Chemokine CCL28 induces apoptosis of decidual stromal cells via binding CCR3/CCR10 in human spontaneous abortion

Chan Sun¹, Yuan-Yuan Zhang², Chuan-Ling Tang¹, Song-Cun Wang¹, Hai-Lan Piao¹, Yu Tao¹, Rui Zhu¹, Mei-Rong Du¹*, and Da-Jin Li¹

¹Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, IBS, Fudan University Shanghai Medical College, Shanghai 200011, China ²Department of Obstetrics and Gynecology, Second Affiliated Hospital of Zhongshan University, Guangzhou 516100, China

*Correspondence address. Fax: +86-21-63457331; E-mail: dmrlq1973@yahoo.com.cn

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ABSTRACT: Spontaneous abortion is the most common complication of pregnancy. Immune activation and the subsequent inflammation-induced tissue injury are often observed at the maternal–fetal interface as the final pathological assault in recurrent spontaneous abortion. However, the precise mechanisms responsible for spontaneous abortion involving inflammation are not fully understood. Chemokine CCL28 and its receptors CCR3 and CCR10 are important regulators in inflammatory process. Here, we examined the expression of CCL28 and its receptors in decidual stromal cells (DSCs) by immunochemistry and flow cytometry (FCM), and compared their expression level in DSCs from normal pregnancy versus spontaneous abortion, and their relationship to inflammatory cytokines production by DSCs. We further analyzed regulation of the pro-inflammatory cytokines on CCL28 expression in DSCs by real-time polymerase chain reaction, In-cell Western and FCM. The effects of CCL28–CCR3/CCR10 interaction on DSC apoptosis was investigated by Annexin V staining and FCM analysis or DAPI staining and nuclear morphology. Higher levels of the inflammatory cytokines interleukin (IL)-1β, IL-17A and tumor necrosis factor-α, and increased CCR3/CCR10 expression were observed in DSCs from spontaneous abortion compared with normal pregnancy. Treatment with inflammatory cytokines differently affected CCL28 and CCR3/CCR10 expression in DSCs. Human recombinant CCL28 promoted DSC apoptosis, which was eliminated by pretreatment with neutralizing antibodies against CCR3/CCR10 and CCL28. However, CCL28 did not affect DSC growth. These results suggest that the inflammation-promoted up-regulation of CCL28 and its receptors interaction in DSCs is involved in human spontaneous abortion via inducing DSC apoptosis.

Key words: CCL28 / decidual stromal cells / apoptosis / inflammatory cytokine / spontaneous abortion

Introduction

Spontaneous abortion is the most common complication of pregnancy, occurring in ~15% of clinically recognized pregnancies. Approximately 1–3% of women may have three or more consecutive spontaneous abortions prior to 20 weeks gestation; this is termed recurrent spontaneous abortion or recurrent pregnancy loss (RPL) (Sierra and Stephenson, 2006; Ford and Schust, 2009; Jaslow et al., 2010). Multiple etiologies, including endocrine, anatomic, genetic, hematological and immunological factors, have been reported for this devastating disease. However, over half of the cases remain unexplained (Kwak-Kim et al., 2010). Immune activation and subsequent inflammatory and thrombotic tissue injury are often observed at the maternal–fetal interface as the final pathological assault in RPL (Kwak-Kim et al., 2009). Exaggerated maternal inflammatory response to the fetus has been suggested as one of the underlying causes of idiopathic spontaneous abortion cases (Babbage et al., 2001; Saini et al., 2011) although the precise mechanisms responsible for spontaneous abortion are not fully understood.

