for the three chains of C1q (A, B, and C) on mRNA extracted from the whole placenta, CTBs, DSCs, the most abundant cells present in decidua, and PBDMs examined as a positive control. The results presented in Fig. 2 show that mRNA for the three chains was expressed in PBDMs and in whole decidual tissue, suggesting local production of C1q. Analysis of decidual cells revealed the presence of the three transcripts in CTBs, whereas DSCs express only the mRNA for the A chain (Fig. 2A).

Because C1q was observed predominantly in invasive trophoblasts, we hypothesized that the expression of C1q in CTBs was correlated with the acquisition of the invasive phenotype by these cells. To confirm this possibility, we analyzed CTBs expressing HLA-G, a marker of invasive trophoblasts, and HLA-negative cells, including villous trophoblasts, by RQ-PCR for the presence of mRNA for C1qC that discriminates C1q-secreting from nonsecreting cells (29). As shown in Fig. 2B, HLA-G^{+} cells expressed amounts of mRNA C1qC essentially similar to those detected in PBDMs, whereas HLA[−] CTBs failed to express the C1qC transcript.

Next, we investigated the ability of CTBs to synthesize and secrete C1q. For this purpose, 10[6] invading CTBs were plated in
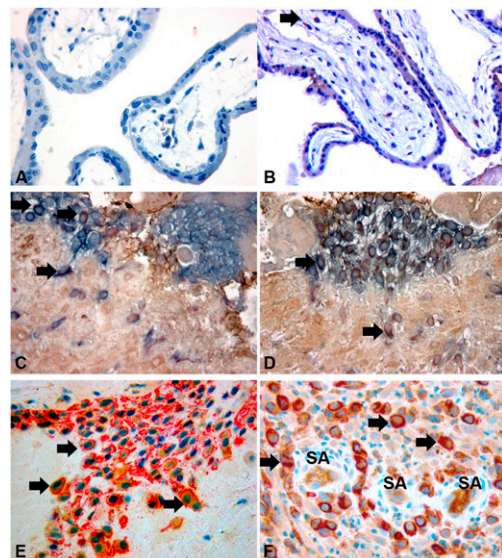

FIGURE 1. Localization of C1q in first-trimester human placenta. Single staining of section of maternal decidua and villi with rabbit IgG anti human-C1q (*B*) or unrelated rabbit IgG (*A*) developed with the Labeled StreptAvidin Biotin + HRP kit (DakoCytomation) and diaminobenzidine as substrate. The sections were counterstained with hematoxylin (DakoCytomation). Double staining of maternal decidua and villi sections with rabbit IgG anti–human-C1q (brown) and mAb OU-TL 12/30 to human cytokeratin 7 (1:50) developed with Alkaline Phosphatase Anti-Alkaline Phosphatase Complex (DakoCytomation) and NBT/5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (DakoCytomation) as chromogen (blue) (*C, D*) or Fast Red (magenta) (*E, F*). Original magnification ×400. Note the failure of villous CTBs and syncytiotrophoblasts to stain for C1q (*B*) in contrast with the positive staining of two positive Hofbauer cells (*B*, arrow) and the wide distribution of C1q staining in the decidual stroma (*C, D*). Note also staining for C1q in EVTs migrating into decidua (C–E, see arrows) or localized around spiral arteries (*F*, see arrows). SA, spiral artery.

multiwell chambers, and the cell supernatants were collected after 48 h of culture and stored at −80°C. The amount of C1q measured by ELISA in these samples varied between 50 and 100 ng/10[6] cells and was approximately half of the amount of C1q secreted by PBDMs (Fig. 2C).

C1q secreted by trophoblasts is used by these cells to invade the decidua

Having found that C1q is secreted by the invading CTBs and widely distributed in decidua, we sought to investigate the role played by this complement component in trophoblast motility. To evaluate cell binding to C1q, freshly isolated CTBs were labeled with the fluorescent probe *FAST* DiI and allowed to adhere to solid phase-bound C1q or purified gC1q in 96-well plates for 30 min at 37°C, using BSA and FN as negative and positive controls, respectively. Approximately 40% of CTBs adhered to C1q as opposed to 20% of cells adhering to BSA and 60% to FN. Interestingly, the percentage of CTBs binding to gC1q was essentially similar to that observed with the whole C1q molecule, suggesting that trophoblast cells interact with the globular domain of the molecule (Fig. 3A).

