Cyclosporin A promotes crosstalk between human cytotrophoblast and decidual stromal cell through up-regulating CXCL12/CXCR4 interaction

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Submitted on August 1, 2011; resubmitted on February 26, 2012; accepted on March 2, 2012

BACKGROUND: Our previous studies have demonstrated that cyclosporin A (CsA) can increase the cell number and invasion by human first-trimester trophoblasts and induce maternal–fetal tolerance. C-X-C chemokine receptor type 4 (CXCR4) and C-X-C chemokine ligand 12 (CXCL12) are important mediators at the maternal–fetal interface during early pregnancy. In this study, we further investigate the molecular mechanisms underlying modulation by CsA of the crosstalk between human cytotrophoblast and decidual stromal cell (DSC).

METHODS: Human first-trimester cytotrophoblast and DSC were treated with CsA in the absence or presence of U0126 pretreatment, and then the mRNA and protein levels of CXCL12 and CXCR4 were measured by RT–PCR, qPCR, in-cell western blots and enzyme-linked immunosorbent assay (ELISA), respectively. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and Matrigel invasion assays were used to determine the invasiveness of cytotrophoblast, respectively. The activity of matrix metalloproteinase (MMP)-9 and MMP-2 was detected by gelatin zymography. A co-culture with direct contact between cytotrophoblast and DSC was established and used to investigate the interaction between these two cells.

RESULTS: CsA up-regulated CXCL12 and CXCR4 expression in human first-trimester cytotrophoblast cells, but not in DSCs. Blocking the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK1/2) signaling by U0126 abrogated the CsA-induced increase in CXCL12 and CXCR4 expression and neutralizing antibodies to CXCL12 or CXCR4 completely inhibited the CsA-induced increase in cell number, invasion and MMP-9 and MMP-2 activity of cytotrophoblast. CsA also significantly promoted the activity of MMP-9 and MMP-2 in DSCs, but this was unaffected by CXCL12 or CXCR4 neutralizing antibody. Furthermore, the CsA-induced MMP-9 and MMP-2 activity and the invasiveness of cytotrophoblast in the cytotrophoblast and DSC co-culture were significantly increased compared with CsA-treated trophoblast cultured alone, and CXCR4 blocking antibody effectively abolished the increased MMP activity and invasion of cytotrophoblasts in the cytotrophoblast-DSC co-culture stimulated by CsA.

CONCLUSIONS: CsA can promote the crosstalk between cytotrophoblast and DSC through up-regulating CXCL12/CXCR4 interaction via MAPK signaling, resulting in the increased numbers of and invasion by human cytotrophoblast cells.

Key words: cyclosporin A / CXCL12/CXCR4 / cytotrophoblast / decidual stromal cell (DSC)

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Introduction

Placentaation requires the complex regulation of cytotrophoblast proliferation, differentiation and invasion, which is an essential process for successful pregnancy and fetal development. An insufficient proliferation and invasion of cytotrophoblast is not only associated with pre-eclampsia and fetal growth restriction, but also with early or late miscarriage (MacLennan et al., 1972; Hustin et al., 1990; Kaufmann et al., 2003; Ball et al., 2006; Hupperetz, 2011; Yang et al., 2011). How to improve the pregnancy outcomes related to cytotrophoblast function disorders is a big challenge to researchers and doctors practicing reproductive medicine. Unfortunately, there are few effective treatments until now (Rumbold et al., 2006).

Cyclosporin A (CsA) is a powerful immunosuppressant that is widely used to prevent organ rejection and to treat several autoimmune diseases, including rheumatoid arthritis, nephritic syndrome and systemic lupus erythematosus (Germano et al., 2011; Hetland, 2011; Fujinaga et al., 2012). CsA prevents the activation of lymphokine genes essential for T-cell proliferation by disrupting calcium-dependent signal transduction pathways in leukocytes (O’Keefe et al., 1992). In addition to the classical action on leukocytes, CsA also exerts different effects on other types of cells, and thus regulates disparate biological functions (Alvarez-Arroyo et al., 2002; Ishimatsu-Tsuji et al., 2010; Periyasamy et al., 2010; Wu et al., 2010; Yang et al., 2010; Lablanche et al., 2011). For example, CsA at a low dosage (0.01 μM) is cytoprotective, but at high dosage of CsA (>1.0 μM) increases apoptosis of endothelial cells (Alvarez-Arroyo et al., 2002). It has also been shown in our previous in vitro studies that CsA at low concentrations (0.0001, 0.001, 0.01 and 1.0 μM) increases cell number of and invasion by human first-trimester cytotrophoblast while inhibiting apoptosis of these cells (Zhou et al., 2007; Du et al., 2007b, 2008). Our previous researches on mice have also demonstrated that administration of CsA (0.1, 1.0 and 10 mg/kg) during the window of implantation reduces the embryo resorption rate of CBA/J x DBA/2 abortion-prone matings by protecting the fetus from maternal immune attack and increasing murine cytotrophoblast cell number and invasion (Du et al., 2007a; Zhou et al., 2008a). These observations suggest that CsA could be used as an effective therapy for some pregnancy complications, such as spontaneous abortion, but the regulatory mechanism underlying the action of CsA on cytotrophoblast cell number and invasion remains poorly understood.