Chemokines are selective mediators in leukocyte migration to inflammatory sites, and have been described as pivotal players in diverse physiological and pathological events, including chemotaxis, cellular proliferation, apoptosis, angiogenesis and inflammatory process/disease (Luster, 1998; Rossi and Zlotnik, 2000; Zlotnik and Yoshie, 2000). We have previously demonstrated that the chemokine/receptor pairs CXCL12/CXCR4 and CXCL16/CXCR6 play important roles in the cross-talk between trophoblasts, decidual stromal cells (DSCs) and decidual leukocytes (DLCs), and are involved in embryo development, placentation and maternal–fetal tolerance (Wu et al., 2005; Zhou et al., 2008; Huang et al., 2008; Du et al., 2012). Recently, we have reported that DSC-secreted CCL2 induces and maintains DLC differentiation toward a Th2 bias in human early pregnancy (He et al., 2012).
Mucosa-associated epithelial chemokine (MEC or CCL28) is a functional ligand for CC chemokine receptors 3 and 10 (CCR3 and CCR10). CCL28 is constitutively expressed in different mucosal sites (Wang et al., 2000; Kunkel et al., 2003; Wilson and Butcher, 2004). CCL28 has been implicated in mucosal immunity by recruiting IgA antibody-secreting cells (ASCs) into the mucosal lamina propria (Kunkel et al., 2003; Nakayama et al., 2004; Wilson and Butcher, 2004; Williams, 2004). CCL28 production is increased by inflammatory cytokines and bacterial products (Nakayama et al., 2004). CCL28 is selectively expressed by epidermal cells, and is highly up-regulated during inflammatory processes (Ezzat and Shaheen, 2009; Ezzat et al., 2009; Shibata et al., 2010; Scanlon et al., 2011). CCL28 is pivotal in mediating the migration of CCR3- and CCR10-expressing, skin-homing, cutaneous lymphocyte-associated antigen-positive (CLA+) T-cells.

The uterine decidua is a pregnancy-specific endometrial/mucosal tissue. DSCs are the major cellular component and are differentiated from fibroblast-like precursor cells in decidua. They are particularly important because of their pleiotropic functions during pregnancy. Not only are DSCs classically considered important for the metabolic environment of the embryo, but DSCs are also involved in immune regulation and mediating extravillous trophoblast (EVT) invasion (Zhou et al., 2008; Du et al., 2012; Piao et al., 2012). The local inflammatory environment plays essential roles in the decidualization, including preventing decidual cell apoptosis and promoting stromal fibroblast differentiation into metabolically active DSCs (Challis et al., 2009; Dekel et al., 2010; Gnainsky et al., 2010; Granot et al., 2010). There is a tightly balanced regulation of decidual cell pro-inflammatory and anti-inflammatory responses. Inappropriately elevated inflammatory cytokines may result in severe consequences (Kwak-Kim et al., 2010; Calleja-Agius et al., 2011; Lee et al., 2011, 2012; Saini et al., 2011; Deftereou et al., 2012; Wu et al., 2012). Since DSCs play an important role in pregnancy maintenance, inflammation-related DSC apoptosis will likely significantly contribute to spontaneous abortion.

In this study, we first examined the expression of CCL28 and its receptors CCR3 and CCR10 in DSCs, and compared the expression of the chemokine/chemokine receptor pairs in DSCs from normal pregnancy versus spontaneous abortion, and their relationship to inflammatory cytokines regulation in DSCs. We further analyzed inflammatory cytokine regulation on CCL28 expression in DSCs. The modulation of CCL28−CCR3/CCR10 interaction on DSC apoptosis was also investigated. Our results show a critical role of CCL28-expressing DSCs in the inflammation-induced spontaneous abortion.

Materials and Methods

Human decidua tissue collection

This study was approved by the Human Research Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University (Shanghai, China). All subjects signed informed written consent for the collection and study of tissue samples. The first-trimester human deciduas were obtained from 26 healthy women in early pregnancy [mean age 29.15 ± 5.27 years; gestational age at sampling 52.77 ± 7.8 days (mean ± standard deviation)] whose pregnancies were terminated for non-medical reasons with artificial abortion-vacuum aspiration. There is no any treatment including misoprostol/mifepristone on the pregnant women before the artificial abortion. The decidual samples from 11 spontaneous abortions that occurred during the first trimester of pregnancy were also obtained (mean age 29.1 ± 6.7 years; gestational age at sampling 51.2 ± 9.2 days). All normal pregnancies and spontaneous abortions were confirmed by ultrasound and blood test. Every pregnancy with spontaneous abortion has presence of fetal pole and was not an anembryonic pregnancy. Exclusion criteria included endocrine, anatomic, genetic, infection and smoking reasons. The placentas with genetic abnormalities were excluded from the study by analyzing the chromosomal karyotype and content of the placental tissues. Tissues were immediately collected in a mixture of 50% Dulbecco’s modified Eagle’s medium and 50% Ham’s F-12 medium (DMEM/F12; Invitrogen Life Technologies) with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) and washed in Hank’s balanced salt solution for DSC isolation.

Isolation and primary culture of DSCs

DSCs were isolated by collagenase IV/DNase-I digestion and discontinuous Percoll gradient centrifugation, and characterized, as we described previously (Zhou et al., 2008; Du et al., 2012; He et al., 2012). DSCs, which ranged in density between 1.042 and 1.062 g/ml, were recovered and cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium) in 5% CO2 at 37°C. After culture for 30 min, non-adherent lymphocytes were removed by washing, leaving DSC cultures of >98% purity, which have been characterized by immunocytochemistry and flow cytometry (FCM).