CTBs attached to C1q exhibited a morphologic appearance different from that of FN-bound cells, being much smaller and displaying wide ruffles that extended in multiple directions, in contrast with the marked spreading of CTBs adherent to FN (Fig. 3B). Staining of FN-bound cells with NBD-phallacidin revealed a high number of actin-containing stress fibers and localization of paxillin at the tips of stress fibers already 30 min after cell adhesion. In contrast, the cells attached to C1q showed diffuse
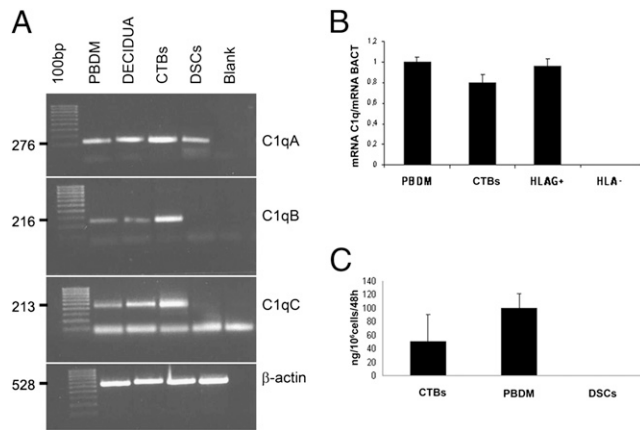
FIGURE 2. CTBs express C1q at mRNA and protein level. RNA prepared from PBDMs, first-trimester decidua, and freshly isolated CTBs and DSCs were analyzed for C1qA, -B, and -C and β-actin expression by RT-PCR (*A*). The relative amount of mRNA for C1qC in PBDMs, total CTBs, HLA-G$^+$, and HLA$^-$ CTBs was evaluated by RQ-PCR and normalized with reference to the β-actin value. The results were expressed as AUs, in which 1 AU represents the value obtained with PBDMs used as a positive control (*B*). Levels of C1q secreted by CTB and, for comparison, by PBDMs and DSCs was measured in the cell supernatant by ELISA after 48 h of culture using solid phase-bound goat IgG anti-C1q to trap C1q and the same biotin-labeled Ab to reveal bound C1q followed by alkaline phosphatase-conjugated streptavidin (*C*). Data from five independent experiments are shown and represent the mean ± SD.

positivity for paxillin and subcortical organization of actin at the same time point (Fig. 3*B*), but some focal plaques were seen after 2 h of CTB adhesion (data not shown).

Next, we examined C1q and FN for their ability to promote CTB migration. The upper side of the transwell inserts was coated with C1q or FN or both, and CTBs were allowed to migrate in the presence or in the absence of DCCMs added to the lower chamber as a chemotactic stimulus. C1q and FN caused migration of ~10% of CTBs in the absence of DCCMs (data not shown). This value raised to 24% when the cells were allowed to migrate through FN in the presence of DCCMs and further increased to 41% through the insert coated with C1q (Fig. 3*C*), suggesting that this complement component was more efficient than FN in promoting CTB migration.

To mimic more closely the situation encountered by CTBs that interact with the ECM while invading the decidua, the insert of the transwell was first coated with FN and then with C1q. FN was selected as one of the ECM molecules largely expressed in decidua and found to strongly interact with C1q (Supplemental Fig. 2). The results presented in Fig. 3*C* clearly show that C1q bound to FN maintains the ability to support migration of 40% CTBs.