It has been gradually recognized that there is a complicated network of chemokines at the maternal–fetal interface, and as an integral part of human placentation, they play important roles in immune and non-immune functions of placenta (Dominguez et al., 2003; Hanna et al., 2003; Hannan et al., 2006; Hannan and Salamonsen, 2007; Kuang et al., 2009; Chen et al., 2010; Dimitriadis et al., 2010; Guo et al., 2010). Of various chemokines and their receptors, chemokine (C-X-C motif) ligand 12 and chemokine (C-X-C motif) receptor 4 (CXCL12/CXCR4) are an extraordinary chemokine/chemokine receptor pair, and involved in several crucial reproductive processes, including uterine natural killer cell recruitment, placentaation, implantation and embryogenesis (Dominguez et al., 2003; Hanna et al., 2003; Wu et al., 2005). Our previous studies have also shown that cytotoxicant-derived CXCL12 not only promotes invasiveness in an autocrine manner, but also recruits peripheral CD56^bright^CD16^−^ CXCR4-positive NK cells into decidua, and facilitates the synchronization between cytotrophoblast cells and decidual stromal cell (DSCs) in a paracrine manner (Wu et al., 2004, 2005; Zhou et al., 2008a; Ren et al., 2012). These results strongly suggest that the CXCL12/CXCR4 axis is an important regulator of the function of fetal-derived cytrophoblast cells and maternal uterine cells crucial for placentation and the establishment of maternal–fetal relationship.

The fact that CXCL12/CXCR4 axis is a key signal for the maternal–fetal crosstalk (Dominguez et al., 2003; Hanna et al., 2003; Wu et al., 2004; Wu et al., 2005; Zhou et al., 2008a; Ren et al., 2012), combined with the fact that both CXCL12/CXCR4 and CsA exert their function via activating the mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK) signals (Wu et al., 2004; Zhou et al., 2007; Du et al., 2007b, 2008; Yano et al., 2009; Siedlecki et al., 2010; Sun et al., 2010), inspired us to investigate whether the regulation by CsA of the maternal–fetal interface is mediated by the CXCL12/CXCR4 axis. Thus, in this study, we first determined the effect of CsA on gene and protein expression of CXCL12/CXCR4 in human first-trimester cytotrophoblast and DSC by CsA. Thereafter the role of CXCL12/CXCR4 in the crosstalk between cytotrophoblast and DSC and its modulation by CsA was investigated by in vitro invasion assay and zymography in the co-culture.

Materials and Methods

Human placental and decidual tissue collection

All procedures involving participants in the study were approved by the Human Research Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University, and all subjects completed an informed consent for the collection of tissue samples. Human placental and decidual tissues were from elective vaginal termination of first-trimester pregnancies (gestational age, 6–9 weeks) for non-medical reasons. All the tissues were immediately collected into ice-cold Dulbecco’s modified Eagle medium (DMEM) with high D-glucose or DMEM/F12 (Gibco, Grand Island, NY, USA), transported to the laboratory within 30 min after surgery and washed in calcium- and magnesium-free Hanks balanced salt solution (HBSS) for trophoblast or DSC isolation.