Treatment of human first-trimester DSC

The freshly isolated DSCs were cultured overnight in complete medium, and further incubated in 12-well tissue culture plates in serum-free medium for 12 h, followed by stimulated with different concentrations of inflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α or IL-17A (PeproTech EC, Ltd, London, UK). At the indicated time points following cytokine treatment, DSCs were collected for mRNA quantification by real-time polymerase chain reaction (PCR), or for protein quantification by In-cell Western assay (detailed below). For growth or apoptosis assay, DSCs were treated with different concentrations of CCL28 (R&D system) for 48 h. In some wells, neutralizing antibody against CCL28 (5 μg/ml), CCR3 (5 μg/ml) or CCR10 (5 μg/ml) (R&D system) were added for 1 h before 100 ng/ml of CCL28 treatment.

FCM for CCR3/CCR10 expression in DSCs

Purified DSCs from normal pregnancy or spontaneous abortion were washed and blocked with 10% horse serum in phosphate-buffered saline (PBS) for 15 min at room temperature before staining. The fluorescence-conjugated antibodies (APC-conjugated anti-CCR3 and PE-conjugated anti-CCR10, both from BioLegend) or isotype-matched control antibodies (eBioscience) were incubated with the cells at the recommended concentrations for 30 min at room temperature in the dark. Cells were washed, resuspended in PBS, and immediately analyzed on a FACSCalibur™ flow cytometer by using CellQuest software (BD Biosciences, Franklin Lakes, NJ).

Immunostaining for CCL28 expression in human decidua

For immunohistochemistry, paraffin sections (5 μm thick) of early human decidua were rehydrated in Tris-buffered saline (TBS), and incubated with hydrogen peroxide and 1% bovine serum albumin (BSA)/TBS to block endogenous peroxidase activity. Sections were incubated with mouse anti-human CCL28 antibody (5 μg/ml, MAB717; R&D Systems, Minneapolis, MN, USA) or mouse isotype-matched IgG overnight at 4°C in a humidified chamber. After washing three times with TBS, sections were overlaid with peroxidase-conjugated rabbit anti-mouse antibody (1:100, 14-13-06;
KPL, Gaithersburg, MD, USA). The reaction was developed with 3,3-
diaminobenzidine (DAB) and counterstained with hematoxylin.

For immunocytochemistry staining, freshly purified DSCs were cultured on
coverslips for 24 h. Coverslips were fixed in 4% paraformaldehyde for
20 min at room temperature, washed in PBS and permeabilized for 10 min
with 0.25% Triton-100 in PBS. Cells were incubated with 1% BSA in PBS/
TWEEN (PBST) for 30 min to block non-specific binding of the antibodies.
Mouse anti-human vimentin monoclonal antibody (1:100, ZA0511, 
Dingguo, Beijing), cytokeratin-7 antibody (1:100, 18-0234, Zymed Labora-
tories, USA) and anti-factor VIII antibody (endothelial cell marker)
(1:14-01, ready to use, Dingguo) were used as markers for DSCs. Mouse anti-
human CCL28 antibody (R&D systems, MAB717) was administrated to
detect whether DSCs express CCL28 protein. The cells were incubated
with primary antibody or isotype-matched IgG, diluted in PBST containing
1% BSA overnight at 4 °C, coverslips were rinsed in PBST, and were incubated with a DAB-labeled affinity-purified antibody to mouse IgG (H+L)
(03-18-06, 1:100, KPL) for 60 min at 37°C. Slides were counterstained with
hematoxylin. The specificity of CCL28 antibody was validated by
mixing the antibody with its peptide before our formal experiments. The
results showed that mixture of CCL28 antibody with CCL28 peptide elimi-
nated the binding of the antibody to CCL28 in the tissue (data not shown).

CCL28 mRNA quantification in DSCs

Total RNA from DSCs treated with different cytokines for 24 h was
extracted and reverse transcribed, and cDNA was amplified by real-time
PCR in a final volume of 50 μl containing 25 μl of Hot-Start PCR Master
Mix (RuiCheng Bio) and 200 nM of each primer probe. The primers and
probes in the study were as follows: human CCL28 (86 bp), forward primer:
5′-AAG GAATTTG TTGGCA ACAGG-3′, reverse primer: 5′-ATGGCTGA TTGGCT GTTG TT-3′; human GAPDH (138 bp),
forward primer: 5′-GCCG TCAAGGCTG AGA AC-3′, reverse primer: 5′-TGGTG AGAGGC CAG TGG A-3′ (Shenggong Corp., Shang-
hai, China). Each sample was analyzed in duplicate using an ABI Prism™
7000 Sequence Detector (Applied Biosystems). The PCR amplification was corre-
lated against a standard curve.