gC1qR and β₁ integrin are involved in trophoblast–C1q interaction

We have previously shown that gC1qR/p33 (gC1qR) on endovascular trophoblasts is involved in the adhesion of these cells to DECs expressing C1q on their surface (29). To evaluate the contribution of gC1qR to CTB–C1q interaction, the adhesion assay was performed using CTBs preincubated with two different Abs to gC1qR, mAb 60.11 or mAb 74.5.2, that recognize distinct epitopes on the molecule, or an Ab to cC1qR for 30 min at 37°C. As shown in Fig. 4*A*, the adhesion of CTBs to C1q was inhibited almost completely by mAb 60.11, whereas the other mAb 74.5.2 to gC1qR and the rabbit IgG to cC1qR were ineffective. Based on our previous findings that trophoblast cells use $\alpha_4\beta_1$ integrin to
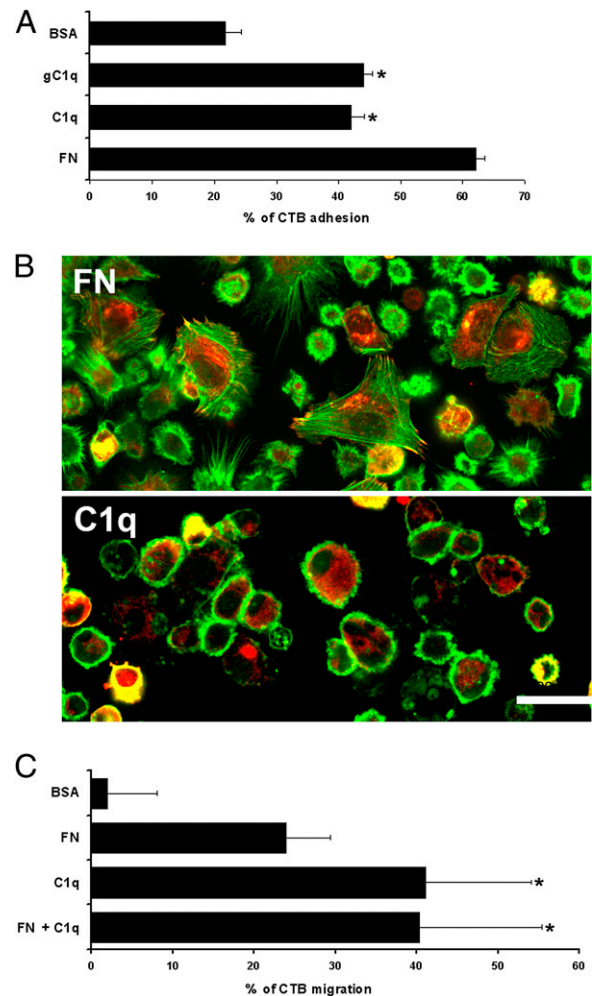

FIGURE 3. C1q mediates CTB adhesion and migration. *A*, Adhesion of *FAST* DiI-labeled (Molecular Probes, Invitrogen) CTBs to wells of 96-well plates coated with C1q, gC1q, FN, or BSA at the concentration of 10 μg/ml was measured after 30 min of incubation at 37°C. The results are given as percent of cell adhesion with reference to a calibration curve established with an increasing number of labeled cells. Data from five independent experiments are presented as mean ± SD. ∗$p < 1 \times 10^{-5}$. *B*, CTBs plated for 30 min on eight-chamber culture slides coated with FN (*upper panel*) or C1q (*bottom panel*), fixed, and stained with NBD-phallacidin (green) and mouse monoclonal anti-paxillin Ab followed by Cy3-conjugated goat anti-mouse IgG (red) were analyzed by confocal microscope. Original magnification ×600. Note the presence of actin-containing stress fibers and focal adhesion formation in cells attached to FN and, conversely, subcortical actin organization and diffuse positivity for paxillin in cells bound to C1q. Confocal microscope images were acquired with a Nikon C1Si confocal system using the Nikon EZ-C1 Confocal Software and a 63× fluorescence objective on a Nikon TE2000-U inverted microscope (Nikon). *C*, Migration of CTBs was evaluated using *FAST* DiI-labeled cells (2×10^5) added to the upper chamber of a transwell and allowed to migrate for 4 h through inserts coated with FN (40 μg/ml), C1q (40 μg/ml), or C1q (20 μg/ml)–FN (20 μg/ml) complex in the presence of DCCMs added to the lower chamber as a chemotactic stimulus. The results obtained from four independent experiments are shown as percent of cell migration and presented as mean ± SD. ∗$p < 0.05$.