Isolation and primary culture of first-trimester human cytotrophoblast cells

The first-trimester human placentas were separated carefully from the decidua under a stereomicroscope, pooled and miniced into small fragments, then treated by repeated trypsin digestions and Percoll gradient centrifugation according to our previous method (Wu et al., 2004). Briefly, the placental tissues obtained from seven to eight separate individuals were pooled and digested by 0.25% trypsin (Bio Basic Inc, BBI, Ontario, Canada) and 0.02% DNase type I (Sigma, Saint Louis, Missouri, USA) at 37°C with gentle agitation for 5 min. The liquid suspension was discarded and the residual tissue was subjected to four cycles of 10-min digestion with the same enzymes. Each time trypsin digestion was stopped with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and the liquid digest was removed. The four digests were, pooled, centrifuged at 300g for 10 min, and the pellet resuspended in 4 ml DMEM with high D-glucose (Gibco, Grand Island). This suspension was carefully layered over a discontinuous Percoll gradient consisting of 70–5% Percoll (vol/vol) and the liquid digest was removed. The four digests were, pooled, centrifuged at 1000g for 20 min, the cells sedimenting at densities...
Isolation and primary culture of human first-trimester DSCs

Isolation of DSCs was performed as published before by our group (Zhou et al., 2008b). Briefly, the specimens were minced and digested in six cycles of 10–20 min using 0.25% trypsin (Bio Basic Inc.) in a shaking water bath at 37°C. The cell digest was then passed through a 38 μm gauze and purified by centrifugation through a discontinuous Percoll gradient (20%/40%/60%). The cells were collected and maintained in DMEM/F12 complete medium with 10% FBS. After primary culture for 30 min at 37°C in 5% CO2, the non-adherent lymphocytes were removed by washing, leaving a 98% purity of DSCs. The characterization of DSCs cultured in vitro could be obtained in our previous publication (Zhou et al., 2008b).

Reverse transcription–polymerase chain reaction

The primary cytotrophoblast cells and DSCs were seeded at a density of 8 × 105 cells/ml in six-cell culture plate. Because CsA was dissolved in dimethyl sulfoxide (DMSO), in the present study, we used DMSO (0.1%, v/v) as a negative control. After treatment with 0.1%, v/v DMSO, 1.0 μM CsA, 30 μM U0126 or 0.6 μM CsA plus 30 μM U0126 for 24 h, total cellular RNA was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The complementary DNA (cDNA) was generated with oligo(dT)18 primers using Revert Aid First Strand cDNA Synthesis Kit (Fermentas Life Science, USA). The cDNA was amplified by PCR reaction in a final volume of 50 μl containing 2 mM dNTP, 0.8 mM specific primers, 1.25 U Taq DNA polymerase, 1 mM MgCl2 and 1 × reaction buffer. A 5-min precycle at 95°C was followed by 30 cycles of 1 min at 94°C, 30 s at 55°C and 30 s at 72°C. After the final cycle, the samples were kept at 72°C for 15 min to complete the synthesis. The primer pairs for cDNA amplification were as follows: 5′-GAA CTT CCT ATG CAA GCC AGT CC-3′ (forward) and 5′-CCA TGA TGT GCT GAA ACT GGA AC-3′ (reverse) for human CXCR4; 5′-ATG AAG GCC AAG GTC GTC GTC G-3′ (forward) and 5′-TGT TGT TGT TGT TGT TCA GCC G-3′ (reverse) for human CXCL12; 5′-GGG GAG CCA AAA GGG TCA TCA TCT-3′ (forward) and 5′-GAG GGG CCA TCC ACA GTG TCT T-3′ (reverse) for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The expected fragment lengths of CXCL12, CXCR4 and GAPDH were 206, 202, and 235 bp, respectively. The PCR reaction products (10 μl) were electrophoresed on 2% agarose gels and ethidium bromide-stained bands were photographed and analyzed by gel imaging systems. The relative intensity of CXCL12 or CXCR4 = absorbance value of the target fragment/that of GAPDH. The samples were run in triplicate and experiments were repeated three times.

Quantitative RT–PCR

The treatment and primers for CXCL12, CXCR4 and GAPDH were the same as in ‘RT–PCR analysis’. Real-time RT–PCR was done as described previously (Huang et al., 2006; Guo et al., 2010).

