IL-33 enhances proliferation and invasiveness of decidual stromal cells by up-regulation of CCL2/CCR2 via NF-κB and ERK1/2 signaling

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ABSTRACT: Interleukin (IL)-33, a newly described member of the IL-1 family, has been reported to facilitate primary tumor progression and metastatic dissemination. However, its biological function on decidual stromal cells (DSCs) remains unclear. In this study, we tested the hypothesis whether IL-33 promotes proliferation and invasion of DSCs, and the possible mechanism. IL-33 and its orphan receptor ST2 was found to be co-expressed by DSCs in human first-trimester pregnancy. Addition of IL-33, enhanced the proliferation and invasion of DSCs in a dosage-dependent manner, concomitantly with increasing expression of proliferation relative gene (PCNA, survivin) and invasion relative gene (titin, MMP2). Blocking IL-33/ST2 signaling by soluble sST2 apparently abolished the stimulatory effect on the proliferation, invasiveness and related gene expression in DSCs. We also demonstrated that chemokines CCL2/CCR2 was significantly increased with IL-33 administration. Moreover, inhibition of CCL2/CCR2 activation using CCL2 neutralizing antibody or CCR2 blocker prevented IL-33-stimulated proliferation and invasiveness capacity of DSCs. Increasing phosphorylation of nuclear factor NF-κB p65 and extracellular signal-regulated kinases ERK1/2 after treatment with IL-33 was confirmed by western blotting. And the IL-33-induced CCL2/CCR2 expression was abrogated by treatment with the NF-κB inhibitor BAY 11-7082 or ERK1/2 inhibitor U0126. Finally, we showed that decreased IL-33/ST2 expression was observed in DSCs from spontaneous abortion compared with normal pregnancy at both gene and protein levels. This study provides evidence for the molecular mechanism of IL-33 in promoting proliferation and invasiveness of DSCs by up-regulation of CCL2/CCR2 via NF-κB and ERK1/2 signal pathways and thus contributes insight to the potential of IL-33 involved in successful pregnancy via inducing DSCs mitosis and invasion.

Key words: decidual stromal cells / IL-33 / CCL2 / proliferation / invasion

Introduction

Embryo implantation, decidualization and placentation are crucial steps for successful establishment of mammalian pregnancy that require a series of regulatory mechanisms involving both fetus-derived trophoblast cells and maternal decidua. The abnormal dialogue between mother and embryo may disturb the biological functions of cells assembling at the materno-fetal interface, resulting in several pregnancy associated diseases, such as implantation failure, miscarriage, preterm birth, pre-eclampsia and intrauterine growth restriction (Carson et al., 2000; Salker et al., 2010).

Decidual tissue, the mother-derived component of the materno-fetal interface, is composed principally of typical stromal-type cells as well as epithelial cells and immunocytes (Bulmer, 1995). Decidual stromal cells (DSCs), comprising 75% of decidual cells, are thought to exert nutritive, endocrine and immune functions that play an important role in embryo implantation and placentation (Zhu et al., 2009; Nancy et al., 2012). Existing data show that even before embryo attaches to uterine epithelium, DSCs start to secrete leukemia-inhibiting factor, a cytokine belonging to IL-6 family, which regulates proliferation and decidualization of DSCs. The efficiency of decidualization of DSCs is vital and impaired decidualization can lead to adverse pregnancy outcomes (Charnock-Jones et al., 1994; Dimitriadis et al., 2000). More interestingly, in embryo co-culture and rat models, it is observed that during the process of embryo implantation, DSCs are not passively invaded by the embryo, instead, they actively penetrate the basal lamina and support trophoblast cells invasion (Schlaeke et al., 1985; Grewal et al., 2008). However, the proliferation and invasive capacities of DSCs in early pregnancy have not been fully understood.