interact with EMILIN1, a member of C1q family proteins sharing a gC1q signature domain (19, 45), we examined the effect of neutralizing Abs to α_4 and β_1 integrin in the adhesion assay. Our results (Fig. 4*A*) suggest that both α_4 and β_1 integrin are also involved in CTB adhesion to C1q, and the same molecules appear to play a role in trophoblast migration through C1q (Fig. 4*B*).
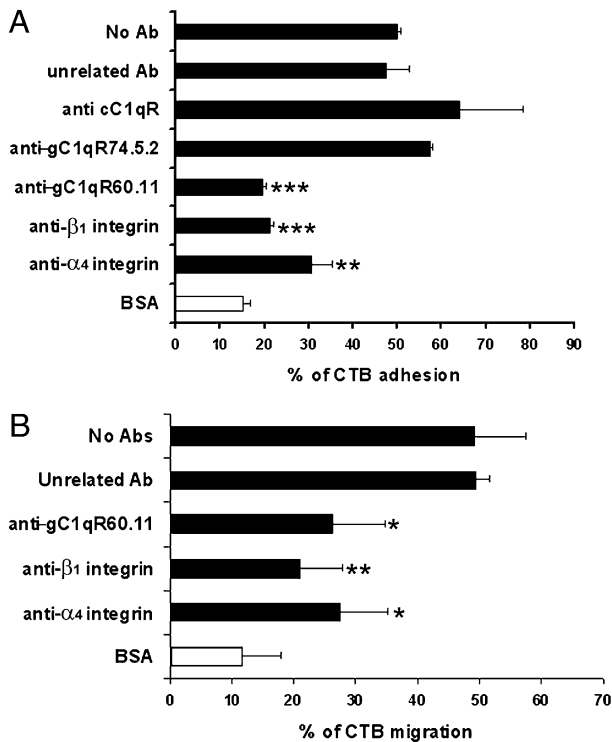
FIGURE 4. Involvement of gC1q R and β_1 integrin in trophoblast–C1q interaction. *A*, *FAST* Dil-labeled (Molecular Probes, Invitrogen) CTBs were allowed to adhere to C1q in the presence of mAbs anti-gC1qR 60.11 and 74.5.2, rabbit IgG anti-cC1qR, mAb P5D2 directed to β_1 integrin, mAb P5H4 to α_4 integrin, mouse IgG1 isotype control, or medium alone. The results are given as percent of cell adhesion. *B*, Migration of CTBs through inserts of transwells coated with C1q was evaluated using DCCMs as a chemotactic stimulus in the presence of mAb 60.11, mAb P5D2, mAb P5H4, or mouse IgG1 isotype control. The results are given as percent of cell migration. Data from five independent experiments are shown and represent the mean $\pm$ SD. $*p < 0.05$; $**p < 0.01$; $***p < 5 \times 10^{-5}$.

Because gC1qR was found to be associated with β_1 integrin on the surface of ECs upon binding to C1q (46), we considered the possibility that this may also happen in our model system. To address this issue, CTBs were incubated with solid phase-bound C1q or FN, lysed, and sequentially immunoprecipitated on protein G-Sepharose beads coupled with mAbs to gC1qR and anti-β_1 integrin. As shown in Fig. 5*A*, immunoblot analysis revealed the presence of a band comigrating with β_1 integrin on gC1qR immunoprecipitates (IPs). Moreover, by using confocal analysis, we found that gC1qR colocalizes with β_1 integrin at points of contact between CTBs and C1q (Fig. 5*B*). Together, these results suggest that CTB binding to C1q results in the association of gC1qR with β_1 integrins.

Binding of trophoblast cells to C1q enhances ERK1/2 MAPK phosphorylation

Based on the ability of C1q to support trophoblast adhesion and migration, we decided to investigate if binding of C1q to trophoblast cells could also result in the activation of signaling molecules involved in the regulation of cellular processes associated with adhesive and migratory functions. For this purpose, freshly isolated CTBs were allowed to adhere to wells coated with C1q, FN, or BSA for different lengths of time at 37°C, and the phosphorylation status of ERK1/2 MAPKs on total cell lysates was evaluated by immunoblotting. As shown in Fig. 6, binding of CTBs to C1q resulted in the activation of ERK1/2 MAPKs that was already evident at 5 min and

still persisted 20 min poststimulation. Interestingly, the levels of ERK1/2 MAPK phosphorylation evaluated by densitometric analysis in CTBs bound to C1q were comparable to those observed in cells adherent to FN and were ~5-fold higher than the levels observed in BSA-stimulated CTBs (Fig. 6). The involvement of ERK1/2 MAPK in the process of trophoblast motility was further supported by the finding that the synthetic inhibitor PD 098059 (25 mM; Calbiochem-Novabiochem), which specifically prevents activation of the ERK-activating kinase MAPK/ERK-1 (47, 48), completely inhibited CTB migration through C1q (data not shown).