Cell number assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Sigma Chemicals] assay was applied to evaluate the effects of CsA on cell number. The isolated cytotrophoblast cells were re-suspended in DMEM-high glucose with 15% FBS, and seeded at a density of 3 × 104 cells/well in 96-well flat-bottom microplates. The cells were starved with DMEM-high glucose containing 2% FBS for 12 h before treatment. The medium was removed once again, and the cells were stimulated with 0.1%, v/v DMSO or 1.0 μM CsA. Before treatment with CsA, the cells were pretreated with 40 μg/ml of CXCL12 or 20 μg/ml CXCR4 neutralizing antibody (R&D Systems) or U0126 (Sigma) (30 μM). DMSO or mouse isotype control antibody (Sino-America Co. Ltd, China) was used as a negative control. MTT reagent (20 μl) was added to each well of 96-well microplates and incubated at 37°C in 5% CO2 for 4 h. The medium was decanted and 100 μl of ethanol was added to solubilize the metabolite of MTT, formazan, only produced by the living cells. Absorbency was measured at a wavelength of 570 nm on an automatic microplate reader. The samples were run in triplicate and experiments were repeated three times.

Matrigel invasion assay

The invasion of cytotrophoblast cells through Matrigel was evaluated objectively based on our previous procedure (Du et al., 2007b, 2008; Zhou et al., 2007, 2008b). Cell culture inserts (8 μm pore size, 6.5 mm diameter; Corning, Corning, NY, USA) precoated with 15 μl matrigel were placed in a 24-well plates. The purified cytotrophoblast cells (1 × 105 in 200 μl DMEM-high glucose with 2% FBS) were seeded in the upper chamber and treated with DMSO (0.1%, v/v) or CsA (1.0 μM). Before treatment with CsA, the cells were preincubated with 40 μg/ml of CXCL12 or 20 μg/ml of CXCR4 neutralizing antibody or isotype control antibody (R&D Systems), and the isotype control antibody, CXCL12 or CXCR4 neutralizing antibody alone were also included. The lower chamber was filled with 800 μl DMEM-high glucose with 10% FBS. The cells were then incubated at 37°C for 48 h.

The co-culture invasion model of cytotrophoblast cells and DSCs was established to observe the invasiveness of cytotrophoblast cells. Briefly, the isolated DSCs were seeded on the opposite side of the upper chamber at a density of 1 × 105/well, and cultured in DMEM with 10% FBS for 4 h. Then, the freshly isolated cytotrophoblast cells (1 × 105 in 200 μl DMEM with 2% FBS) were added to the upper surface of the upper chamber, and incubated at 37°C for 48 h. Before co-culture, the DSCs or cytotrophoblast cells in wells were pretreated with CXCR4 (20 μg/ml) neutralizing antibody for 30 min. Cultures of cytotrophoblast cells alone were used as control. After 48 h of incubation in 5% CO2 at 37°C, the inserts were removed, washed in PBS and the non-invading cells together with the Matrigel were removed from the upper surface of the filter by wiping with a cotton bud. The remaining cells on the lower surface were fixed in methanol for 10 min at room temperature, and then stained with hematoxylin. For co-culture, the inserts were first incubated with anti-human CK7 monoclonal antibody to identify cytotrophoblast cells, and then counter stained with hematoxylin to show DSCs. Cell invasive ability was determined by counting the number of stained cells on the membranes in 10 randomly selected, non-overlapping fields.
at a magnification of 200× in each group with the counter ‘blinded’ to sample identity to avoid experimental bias using Olympus BX51tDP70 fluorescence microscope (Olympus, Tokyo, Japan). Each experiment was carried out in triplicate, repeated three times. The invasion index was calculated as the ratio of the invaded cells of the experimental group to that of the DMSO control.

**Gelatin zymography**

MMP-9 and MMP-2 are the key enzymes for the degradation of extracellular matrix, and can facilitate trophoblast penetration into the decidua (Shimono-vitz et al., 1994; Staun-Ram et al., 2004). Gelatin zymography is a simple and economic method which can directly observe the proteolytic activity of MMP-9 and MMP-2. Therefore, to explore whether the change of cytotrophoblast invasiveness was accompanied by an alteration in the ECM degrading MMP’s 9 and 2 activity, we conducted zymography analysis as performed by our previous publications (Zhou et al., 2007, 2008b).