Interleukin-33 (IL-33) is a novel member of the IL-1 superfamily that can induce synthesis of chemokine and Th2-associated cytokine via its
orphan receptor ST2 (Komai-Koma et al., 2007). ST2 exists in several forms, including transmembrane bound (ST2L) and soluble variants (sST2). ST2L is considered to be the functional component for induction of IL-33 bioactivities, while sST2 acts as a decoy receptor for IL-33 (Palmer and Gabay, 2011). IL-33 has been shown to be involved in the pathophysiology of many diseases, such as asthma, arthritis, obesity and atherosclerosis (Miller et al., 2008, 2010; Mu et al., 2010; Oboki et al., 2011; Ohno et al., 2012). It has been reported that dysregulation of IL-33/ST2 prolongs uterine receptivity in recurrent pregnancy loss (RPL) patients and disordered levels of serum IL-33/ST2 are associated with miscarriage and pre-eclampsia (Gnanne et al., 2011; Kaitu’U-Lino et al., 2012; Salker et al., 2012). However, to the best of our knowledge, there is no published literature focusing on the roles of IL-33 in biological functions of DSCs and the related mechanisms. Recent studies also show that IL-33 drives production of chemokine and Th2 cytokine via activation of several intracellular signaling pathways, including extracellular signal-regulated kinases ERK1/2, c-Jun NH(2) terminal kinase JNK, p38 and nuclear factor-kappaB (NF-kB) (Schmitz et al., 2005; Funakoshi-Tago et al., 2008; Chow et al., 2010; Tare et al., 2010; Yndestad et al., 2010). Since chemokine secretion is a complex and tightly controlled process, and intracellular pathways regulating secretion are often uniquely tailored to each chemokine and cell type, whether IL-33 can activate the above-mentioned signaling pathways in DSCs is unclear.

Therefore, here we sought to examine the pattern of IL-33 and ST2 expression at maternal–fetal interface and to analyze the regulatory effects of IL-33 on the biological behaviors of DSCs. Our current results demonstrate that IL-33 stimulates the activation of NF-kB and ERK1/2 to increase the expression of CCL2/CCR2, thereby promoting the proliferation and invasion of DSCs, which may play a role in establishing and maintaining physiological pregnancy.

Materials and Methods

Decidual tissue collection

The human decidual samples were obtained from 30 healthy women (age, 23 ± 1.9 years, mean ± SD) in first-trimester pregnancies (group of normal pregnancy (NP); 54 ± 3.5 days, mean ± SD) who underwent elective vaginal termination for non-medical reasons at Obstetrics and Gynecology Hospital, Fudan University. Subjects attended the hospital for RPL were also investigated according to the standard clinical protocols (group of RPL; age, 22 ± 1.45 years, mean ± SD; gestational age at sampling, 52 ± 3.6 days, mean ± SD). RPL was defined as three or more consecutive pregnancy losses occurred during first or second trimester. Decidual samples for immunohistochemistry were obtained after curettage and immediately fixed with 4% formalin. This study was approved by Fudan University Human Investigation Committee, and informed consent was obtained from each woman enrolled.

Isolation and culture of DSCs

Decidual tissues were collected under sterile conditions and transported to the laboratory in ice-cold Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Gibco, USA) with 10% fetal calf serum (FCS; HyClone, Logan, UT, USA). DSCs were isolated by trypsin-DNase I digestion and discontinuous percoll gradient centrifugation according to the methods described in our previous study (Wu et al., 2004). DSCs, which ranged in density between 1.042 and 1.062 g/ml, were recovered and cultured in DMEM/F12 complete medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO2 at 37°C. Through this method, we obtained >98% vimentin-positive and cytokeratin-negative DSCs (Supplementary data, Fig. S2).

Enzyme-linked immunosorbent assay for determination of IL-33 and CCL2

The purified DSCs were treated with trypsin, and transferred into 24-well plates at 2 × 10^5, 4 × 10^5 or 8 × 10^5 cells/well. The supernatants were harvested at 24, 48 and 72 h of culture for IL-33 determination. To evaluate the secretion of CCL2 after treatment with rhIL-33, rsST2-Fc fusion protein (sST2) or signaling pathway inhibitors of NF-kB (BAY 11-7082, ERK1/2 (U0126) and JNK (SP600125), DSCs (2 × 10^5 cells/well) were seeded in 24-well plates with or without rhIL-33(0.01–10 ng/ml in a 10-fold dilution series, 3625-IL, R&D Systems, Minneapolis MN, USA), sST2 (100–400 ng/ml in a 2-fold dilution series, 523-ST, R&D Systems), BAY 11-7082 (2.5 μM, Beyotime Institute of Biotechnology, Jiangsu, China), U0126 (10 μM, Beyotime Institute of Biotechnology) and SP600125 (10 μM, Beyo-time Institute of Biotechnology). The supernatant was then collected after 48 h. At the same time, we detected the protein concentration, and the CCL2 level of each group was calculated as the ratio of the CCL2 concentration of supernatant to the protein concentration. Each supernatant was centrifuged to remove cellular debris and stored at −80°C until being assayed. IL-33 and CCL2 concentrations in the supernatant were measured by IL-33 enzyme-linked immunosorbent assay (ELISA) kits (435 907, BioLegend, San Diego, USA) and CCL2 ELISA kits (Dakewei Biotech Co., Ltd, Shenzhen, China), respectively, according to the instructions of manufacturer. Each experiment was carried out in triplicate.