Defective invasion of CTBs in C1q$^{-/-}$ mice associated with embryo growth restriction

To examine the actual contribution of C1q to pregnancy outcomes, we performed a gross and microscopic time-course analysis of implantation sites from C1q$^{-/-}$ mice and wild-type (WT) controls.

Increased fetal resorption frequency (Fig. 7*A*) and diminished fetal weight (Fig. 7*B*) were observed in C1q-deficient mice at day 15 of pregnancy compared with WT mice. In accordance with the increased fetal death observed at day 15 of pregnancy, C1q-deficient mice showed smaller litter sizes at birth when compared with WT mice (Fig. 7*C*).

Histopathologic analysis of implantation sites from C1q$^{-/-}$ mice at different time points of gestation from 7.5–14.5 d revealed signs of impaired development as compared with WT controls matched for gestational age. Changes were mostly noticed starting from day 9.5 and were characterized by impaired labyrinth development (Fig. 8, *upper panels*), lower degree of vascular remodeling (Fig. 8, *lower panels*), and marked edema in the interstitium of decidual stroma. In addition, staining of decidua from C1q$^{-/-}$ mice for cytokeratin revealed marked reduction in trophoblast invasion and nearly complete absence of periarteriolar clusters of trophoblasts compared with the decidua obtained from gestational age-matched WT mice.

Discussion

Trophoblast invasion of decidua is a critical process for placentation and embryo implantation and is regulated by a series of integrated adhesion and signaling events that include the cross talk between trophoblast and decidual cells requiring interaction between cell-surface adhesion molecules and ECM proteins. The data presented in this study suggest that C1q synthesized and secreted by EVTs is part of this complex network and is used by EVTs to migrate inside the decidua.