In-cell Western Assay for CCL28 protein level in DSCs

According to the description by Egorina et al. (2006) and our previous pro-
cedure (Zhou et al., 2008; Du et al., 2012), we used In-cell Western Assay
to determine the intracellular protein level of CCL28. DSCs cultured in
96-well tissue culture plates were stimulated with different cytokines for
48 h. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature.
After washing with 0.1% Triton-X-100, cells were blocked with
150 μl LI-COR OdysseyTM Blocking Buffer (LI-COR Biosciences, Lincoln,
NE, USA) for 90 min at room temperature, and incubated with mouse anti-
human CCL28 (R&D Systems) with rabbit anti-human actin (1:80, Santa Cruz
Biotechnology, Santa Cruz, CA, USA) as a control. After overnight primary
antibody treatment at 4°C, the wells were incubated with corresponding
second IRDyeTM700DX-conjugated affinity-purified anti-mouse antibody
(red fluorescence) and IRDyeTM800DX-conjugated affinity-purified anti-
rabbit antibody (green fluorescence) at the recommended concentration
(Rockland, Inc., Gilbertsville, PA, USA) in the dark. Images of the target mol-
ecule were obtained by using the Odyssey® Infrared Imaging System
(LI-COR Biosciences GmbH). The expression level of the correspondent
molecules was calculated as the ratio of the fluorescence intensity of target
proteins to actin.

DSC cell growth assay

We used a commercial cell counting assay (Cell Counting Kit-8, CCK-8;
Dojindo, Japan) to evaluate cell growth. The CCK-8 kit uses a water-soluble
tetrazolium salt that is reduced by cellular dehydrogenase activity to give
a yellow formazan dye, in proportion to living cell number. DSCs (1 × 10^4/
well) were cultured in 96-well plates and stimulated for 48 h with different
cytokine treatments. Thereafter, 10 μl CCK-8 was added into each well
and incubated at 37°C for 2–4 h. The optical density at 450 nm was deter-
mined using an enzyme-linked immunosorbent assay (ELISA) Reader (Mo-
olecular Devices, USA), to measure cell number.

Annexin V and propidium iodide staining

We used a commercial Apoptosis Detection Kit (Bender, Burlingame, CA)
to evaluate apoptosis by annexin V binding and propidium iodide (PI) staining.
The DSCs were washed and resuspended in 80 μl binding buffer (10 mM
HEPES, 140 mM sodium chloride, 2.5 mM calcium chloride, pH 7.4). To
each cell suspension, 10 μl Alexa Fluor 488-conjugated annexin V (10 μg/
ml) and 10 μl PI reagent (50 μg/ml) were added. The cells were mixed
and incubated in the dark for 15 min at room temperature. At the end of
the incubation, a further 400 μl binding buffer was added and the cells
were analyzed immediately by FCM (BD Biosciences). Control tubes of
unstained cells, cells stained with PI alone and cells stained with annexin V
only were included for setting the flow cytometer compensation. The apop-
tosis index was calculated as the percentage of annexin V^+ PI^- cells in each
DSC population.

Assessment of apoptosis by DAPI staining

The cells were grown on BD Falcon™ culture slides and exposed to different
concentrations of CCL28 or/and the neutralizing antibodies against CCL28
and its receptors for the indicated time. At the end of different treatment,
the culture media were removed, and the cells were permeabilized with 0.1%
Triton X-100 after being fixed with 4% paraformaldehyde. Thereafter, the
cells were stained with 4–6-diamidino-2-phenylindole (DAPI) (1:50; Invitro-
gen). Fluorescence images were observed by using an Olympus BX51 fluori-
escence microscope (Tokyo, Japan), and recorded with a high-resolution
DP70 Olympus digital camera. The percentage of apoptosis was determined
by nuclear morphology. At least 400 cells were counted in each group with
the counter ‘blinded’ to sample identity to avoid experimental bias.