The collection of culture medium for zymography analysis was divided into two steps. First, cytotrophoblasts or DSCs (3 × 10^5 cell/ml) were seeded on 24-well plates, and treated with 1.0 μM CsA, 40 μg/ml CXCL12 neutralizing antibody, 20 μg/ml CXCR4 neutralizing antibody or 1.0 μM CsA combined with 40 μg/ml CXCL12 or 20 μg/ml CXCR4 neutralizing antibody for 48 h. DMSO (0.1%, v/v) and isotype antibody (Sino-America Co. Ltd., China) were used as controls. Second, the culture medium of the invasion assay was harvested, and stored at −70°C for zymography. The proteolytic activity of both MMP-9 and MMP-2 was measured by the technique of gelatin zymography described previously (Yoshizaki et al., 1997; Zhou et al., 2008b). Briefly, the collected culture supernatants containing 10 μg of total protein were mixed with sodium dodecyl sulfate (SDS) loading buffer and electrophoresed on 10% SDS-polyacrylamide gels copolymerized with 0.2% gelatin. After electrophoresis, the gel was rinsed in 2.5% Triton-X 100 for 1 h to remove SDS, then incubated for 12 h at 37°C in 50 mM Tris–HCl (pH 7.5), 200 mM NaCl, 10 mM CaCl2 and stained with 2.5% Coomassie Blue R250 (Sigma Chemical Co.) dissolved in 40% (v/v) methanol and 10% acetic acid. The gels were then rinsed in three different decolorant solutions (A: 30% methanol, 10% acetic acid; B: 20% methanol, 10% acetic acid; C: 10% methanol, 5% acetic acid), respectively. The

![Figure 1](https://academic.oup.com/humrep/article-abstract/27/7/1955/794010/103166384)
gelatinolytic activity was visualized as a clear white band against a dark background of stained gelatin. The gel was photographed and assayed by the Odyssey Infrared Imaging System. The relative MMP activity was calculated as the ratio of the white band intensity of the experiment group to that of the DMSO control. The samples were run in triplicate, and experiments were repeated three times.

Enzyme-linked immunosorbent assay

The purified cytotrophoblast cells were seeded in a 24-well plate precoated with matrigel at a density of $1 \times 10^6$ cells/ml. The cytotrophoblast supernatants were collected after treated with vehicle (0.1%, v/v DMSO), 1.0 μM CsA, 30 μM U0126 or 1.0 μM CsA combined with 30 μM U0126 for 48 h. Each supernatant was centrifuged at 2000 g and stored at −80°C. Human CXCL12 ELISA kit (R&D Systems) was used to measure chemokine production in each supernatant according to the manufacturer’s instructions. The CXCL12 assay demonstrated a sensitivity of 18 pg/ml and an intra-assay coefficient of variation of 3.4–3.9%. The ELISA assay was carried out in duplicate in three separate experiments.

In-cell western

In-cell western is a simple and cost-effective method for direct semi-quantification of intracellular molecules in cells. According to the description by Egorina et al. (2006) (Zhou et al., 2007; Li et al., 2010; Li et al., 2011), we detected the relative protein level of CXCL12 and CXCR4 in cytotrophoblast cells and DSCs. The procedure was as follows: 0.1%, v/v DMSO or 30 μM U0126 was added to cytotrophoblast culture in 96-well plates for 30 min, the cells were then incubated with 0.1%, v/v DMSO or 1.0 μM CsA for 48 h. After that, the cells were immediately fixed with 4% formaldehyde in PBS for 20 min at room temperature. After washing with 0.1% Triton, these cells were treated by adding 150 μl of LI-COR Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, Nebraska, USA) for 90 min at room temperature, and then incubated with mouse anti-human CXCL12 (20 μg/ml) or CXCR4 (40 μg/ml) (R&D Systems, Abingdon, UK) primary antibody, respectively. The housekeeping protein, actin served as a control and rabbit anti-human actin (1:50) (Santa Cruz, CA, USA), was added to each well at the same time. After overnight treatment at 4°C, the wells were incubated with the corresponding second IRDyeTM700DX conjugated affinity purified (red fluorescence) anti-mouse (1:500) or IRDyeTM800DX conjugated affinity purified (green fluorescence) anti-rabbit
fluorescence antibody (1:1000) as recommended by the manufacturers (Rockland, Inc., Gilbertsville, PA, USA). This procedure was done in the dark. Images of CXCL12 and CXCR4 were obtained using the Odyssey Infrared Imaging System (LI-COR Biosciences GmbH). The relative protein amounts of CXCL12 or CXCR4 were presented as the ratio of the fluorescence intensity of CXCL12 or CXCR4 (red) to that of actin (green). The experiments were carried out in triplicate, repeated three times.