While searching for complement involvement in tissue remodeling that occurs in maternal deciduas following embryo implantation, we observed deposits of C1q on decidual stroma in the absence of C4 and C3. This finding was unexpected because C1q is usually detected on cells and tissues in pathological conditions associated with complement activation through the classical pathway. As a result, biologically active products that are primarily involved in the removal of immune complexes, apoptotic cells, and infectious and other harmful agents are released. Even more surprising was the observation that the deposits of C1q in maternal decidua were mainly seen in areas of trophoblast invasion, suggesting that C1q may have been synthesized locally and used for some special functions unrelated to complement activation. Staining for C1q was also observed in the villi, particularly in the Hofbauer cells that displayed a strong signal. This is not surprising because they belong to the monocyte/macrophage lineage known to be a major source of C1q (49–51). A low level of staining was also apparent in villous trophoblast cells, most likely due to C1q deposits from maternal blood, as these cells were found to lack the C1qC message required for the assembly of C1q.
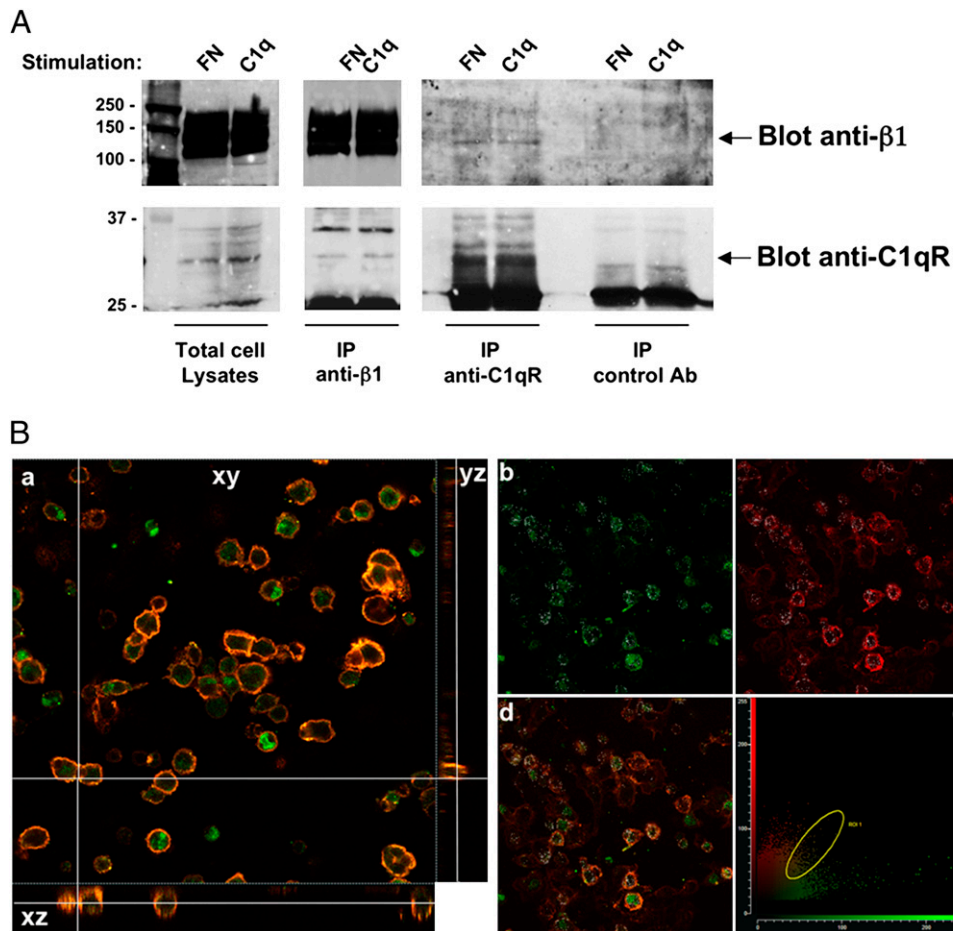
FIGURE 5. Colocalization gC1qR/β_1 integrin. *A*, Western blot analysis of cell lysates from CTBs left to adhere to C1q (20 μg/ml) or FN (20 μg/ml) for 30 min at 37˚C and immunoprecipitated first with normal mouse IgG and then either with mAb 60.11 anti-gC1qR or mAb 4B4 anti-β_1 integrin subunit. The presence of β_1 integrin subunit (130 kDa) and gC1qR (33 kDa) was revealed using rabbit polyclonal Abs. These results are representative of one out of three independent experiments. *B*, Surface distribution of gC1qR and β_1 integrin molecules on CTB cells allowed to adhere to C1q for 1 h at 37˚C. Postfixation, the cells were immunostained for gC1qR (green) and for β_1 integrin (red), as detailed in *Materials and Methods* and then examined by confocal microscopy. *Ba*, A representative field of a z-step (*xy*) analysis and the corresponding z projections (*yz* and *xz*). A representative colocalization analysis of gC1qR (*Bb*) and β_1 integrin (*Bc*). *Bd*, Merged image for gC1qR and β_1 integrin showing several spots (pseudocolored in gray by the software) of evidence of the presence of both signals in the plasma membrane of several cells. *Be*, The relative colocalization graph indicating the pixels of the colocalized signals (yellow ellipse, ROI) calculated by colocalization function of Leica Confocal Software (Leica Microsystems). Original magnification ×200. ROI, region of interest.

The extracellular deposition of C1q in close proximity to the surface of trophoblasts invading the decidua and, more importantly, the cytoplasmic staining of EVTs for this complement component,
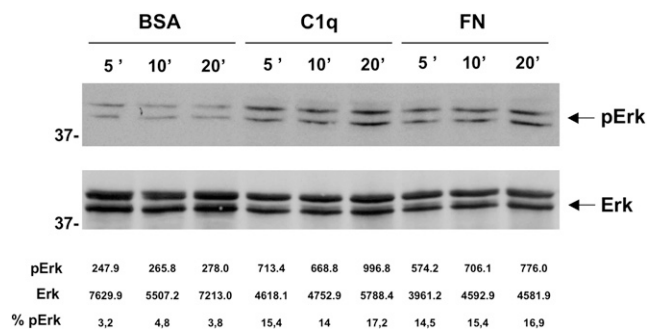