BrdU cell proliferation assay

Bromodeoxyuridine (5-bromo-2′-deoxyuridine, BrdU) cell proliferation assay was applied to evaluate the effect of IL-33 on cell proliferation with or without sST2, anti-CCL2 neutralizing antibody (MAB279, R&D Systems) and CCR2 blocker RS102895 (R1903, Sigma-Aldrich, Santa Clara, USA). The isolated DSCs were seeded at a density of 1 × 10^5 cells/well in 96-well flat-bottom microplates. After 70–80% confluence, the cells were starved with DMEM containing 1% FCS for 12 h before treatment. The medium was removed once again, and the cells were treated with rhIL-33 (0.01–10 ng/ml), sST2 (100–400 ng/ml in a 2-fold dilution series), CCL2 neutralizing antibody (1 μg/ml) or CCR2 blocker RS102895 (100 ng/ml) with vehicle as control. After stimulation with rhIL-33 (1 ng/ml), the cells were treated with CCL2 neutralizing antibody (1 μg/ml) or RS102895 (100 ng/ml) for another 48 h. Mouse isotype (1 μg/ml) (Sino-America Co. Ltd.) or vehicle as negative control was added. The ability of DSCs proliferation was detected by BrdU cell proliferation assay kits (Calbiochem, Germany) according to the manufacturer’s instruction. Each experiment was carried out in triplicate, and repeated four times.

Matrigel invasion assay

The invasion of DSCs across matrigel was evaluated objectively in an invasion chamber, according to our previous procedure (Li et al., 2010). Briefly, the cell inserts (8-μm pore size, 6.5-mm diameter, Corning, USA) coated with 15–25 μl of matrigel were placed in a 24-well plate. The primary DSCs at a density of 2 × 10^5 cells/well were plated in the upper chamber. rhIL-33 (at 0.01, 0.1, 1 and 10 ng/ml), sST2 (100, 200 and 400 ng/ml), CCL2 neutralizing antibody (1 μg/ml) and CCR2 blocker RS102895 (100 ng/ml) were added or not. The lower chamber was filled with 800 μl of DMEM/F-12 containing 1% charcoal stripped FCS. The cells were then incubated at 37°C for 48 h. Then the inserts were removed, washed in phosphate-buffered saline (PBS) and the non-invading cells together with the matrigel...
were removed through wiping the upper chamber with a cotton bud. The inserts were then fixed in 4% methanol for 10 min at room temperature and stained with hematoxylin. The cells migrating to the lower surfaces were removed through wiping the upper chamber with a cotton bud.

**Cell cycle assay and CCR2 detection by flow cytometry**

A change of cell cycle would affect cell proliferation. Since IL-33 showed a dose-dependent effect on DSCs proliferation, we investigated whether IL-33 influences the cell cycle of DSCs after treatment with rhIL-33, sST2, CCL2 neutralizing antibody or CCR2 blocker RS102895. DSCs were treated as same as noted before except they were cultured at a density of 3 × 10^5 cells/well in 12-well microplates. 48 h later, DSCs were harvested and washed with PBS, fixed with cold 70% ethanol, stained with propidium iodide (Sigma-Aldrich), ribonuclease-A (Sigma-Aldrich) treated and subjected to flow cytometry analysis. The degree of proliferation in primary cultures were evaluated by measuring proliferation index, i.e. (S% + G2%)/ (G1% + G2%). Both experiments were performed in triplicate, and repeated five times.

To evaluate the CCR2 expression level, DSCs were treated as same as mentioned above and cultured for 48 h, treated with trypsin, then transferred into plastic tubes. They were then washed and suspended in PBS, incubated in CCR2-allophycocyanin (APC) labeled antibody for 30 min at room temperature. Next, cells were washed and suspended in PBS, and immediately analyzed by a four-color flow cytometry using CellQuest software (FACSCalibur, BD Biosciences, Rockville MD, USA) with an isotypic control.