Cytokine quantification in DSC supernatants by ELISA

To detect cytokine secretion, DSCs (1 × 10^6/well) were co-cultured with
phorbol myristate acetate (PMA, 25 ng/ml) and ionomycin (1 μg/ml) for
6 h in 6-well tissue culture plates before the end of the 72-h culture. There-
after, the levels of secreted TNF-α, IL-1β and IL-17A in the supernatant from
each experiment were quantified by using the commercially available ELISA
kit (Dakewe Biotech, China) following the manufacturer’s instructions.

Statistical analysis

Statistical comparisons were performed by using one-way analysis of variance
(ANOVA) or two-way ANOVA or t-test with SPSS software version 15.0
(Chicago, IL, USA). All error bars in figures indicate standard error (SE). Stat-
istical significance was accepted at P < 0.05.
Results

Characterization for the purity of human first-trimester DSC by immunocytochemistry and FCM

After 24 h of culture, we characterized the expression of vimentin, cytokeratin 7 (CK7) and anti-factor VIII in these cells. As shown in Fig. 1A, the cells we isolated were almost all stained for vimentin, whereas no cells were found stained with anti-CK7, anti-smooth actin or anti-factor VIII antibody, which is epithelial cell marker, smooth muscle cell marker and vascular endothelial cell marker, respectively. We observed that purity of the isolated DSC was ≏ 98%. To exclude the contamination of immune cells (CD45-positive), we further characterized the purity of isolated DSCs by FCM. The results in Fig. 1B showed that around 98% of the primary DSC was vimentin$^+$ CK7$^2$ CD45$^2$, which was in agreement with immunocytochemistry.

Expression of CCL28 and its receptors CCR3/CCR10 in first-trimester human DSCs

We first examined CCL28 expression in first-trimester human decidual tissue. Immunohistochemistry was performed on paraffin-embedded decidua to stain for CCL28, vimentin, CK7 and isotype-matched antibody as control. Specific brown-colored staining of CCL28 was observed in the cytoplasm and the cytomembrane of DSCs and some gland epithelial cells (Fig. 2A, upper). We also verified CCL28 expression in DSCs through immunocytochemistry. DSCs were positive for CCL28 and vimentin with negative CK7 (Fig. 2A, lower). The expression of CCL28 in DSCs was further confirmed by In-cell Western (Fig. 2B). In addition, we detected the expression of CCL28 and its receptors CCR3/CCR10 in DSCs by FCM. The results in Fig. 2C showed that > 95% of vimentin$^+$ CD45$^-$ DSCs expressed CCL28, and 9.11 ± 1.34% of were CCR10-positive and 5.11 ± 1.15% were CCR3-positive from three independent experiments. These data demonstrate the co-expression of CCL28 and its receptors CCR3 and CCR10 in DSCs.

Up-regulation of CCR3/CCR10-expressing DSCs is accompanied by increased inflammatory cytokines in human DSCs from spontaneous abortion

Because inflammatory stimulation can cause spontaneous abortion (Challis et al., 2009; Kwak-Kim et al., 2010; Calleja-Agius et al., 2011; Wu et al., 2012), and CCL28 and its receptors are involved in some inflammatory processes (Ezzat and Shaheen, 2009; Ezzat et al., 2009; Shibata et al., 2010; Scanlon et al., 2011), we compared the expression of chemokine/chemokine receptor pairs and inflammatory cytokines in DSCs from normal pregnancy versus spontaneous abortion. As shown in Fig. 3A, the percentage of CCR3-positive and CCR10-positive DSCs from spontaneous abortion was significantly higher than that of normal pregnancy. However, there was no difference between normal pregnancy and spontaneous abortion of CCL28 expression in DSCs (data not shown). Meanwhile, we observed increased TNF-α and IL-1β and IL-17A production in DSCs from spontaneous abortion versus normal pregnancy (Fig. 3B).

Inflammatory cytokines up-regulate CCL28 expression in DSCs

To test whether inflammatory stimuli modulate expression of CCL28 in DSCs, we measured CCL28 mRNA and protein levels in DSCs after treatment with different concentrations of TNF-α, IL-17A and IL-1β for 24 or 48 h. As shown in Fig. 4A and B, TNF-α, IL-1β and Th17A alone significantly up-regulated CCL28 mRNA and protein production in DSCs. Different combinations of two kinds of inflammatory cytokines (10 ng/ml) amplified CCL28 protein up-regulation in DSCs (Fig. 4B). However, there is no further increase of CCL28 expression after...
treatment of DSCs with three types of inflammatory cytokines together. We also detected the expression of CCR3 and CCR10 on DSCs treated by these cytokines and their combinations. Figure 4C showed that 10 ng/ml of IL-1β alone significantly up-regulated CCR3 and CCR10 expression on DSCs. Combinations of any two inflammatory cytokines (10 ng/ml) increased CCR10, but not CCR3, expression on DSCs. Treatment with IL-17A alone up-regulated CCR3 expression on DSCs. However, combination of three inflammatory cytokines (10 ng/ml) showed no further regulation of CCR3 or CCR10 expression on DSC cells.