FIGURE 6. Binding of trophoblast cells to C1q enhances ERK1/2 MAPK phosphorylation. CTBs were allowed to adhere to C1q-, FN-, or BSA-coated wells for different lengths of time, and the phosphorylation status of ERK1/2 MAPKs (42 and 44 kDa) was evaluated on total cell lysates by immunoblotting. The amount of ERK and p-ERK1/2 MAPK was quantified by densitometric analysis using the ImageJ 1.37v program (National Institutes of Health) and expressed as absolute value as well as percentage of p-ERK.

suggest that EVT may be a good candidate for the local production of C1q. Evidence supporting this conclusion was obtained by the finding that EVTs expressed the messages for the three chains of C1q and secreted a detectable amount of the molecule in the culture supernatant. C1q is one of the few complement components synthesized at the extrahepatic sites, as suggested by the observation that bone marrow transplants from complement-sufficient mice restore C1q levels in C1q-deficient animals (52). Besides cells of the monocyte/macrophage lineage, other cell types contribute to the production of C1q, including follicular dendritic cells and interdigitating cells (53), fibroblasts (54), and epithelial cells of the intestinal and urogenital tracts (55). Our data suggest that decidual tissue is an important source of C1q in pregnancy and contributes to its local synthesis both with EVTs and, as recently reported, with the ECs of spiral arteries (29). C1q secreted by DECs binds avidly to the cell surface and acts as a physical link between endovascular trophoblasts and DECs (29).

The strong staining of EVTs for C1q explains the preferential localization of this complement component at sites of trophoblast invasion. The association of C1q with the invasive phenotype of trophoblast cells is further supported by the finding that this protein is synthesized exclusively by trophoblasts expressing HLA-G, a well-
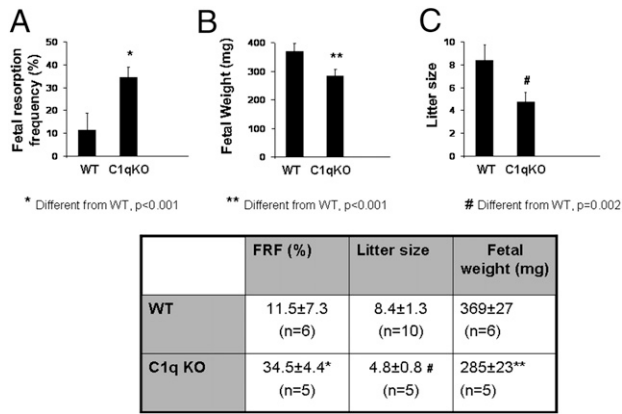
	FRF (%)	Litter size	Fetal weight (mg)
WT	11.5±7.3 (n=6)	8.4±1.3 (n=10)	369±27 (n=6)
C1q KO	34.5±4.4* (n=5)	4.8±0.8 # (n=5)	285±23** (n=5)

FIGURE 7. Pregnancy outcomes in C1q-deficient mice. *A*, Fetal resorption frequency. *B*, Fetal weight. *C*, Litter size at birth. Increased fetal resorption frequency (*A*) and diminished fetal weight (*B*) was observed in C1q-deficient mice at day 15 of pregnancy compared with WT mice. In accordance with the increased fetal death observed at day 15 of pregnancy, C1q-deficient mice showed smaller litter sizes at birth when compared with WT mice (*C*).

known marker of EVT. Thus, C1q can be considered as an additional marker of EVT, and its synthesis can be added to the list of phenotypic changes of decidual trophoblasts, including the expression pattern of adhesion molecules (56) and the secretion of hyperglycosylate human chorionic gonadotropin (57).

The recognition that C1q secreted by EVT as a soluble molecule is deposited on decidual stroma in the absence of C4 and C3 led us to hypothesize that trophoblast cells may use C1q to invade the decidua. This process requires interaction of C1q with proteins of the ECM present in the decidua with a characteristic distribution in areas of trophoblast invasion and around the spiral arteries. Binding of C1q to several of these proteins was documented by the results of the immunoenzymatic assay and is in line with previous reports showing interaction of C1q with FN (58), laminin (59), small leucine-rich repeat proteins, and mucopolysaccharides including heparin sulfate and dermatan sulfate (60).