**Statistical analysis**

All values were expressed as the mean ± SEM. Data were analyzed by using one-way or two-way ANOVA, with application of Dunnett’s test. Differences were considered as statistically significant at \( P < 0.05 \).

**Results**

CsA increases mRNA and protein expression of CXCL12 and CXCR4 in human first-trimester cytotrophoblast cells through MAPK/ERK1/2 signaling pathway

CsA (1.0 \( \mu \)M) significantly increased the mRNA and protein levels of CXCL12 and CXCR4 in first-trimester human cytotrophoblast cells compared with the control. U0126, a MAPK/ERK signaling specific blocker, completely inhibited the increase in CXCL12 and CXCR4 induced by CsA (Fig. 1A and B). The secretion of CXCL12 was further confirmed by measuring the CXCL12 level in the supernatant of trophoblasts with ELISA (Fig. 1C). However, treatment with CsA

![Figure 4](https://academic.oup.com/humrep/article-abstract/27/7/1955/794010/1960 Du et al)

**Figure 4** CXCL12/CXCR4 is involved in CsA-promoted invasion by human first-trimester cytotrophoblast cells. (A) Pictures (200 \( \times \) magnification) of cytotrophoblasts invaded through matrigel-coated membranes. Human primary cytotrophoblast was incubated in the presence of (a) 0.1% DMSO (control), (b, c) 20 \( \mu \)g/ml anti-CXCR4 or 40 \( \mu \)g/ml anti-CXCL12 neutralizing antibodies alone, (d) 1.0 \( \mu \)M CsA, (e, f) 1.0 \( \mu \)M CsA and pretreatment with 20 \( \mu \)g/ml anti-CXCR4 or 40 \( \mu \)g/ml anti-CXCL12 neutralizing antibodies for 48 h. Isotype IgG was used as control. The bar chart to the left shows the invasive index of the cells under the same conditions. (B) The proteolytic activity of MMP-9 (92 kD) and MMP-2 (72 kD) in the supernatant from the upper well in the cytotrophoblast invasion assay. The upper panel shows a representative gelatin zymogram. The histogram represents the band intensity of MMP-9 and MMP-2 in treatment groups relative to that of the respective control. Data are mean ± SEM of three experiments performed in triplicate wells with three different samples, \( *P < 0.05 \), \( **P < 0.01 \) compared with the respective control; \( ##P < 0.01 \) compared with the respective CsA treatment (ANOVA plus Dunnett’s test).
failed to change the protein level of CXCL12 or CXCR4 in DSCs (Fig. 2).

**CsA increases cell number of human first-trimester cytotrophoblast cells via CXCL12/CXCR4 autocrine manner**

Figure 3 shows that CsA enhanced the cell number of human first-trimester cytotrophoblasts, and the increase was blocked by CXCL12 or CXCR4 neutralizing antibody. Consistent with our previous study (Du et al. 2007b, 2008) pretreatment with U0126 was also able to block the increase in cell number of cytotrophoblasts induced by CsA. Neutralizing antibody to CXCL12 or CXCR4 or U0126 also decreased the cell number in the absence of CsA.

**CsA enhances invasion in vitro of human first-trimester cytotrophoblast cells via CXCL12/CXCR4 in an autocrine manner**

CXCL12 or CXCR4 blocking antibodies could eliminate the CsA-induced invasion increase of human cytotrophoblast cells. Moreover, even in the absence of CsA the invasive index following neutralizing antibody treatment was significantly lower than that of the control (0.1% DMSO; Fig. 1A).

CXCL12/CXCR4 neutralizing antibodies significantly diminished the enhanced proteolytic activity of MMP-9 and MMP-2 of cytotrophoblasts induced by CsA (Fig. 4B), while there was no difference in the protein expression of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2, the key inhibitors for MMP-9 and MMP-2, in various experimental groups (data not shown).

**CsA promotes the crosstalk between human first-trimester cytotrophoblast and DSC via CXCL12/CXCR4 signal**

Exposure of DSCs to CsA increased the activity of MMP-9 and MMP-2. However, pretreatment with neutralizing antibodies to CXCL12 and CXCR4 did not affect the CsA-increased activity of MMP-9 and MMP-2 in DSCs (Fig. 5).