**CCL28 induces DSC apoptosis via interaction with CCR3**

After we had found that DSCs expressed the CCL28 receptors, CCR3 and CCR10 (Fig. 2), we investigated whether CCL28 could affect

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**Figure 2** Expression of CCL28 and its receptors CCR3/CCR10 in human first-trimester DSCs. (A) Immunohistochemical and immunocytochemical characterization of CCL28 in human first-trimester decidual tissue and DSCs. Specific brown staining for CCL28 was observed in the cytoplasm and on the cytomembrane of the decidual tissue cells and DSCs. No background staining was detected in the isotype-matched controls. Data were from three independent experiments with three deciduas. The picture is a representative one. (B) DSCs were seeded at $2 \times 10^6$ cells/200 μl in 96-well plates, and cultured for 48 h. CCL28 protein expression was detected by an In-cell Western assay. CCL28 (red); β-actin (green). The Histogram shows the ratio of the protein fluorescence intensity of CCL28 to that of actin. Each dot represents results from different samples. Data represent the mean ± SEM of six experiments performed in triplicate wells with six different samples. (C) FCM was used to analyze the expression of CCL28 and its receptors CCR3 and CCR10 on Vimentin^+^CD45^-^ DSCs. Results were highly reproducible in three independent experiments with three decidual tissue samples. The FCM plot shown is from one representative experiment.
growth and apoptosis of DSCs. We examined the effects of different concentration of CCL28 on cell growth of human first-trimester DSCs. Figure 5A showed no effect of CCL28 on DSC growth. However, treatment with CCL28 promoted DSC apoptosis, especially at the concentration of 100 ng/ml (Fig. 5B). Addition of neutralizing antibody against CCR3 alone or with antibody against CCR10 antibody significantly reduced the pro-apoptotic effect of exogenously administrated CCL28. When DSCs were pretreated with neutralizing antibody against CCL28, the CCL28-induced DSC apoptosis was almost completely abolished (Fig. 5C). There was no further change of cell death by treatment of CCL28 with all the neutralizing antibodies of CCL28 and its receptors when compared with the treatment of CCL28 and CCL28 antibody. The apoptosis of DSC cells under the combined treatment with CCL28 and the three kinds of antibodies was similar to that of the control. Blocking with antibody to CCR3 was more effective than antibody to CCR10 (Fig. 5B and C) suggesting that CCL28 induces DSC apoptosis mainly via binding CCR3. To confirm the effect of CCL28 on DSC cells, we further conducted the apoptosis assay by counting the apoptotic cells under microscope after DAPI staining. The results in Fig. 5B and C showed that treatment with CCL28-induced apoptosis, and the effect of CCL28 at 100 ng/ml appeared more prominent than effect at 500 ng/ml. Blocking the signaling of CCL28–CCR3/CCR10 inhibited CCL28-induced apoptosis.

Discussion

We have demonstrated that the chemokine CCL28 and its receptors CCR3 and CCR10 are co-expressed in human first-trimester DSCs. Interestingly, higher levels of inflammatory cytokines secretion and CCR3/CCR10 expression are observed in DSCs from spontaneous abortion versus normal pregnancy. The expression of CCL28 and its receptors in DSCs is increased upon exposure to IL-1β, TNF-α and IL-17A. The expression of CCL28 is further enhanced by combined treatment of these inflammatory factors. Cell growth and apoptosis study reveals that CCL28 induces cell apoptosis, but not growth of DSCs. Surprisingly, the effect of CCL28 at 100 ng/ml appeared more prominent than effect at 500 ng/ml. The higher dose of CCL28 induces significantly increased but relatively lower apoptosis of DSCs. It might be possible that different CCL28 receptor might have different affinity with CCL28, and the affinity of CCL28 to CCR3 and CCR10
probably is responsible for the modulation of CCL28-induced different cell biological behaviors. CCL28 might bind CCR3 in relative low concentration and induce apoptosis. Higher concentrations of CCL28 could bind CCR10 or some unknown molecular and show other effects. However, this needs to be investigated in a future study. The action of CCL28 on DSCs apoptosis is inhibited mainly by neutralizing antibodies against CCR3, especially in the pretreatment with anti-CCL28 antibody.