**Immunohistochemistry**

Paraffin sections (5 μm) of decidua from group of NP (n = 6) and RPL (n = 6) were dewaxed in xylene, rehydrated in graded ethanol and in Tris-buffered saline (TBS). To block endogenous peroxidase, tissue sections were incubated with hydrogen peroxide and 1% bovine serum albumin (BSA)/TBS. Thereafter, the samples were incubated with mouse anti-human IL-33 antibody (20 μg/ml, AB9362S, R&D Systems), rabbit anti-human ST2 antibody (10 μg/ml, PRS3363, Sigma-Aldrich) or mouse/rabbit IgG isotype overnight at 4°C in a humid chamber. After washing three times with TBS, the sections were overlaid with peroxidase-conjugated goat anti-mouse/rabbit IgG (Golden Bridge International, Inc., Beijing, China); the reaction was developed with 3,3’-diaminobenzidine and counterstained with hematoxylin. The immunohistochemical results were evaluated by a pathologist. The experiments were repeated five times.

**Quantitative real-time polymerase chain reaction**

Total RNA from DSCs treated with IL-33 or sST2 for 48 h was extracted using the TRIzol reagent (TaKaRa Biotechnology Co., Ltd, Tokyo, Japan), according to the manufacturer’s instructions. Besides, the IL-33/ST2 mRNA levels in normal DSCs from healthy controls (NP, n = 6) and DSCs from RPL patients (n = 6) were also evaluated by performing quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA (1 μg) was reverse transcribed into first-stand cDNA (RR036A, TaKaRaBiotechnology Co., Ltd, Tokyo, Japan) following the manufacturer’s procedure. The synthesized cDNA was used as a template for PCR amplification. Real-time PCR was performed using ABI PRISM® 7900 Sequence Detector (Applied Biosystems, Warrington, UK). The primer sequences are indicated in Table I. PCRs were carried out for 40 cycles using the following conditions: denaturation at 95°C for 30 s, annealing at 95°C for 5 s and elongation at 60°C for 34 s. The expression levels of the samples were expressed as arbitrary units defined by the 2^(-ΔΔCt) method. All measurements were performed in triplicate. The specificity of the product was assessed from melting curve analysis.

**Western blotting**

Whole-cell protein extracts were prepared by lysing cells in RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors (Beyotime Institute of Biotechnology). Protein yield was quantified using the BCA protein assay. After denaturation, equal amounts of protein were separated by SDS-PAGE before wet-transfer onto PVDF membranes (Amersham Biosciences, UK). Non-specific binding sites were blocked by 5% BSA in TBS with 0.1% Tween (TBS-T). Following washing, the membranes were incubated with specific antibodies against ST2 (1:5000), rabbit anti-human ST2 antibody (2 μg/ml, PRS3363, Sigma-Aldrich), IL-33 (2 μg/ml, MAB3625, R&D Systems), GAPDH and Tubulin (1:1000, Beyotime Institute of Biotechnology). p-ERK1/2 Thr202/Tyr204, p-JNK Thr183/Tyr185, p-p38 Tyr187, p-ERK1/2 Thr202/Tyr204, p-NFκB p65, p-JNK Thr183/Tyr185, p-p38, ERK1/2 Thr202/Tyr204, NFκB p65, JNK and p38 (1:1000, Cell Signaling Technology, Danvers, MA) diluted in blocking buffer (5% BSA, 1 × TBS, 0.1% Tween) overnight at 4°C. Primary antibody was removed by washing the membranes four times in TBS-T, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:5000). Following three times of washing in TBS-T, immunopositive bands were visualized with the ECL detection system (Pierce, Rockford, USA). Quantitative analysis of the relative density of the bands in western blots was performed using Image J.

**Statistical analysis**

All studies were set up to include three wells per condition, and each experiment was independently repeated a minimum of three times. Data collected from each independent experiment were analyzed with the statistical package Graphpad Prism (Graphpad software Inc.). T-test or one-way analysis of variance was used when appropriate. Differences were considered as statistically significant at P < 0.05.
Results

IL-33/ST2 is co-expressed by DSCs in human first-trimester pregnancy

To determine the expression and distribution of IL-33/ST2 at human maternal–fetal interface, we performed immunohistochemistry with paraffin-embedded decidua. As shown in Fig. 1A, the enlarged round-shaped DSCs were moderately stained for IL-33 and ST2.

To further study the secretion level of IL-33 by primary DSCs, ELISA analysis was conducted. Our data showed that there was no significant time-dependent manner of IL-33 production during 24–72 h. Moreover, although the increase of IL-33 secretion seemed paralleled with the increase of culture cell density (Fig. 1B and Supplementary data, Fig. S1, time-dependent manner of IL-33 production during 24–72 h. Moreover, the inhibitory effect of IL-33/ST2L signaling pathway. As shown, IL-33 could stimulate the proliferation (Fig. 2A) and invasiveness of DSCs, the effect of specific sST2 did not appreciably enhance the inhibition effect (Fig. 2A and B).