Several pieces of evidence argue for gC1q as the portion of C1q molecule involved in the promotion of trophoblast adhesion and migration, and the most compelling one is that the effect of C1q was reproduced with purified gC1q. Strictly related to this point is
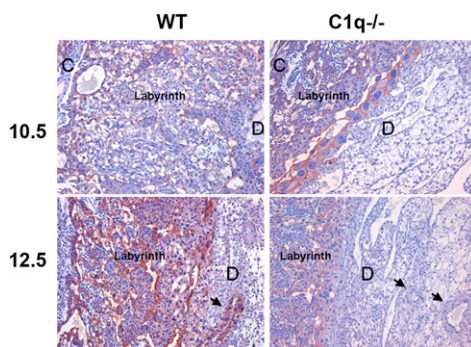
the finding that EMILIN1, an important component of decidual stroma and a member of C1q and TNF superfamily characterized by a common gC1q signature domain (45), is also implicated in trophoblast invasion of decidua (19). Equally important in this context is our previous observation that CTBs only express gC1qR, which selectively recognizes gC1q while failing to express the receptor for the collagen portion of C1q (29). The role played by gC1qR–gC1q interaction in trophoblast adhesion and migration is further supported by the inhibition obtained with mAb 60.11 to gC1qR, which recognizes a C1q binding site on this receptor, whereas mAb 74.5.2, which reacts with an epitope of gC1qR involved in the reaction with the high m.w. kininogen (61), was ineffective.

Interaction of C1q with CTBs results in cell activation, as suggested by the enhanced phosphorylation of ERK1/2 MAPK to a level essentially similar to that observed in CTBs bound to FN. This finding extends previous observation by Liu et al. (62), who showed that activation of PI3K/AKT, ERK, and JNK pathways is required for the chemotaxis of immature dendritic cells to C1q, whereas activation of AKT and p38 pathways is needed for C1q-mediated enhancement of mature dendritic cell chemotaxis to CCL19. However, because gC1qR lacks direct access to the intracellular compartment, the sole engagement of this receptor by bound C1q is unlikely to deliver the activation signal to CTBs for which the contribution of β_1 integrin is required. The involvement of $\alpha_4\beta_1$ integrin expressed on the surface of invasive trophoblasts (19, 20, 63) in C1q-mediated cell adhesion and migration is suggested by the inhibition obtained with mAbs to α_4 and β_1 integrins, the colocalization of this integrin with gC1qR on CTBs adhering to bound C1q, and coprecipitation with the same receptor in extract of these cells. Similar results were obtained by Feng and colleagues (46), who showed that EC adhesion and spreading on C1q-coated microtiter wells was prevented both by mAb 60.11 to gC1qR and mAb to β_1 integrins and that gC1qR and β_1 integrin cocaptured on C1q-bound ECs. Involvement of integrins in cell activation by C1q has also been established on murine mast cells that have been shown to express $\alpha_2\beta_1$ integrin as a novel receptor for the collagen-like sequence of C1q (64–66).

The best way to prove the in vivo relevance of our observations is to evaluate pregnancy outcome in C1q-deficient patients. Unfortunately, this is difficult to ascertain because C1q deficiency is a rare genetic defect and is often associated with a severe form of systemic lupus erythematosus. However, judging from the reduced litter size, the decreased fetal weight, and, more importantly, the increased number of fetal losses observed in C1q knockout mice, one can confidently conclude that the absence of C1q may impair pregnancy outcome. This is in line with the deficient trophoblast invasion and reduced labyrinth development and remodeling of spiral arteries observed in pregnant C1q$^{-/-}$ mice compared with WT animals of the same gestational age.

In summary, we have shown that C1q secreted by migrating CTB cells acts on the same cells through an autocrine mechanism to promote decidual invasion and more generally regular pregnancy outcome.

Acknowledgments

Disclosures
The authors have no financial conflicts of interest.

FIGURE 8. Trophoblast invasion and labyrinth development in C1q$^{-/-}$ and WT pregnant mice. Sections of implantation sites collected from C1q$^{-/-}$ and WT mice at days 10.5 (*top panel*) and 12.5 (*bottom panel*) of pregnancy stained with rabbit IgG anti-cow cytokeratin wide spectrum recognizing mouse cytokeratin and counterstained with hematoxylin. C and D represent the conceptus and the decidual sides, respectively. Note the impaired labyrinth development, reduced trophoblast invasion of decidua, and absence of perivascular trophoblast cells (arrows) in C1q$^{-/-}$ mice compared with WT animals at days 10.5 and 12.5 of pregnancy.

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