These data suggest that inflammation-induced CCL28 up-regulation and interaction with CCR3/CCR10 in DSC is likely associated with pregnancy failure. Our study adds new insight that excessive inflammatory stimulation might cause pregnancy loss via DSC apoptosis. We have revealed a novel mechanism in which a local inflammation cytokine exerts a deleterious effect on pregnancy via CCL28-mediated DSC apoptosis.

Figure 4  Inflammatory cytokines regulate expression of CCL28 and its receptors CCR3/CCR10 in DSCs. (A) DSCs were seeded at 2 × 10^5 cells/ml in 6-well plates, and were stimulated with indicated concentrations and combinations of the inflammatory cytokines IL-1β, TNF-α and IL-17A, for 24 h. Cells were assessed for CCL28 mRNA expression by real-time RT–PCR. (B) DSCs were seeded at 2 × 10^4 cells/200 μl in 96-well plates, and treated with 10 ng/ml of IL-1β, TNF-α, IL-17A or their different combinations for 48 h. CCL28 protein expression was detected by an In-cell Western assay. CCL28 (red); β-actin (green). (C) DSCs were seeded at 2 × 10^5 cells/ml in 24-well plates, and treated with 10 ng/ml of IL-1β, TNF-α, IL-17A or their combinations for 48 h. Protein expression of CCR3/CCR10 was detected by FCM. Results were highly reproducible in three independent experiments in triplicate with three decidual tissue samples. Each bar shows the mean ± SE. *P < 0.05, **P < 0.01, compared with the control. One-way ANOVA and Tamhane’s test in data analysis of this figure.
The anatomical structure of the maternal–fetal unit places the uterine decidua as a first-line protector against invasion of external infection. At the same time, decidual cells also possess the capacity for tolerance of the fetal allograft and to avoid excessive reaction to microbial pathogen. DSCs, comprising 75% of decidual cells, are the major cellular component at the maternal–fetal interface. DSCs are particularly important because of their pleiotropic functions during pregnancy. Except for their traditionally metabolic and supportive roles in pregnancy,
Challis et al. 2009). However, excessive decidual inflammation is associated with spontaneous abortion and other pregnancy complications such as pre-eclampsia and premature labor (Clark et al., 2004).

A feature of inflammatory reaction is up-regulation of a series of cytokines. It has been shown that the expression of TNF-α, IFN-γ and IL-2 is significantly higher in placenta and peripheral blood mononuclear cells of abortion-prone pregnancy compared with normal pregnancy (Tangri et al., 1994; Callej-Agius et al., 2012). Additionally, T-helper cells from women with RPL have significantly elevated Th1/Th2 and TH17/regulatory T cell cytokine ratios (Wang et al., 2002; Lee et al., 2011, 2012). Such Th1 immunity against trophoblast is inhibited by progesterone (Blois et al., 2004; Ramhorst et al., 2012). Therefore, inflammatory cytokines such as IL-1β, TNF-α and IL-17A play important roles in spontaneous abortion (Lee et al., 2011, 2012; Giannubilo et al., 2012). TNF-α and IFN-γ stimulates the programmed death of human primary villous trophoblast cells (Hills et al., 2006). In addition, IFN-γ and TNF-α promote Fas expression and sensitize trophoblast cells to Fas-mediated apoptosis. TNF-α also inhibits trophoblast migration by elevating plasminogen activator inhibitor-1 expression in first-trimester villous explant cultures, causing abnormal trophoblast invasion (Bauer et al., 2004). TNF-α also down-regulates trophoblast endocrine function, leading to pregnancy failure (Monzon-Bordonaba et al., 2002). Recently, it has been reported that the inflammatory cytokines IL-1β and TNF-α significantly enhance macrophage colony-stimulating factor expression in first-trimester decidual cells and initiate caspase-dependent EVT apoptosis through macrophages (Wu et al., 2012). Unlike these reports, our current data demonstrate that inflammatory cytokines induce DSC apoptosis via CCL28–CCR3/CCR10 interaction, participating in pregnancy failure.