As expected, CsA alone significantly increased the invasiveness of cytotrophoblast, and the increase it produced was much higher in co-culture (Fig. 6A). Moreover, anti-CXCR4 blocking antibody significantly decreased (P < 0.05) the CsA-induced invasiveness of cytotrophoblasts in the co-culture.

The supernatant in the upper chamber of the invasion assay was collected, and the activity of MMP-9 and MMP-2 was detected by gelatin zymography. As shown in Fig. 5B, CsA significantly increased the activity of MMP-9 and MMP-2 in both single and co-culture models but activity was markedly greater in the co-cultures. The anti-CXCR4 blocking antibody in the co-culture significantly decreased the activity of MMP-9 and MMP-2 induced by CsA.

**Discussion**

Based on our studies on CsA and CXCL12/CXCR4 in the regulation of maternal–fetal relationship (Du et al., 2007a, b, 2008; Zhou et al., 2007, 2008a), here we have further demonstrated that CsA can induce the expression of chemokine CXCL12 and its receptor CXCR4 in human first-trimester cytotrophoblast cells through the MAPK/ERK signalling pathway. The neutralizing antibody to CXCL12 or CXCR4 abrogates the CsA-increased cell number and invasion of the first-trimester human cytotrophoblast cells. The increase in cell numbers could be the result of increased cell survival or decreased apoptosis, and this has been indicated in other studies (Alvarez-Arroyo et al., 2002; Du et al., 2007b, 2008). The abrogation of invasion by neutralizing antibodies is associated with the decreased activity of MMP-9 and MMP-2. By contrast, although CsA treatment does not affect CXCL12/CXCR4 expression in DSCs, it promotes MMP-9 and MMP-2 activity of DSCs, by a pathway unaffected by CXCL12 or CXCR4 neutralizing antibodies. In addition, cytotrophoblast-DSC co-culture magnifies the CsA-increased MMP-9 and MMP-2 activity of DSCs, by a pathway unaffected by CXCL12 or CXCR4 neutralizing antibodies. This is an essential step in establishing and maintaining a successful pregnancy. In pre-eclampsia, intrauterine growth restriction and spontaneous miscarriage, there seems to be decreased proliferation and invasion and an increased apoptosis of human villous cytotrophoblasts.
Cytotrophoblast invasion depends on the complex crosstalk between the fetal and maternal tissues at maternal–fetal interface (Irwin et al., 2001; Zhou et al., 2008a; Munoz-Suano et al., 2011). Cytotrophoblasts and maternal decidual cells secrete products that regulate cytotrophoblast differentiation and migration into the maternal decidua (Hempstock et al., 2004; Guzeolgolu-Kayisli et al., 2009). Paracrine interactions between the extravillous cytotrophoblast and maternal decidua are important for successful implantation, including the establishment of the placental vasculature, anchoring the placenta to the uterine wall, as well as maternal restraints and facilitators of invasion into the decidua (Irwin et al., 2001; Jones et al., 2006; Strowitzki et al., 2006; Guzeolgolu-Kayisli et al., 2009; Salker et al., 2010; Teklenburg et al., 2010; Yoshinaga, 2010; Fukui et al., 2011). In addition to their complex interactions with cytotrophoblasts, human decidualized endometrial stromal fibroblasts play an important role in orchestrating the repertoire and behaviors of immune cells within the decidua during the early pregnancy (Blanco et al., 2009; Chen et al., 2011; Vacca et al., 2011). It was reported that the culture media from human cytotrophoblasts could promote the production of proinflammatory cytokines, chemokines and angiogenic/angiostatic factors form DSCs (Hess et al., 2007). The data suggest that cytotrophoblast acts to alter the local environment of the decidua to facilitate the process of implantation and placentation.

Our previous study has demonstrated that CXCL12 promotes crosstalk between human cytotrophoblasts and DSCs through binding to CXCR4, which is beneficial to placental development (Zhou et al., 2008b). In addition, CXCR4 and its specific ligand CXCL12 play a key role in lymphocyte trafficking and recruitment at sites of inflammation and in hematopoiesis and development processes, such as organogenesis, vascularization and embryogenesis (Wu et al., 2004; García-Andrés and Torres, 2010; Sun et al., 2010; Delano et al., 2011; Ping et al., 2011; Sharma et al., 2011). Recently, it has been reported that CXCL12 can cause CXCR4+ Treg cells to migrate into the pregnant mouse uterus and establish a beneficial microenvironment for the fetus (Lin et al., 2009). The first-trimester human cytotrophoblast cells produce CXCL12 which endows the...
cytotrophoblast cells with the capacity to direct the migration of CD56<sup>+</sup>CD16<sup>+</sup> NK cells into the decidua (Wu et al., 2005). More recently, it was reported that CXCL12/CXCR4 signaling could regulate the biology and development of natural killer cells (Noda et al., 2011). These results suggest that CXCL12/CXCR4 signal plays a critical role in the maintenance of successful pregnancy.

It is well known that CXCR4 triggers multiple intracellular signals in response to CXCL12, especially calcium mobilization and phosphorylation of ERK1/2 (Kremer et al., 2003; Roland et al., 2003). The ERK1/2/MAPK pathway has been associated with the regulation of gene expression, cellular proliferation, differentiation, angiogenesis, embryo development and tumor invasion (Reddy et al., 2003; Fang et al., 2011; Sato et al., 2011; Waddell et al., 2011; Wang et al., 2011). It was reported that ERK1/2 were widely expressed throughout early-stage embryo, especially in villous cytrophoblasts and extra-villous cytrophoblasts in the first-trimester gestation. Disruption of the ERK2 locus leads to embryonic lethality early in mouse development after implantation (Kita et al., 2003; Saba-El-Leil et al., 2003). U0126 is a highly selective inhibitor of both MEK1 and MEK2, types of MAPK/ERK kinases, and has been widely used to investigate the role of MAPK/ERK signals (Nishimoto and Nishida, 2006). Our previous studies have found that CsA causes the phosphorylation of ERK1/2, and U0126 effectively inhibits the increased phosphorylation of ERK1/2, which confirms that CsA is able to activate MAPK/ERK signaling (Zhou et al., 2007; Du et al., 2008). Here we have found that CsA enhances CXCL12/CXCR4 expression in human primary cytrophoblasts. We wonder whether the up-regulation of CsA on the expression of CXCL12/CXCR4 of cytrophoblasts is mediated by the MAPK/ERK signal pathway. In the present study, U0126 was used to block MAPK/ERK kinases, resulting in inhibition of MAPK/ERK signaling. Under this condition, we observed that the enhanced expression of CXCL12/CXCR4 induced by CsA was eliminated. Thus, it is suggested that CsA promotes CXCL12 and CXCR4 expression in cytrophoblasts through the activation of the ERK1/2 pathway. Interestingly, CsA has no effect on the CXCL12/CXCR4 expression in DSCs, but improves the MMP activity. The effects of CsA on cytrophoblasts and DSCs are different, which deserves to be explored further. Our study supplies a clue of CsA in therapeutics for pregnancy wastage and other pregnant complications.

The concern is whether CsA administration can induce unlimited invasion of cytostrophoblast associated with hydatidiform moles and choriocarcinomas. Both our previous reports (Li et al., 2010, 2011) and unpublished data show that CsA-treated human cytrophoblasts can promote KAI/CD82 expression in DSCs via CXCL12, which in turn inhibit the over-invasion of cytostrophoblasts. Combining with our results in an animal model that CsA treatment increases cell number and invasion of cytostrophoblast only in the early pregnancy not in the mid- or late-pregnancy (Du et al., 2007a), we propose that CsA might play two-way regulatory role in the crosstalk between human cytostrophoblasts and DSCs through up-regulating CXCL12/CXCR4 interaction, which is beneficial to the maintenance of normal pregnancy.

**Authors’ roles**

M.R.D. participated in research design, analysis, manuscript drafting and critical discussion and manuscript revision. W.H.Z. participated in research design, execution, analysis and manuscript revision. H.L.P. participated in experimental execution. M.Q.L. participated in experimental design. D.J.L. participated in research design, analysis and manuscript editing and critical discussion.

**Funding**

This work is supported by National Basic Research Program of China 2006CB944007, Key Project and Major International Joint Research Project of NSFC 30730087, 309103909, NSFC 30872768 and the Program for Outstanding Medical Academic Leader (all to D.J.L.), NSFC 81070537, NSFC 31171437 (to M.R.D.) and Shanghai Pujiang Talent Program 10PJ1401600 (to M.R.D.), NSFC 30801240 (to W.H.Z.).

**Conflict of interest**

None declared.

**References**