Chemokines consist of a super-family of small chemotactic cytokines that are well known for their functions in leukocyte recruitment and inflammatory regulation. Based on a structural motif of the number and position of two conserved cysteine residues, chemokines are categorized into subfamilies based on the number and spacing of the NH2-terminal cysteines. Chemokine CCL28 is expressed in both murine and human mucosal tissues (Pan et al., 2000; Wang et al., 2000), and displays chemotactic activity for eosinophils and both CD4+ and CD8+ T-cells through CCR3 and CCR10 receptors (Eksteen et al., 2006; Xiong et al., 2012). Human CCL28 has a coding sequence of 373 nucleotide base pairs encoded by four exons separated by introns of varying size. There is 76% nucleic acid identity between mouse and human sequences, which results in 83% similarity at the amino acid level (Pan et al., 2000; Wang et al., 2000). CCL28 plays important roles in mucosal immunity by recruiting IgA ASCs into mucosal lamina propria. Tumor hypoxia promotes the recruitment of regulatory T cells through induction of CCL28 expression, which in turn promotes tumor tolerance and angiogenesis (Facchiabene et al., 2011). CCL28 production is induced by inflammatory cytokines and bacteria (Limura et al., 2004). Estrogen regulates MEC/CCL28 expression in the uterus, which is crucial for IgA ASC migration into the uterus after mucosal vaccination, thus protecting the endometrium from microbial infections (Cha et al., 2011). We have found that DSCs express CCL28 and its receptors CCR3 and CCR10. Inflammatory cytokines such as TNF-α, IL-1β and IL-17A significantly up-regulate CCL28 and CCR3/CCR10 production in DSCs, suggesting that CCL28 might mediate inflammatory cytokine action on DSCs. Indeed, DSC-produced chemokine CCL28 induces DSC apoptosis via binding CCR3/CCR10. Previous studies focused on the toxic role of inflammatory cytokines on placental trophoblasts. The unique finding in our study is that inflammatory cytokine-mediated CCL28 induces DSC apoptosis via binding CCR3 and CCR10. Interestingly, DSCs from spontaneous abortion expresses higher CCR3/CCR10 than that of normal pregnancy, which is accompanied by higher levels of inflammatory cytokines. These data suggest that local inflammation might exert cytotoxic effects on DSCs by inducing CCL28 production and promoting CCL28–CCR3/CCR10 interaction. However, the expression of CCL28 shows no significant difference between the normal pregnancy and spontaneous abortion on CCL28 mRNA and protein level (unpublished data). It might be possible that the observed effects in our study are a consequence of spontaneous abortion and not a cause.

Because DSCs play important roles in pregnancy maintenance, inflammation-related DSC apoptosis can significantly contribute to spontaneous abortion. It is worthy to further investigate the role of different apoptotic pathways in this context that are involved in the pregnancy wastage.

**Figure 5** CCL28 promotes DSC apoptosis via binding CCR3 and CCR10. (A) Primary DSCs were seeded at 2 × 10⁴ cells/200 µl in 96-well plates, and treated with different indicated concentrations of CCL28 for 48 h. A commercial cell enumeration kit was used to analyze DSC growth. (B) DSCs were seeded at 2 × 10⁵ cells/ml in 24-well plates, and cultured with different indicated concentrations of CCL28 for 48 h. FCM was used to analyze DSC apoptosis. Apoptotic cells were defined as AnnexinV+PI− cells. The nuclear morphology of DSCs was determined by DAPI staining. Data represent the mean ± SEM of three independent experiments with three different the results. (C) DSCs were seeded at 2 × 10⁵ cells/ml in 24-well plates, and cultured with control, 100 ng/ml CCL28, 100 ng/ml CCL28 with neutralizing antibodies against CCR3 (5 µg/ml), against CCR10 (5 µg/ml), against CCR3 (5 µg/ml) and CCR10 (5 µg/ml) against CCL28 (5 µg/ml) or against CCL28 (5 µg/ml) with CCR3 (5 µg/ml) and CCR10 (5 µg/ml) together for 48 h. FCM was used to analyze DSC apoptosis. Apoptotic cells were defined as AnnexinV+PI− cells. The nuclear morphology of DSCs was determined by DAPI staining. Data represent the mean ± SEM of three independent experiments with three different the results. One-way ANOVA and Tamhane’s test in data analysis of (A) and (B); two-way ANOVA and Tamhane’s test in data analysis of (C).
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Authors’ roles

C.S. designed the experimental study and carried out most of the experiment and the analysis; Y.-Y.Z. participated in qPCR, in-cell Western; C.-L.T. assisted with the experiment design and analysis; S.-C.W. and H.-L.P. assisted with sample collection and FCM; Y.T. and R.Z. assisted with ELISA; M.-R.D. participated in experimental design and analysis, manuscript drafting and critical discussion. D.-J.L. conceived and analyzed the study, critically discussed and edited the manuscript.

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Conflict of interest

None declared.

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