In order to investigate the effects of IL-33 on DSCs, BrdU proliferation assay, cell cycle and Matrigel invasion assay were performed after rhIL-33 administration. Before stimulation with rhIL-33 (1 ng/ml), DSCs were preincubated with sST2 (100–400 ng/ml) to block IL-33/ST2L signaling pathway. As shown, IL-33 could stimulate the proliferation (Fig. 2A) and invasiveness (Fig. 2B) of DSCs, especially at the concentration of 1 ng/ml (P < 0.001), while administration of sST2 could apparently abolish the stimulatory effect of IL-33 on DSCs growth (Fig. 2A, P < 0.001) and invasion (Fig. 2B, P < 0.001). However, the inhibitory effect attained saturation at 100 ng/ml, a further higher concentration of sST2 did not appreciably enhance the inhibition effect (Fig. 2A and B). In parallel, we could observe that the treatment of sST2 alone had no significant impact on the proliferation and invasiveness of DSCs (data not shown).

Cellular proliferation is regulated primarily by the regulation of cell cycle, which consists of three distinct sequential phases, prereplicative (G0/G1), DNA synthesis (S) and mitosis (G2/M) states. To figure out whether IL-33 influences the cell cycle parameters of DSCs, we performed cell cycle assay and the ratio of (S% + G2%) to (G1% + S% + G2%) were measured to evaluate the proliferation index. As seen in Fig. 2C, the proliferation index increased with increasing IL-33 concentration and showed a peak at 1 ng/ml (Fig. 2C, group d versus group a, P < 0.001). As demonstrated in BrdU proliferation assay, after adding sST2, the proliferation index decreased significantly (Fig. 2C, group f versus group d, P < 0.01).

IL-33 up-regulates the expression of PCNA, survivin, titin and MMP2 in DSCs

To test the effects of IL-33 on the proliferation-relative and invasion-relative molecules of DSCs, qRT–PCR was applied to detect the expression of proliferating cell nuclear antigen (PCNA; a cellular marker for proliferation), survivin (a member of the inhibitor of apoptosis protein family), titin (also known as connectin, a protein that is related to invasion) and matrix metalloproteinase 2 (MMP2) (a kind of endopeptidase that acts as a mediator of metastases). DSCs were incubated for 48 h with different concentrations of IL-33 (0, 0.01, 0.1, 1 and 10 ng/ml) or sST2 (100 ng/ml). As shown in Figure 3, we found that treatment of DSCs with IL-33 significantly elevated the mRNA expression of PCNA, survivin, titin and MMP2 in a dose-dependent manner, and peaked at 1 ng/ml (Fig. 3A–C, group d versus group a, P < 0.001; Fig. 3D, group d versus group a, P < 0.01), which could be reversed by sST2 administration (Fig. 3A–C, group f versus group d, P < 0.001; Fig. 3D, group f versus group d, P < 0.01).

Effects of IL-33/ST2L/sST2 axis on CCL2/CCR2 expression in DSCs

To test the conjecture that IL-33 might increase the expression of CCL2 and CCR2, we used ELISA and flow cytometry to evaluate CCL2 and CCR2 expression of DSCs, respectively. Our data indicated that after IL-33 treatment, CCL2 secretion increased significantly and peaked at 1 ng/ml (Fig. 4A, group d versus group a, P < 0.001), while this effect was blocked by the addition of sST2, the IL-33 blocker (Fig. 4A, group f versus group d, P < 0.001). Meanwhile, the expression of CCR2 on DSCs also obviously elevated and then declined after sST2 treatment (Fig. 4B, group d versus group a, P < 0.001; group f versus group d, P < 0.001).

The increased proliferation and invasiveness of DSCs induced by IL-33 are dependent on CCL2/CCR2 interaction

In order to evaluate whether CCL2/CCR2 signaling is involved in regulating the IL-33-mediated growth and invasion of DSCs, we treated DSCs with IL-33 and anti-CCL2 neutralizing antibody or CCL2 blocker (RS102895) for 48 h. As shown in Figure 5A and C, both CCL2 neutralizing antibody and CCR2 blocker decreased the proliferation and invasiveness of DSCs (Fig. 5A, group c versus group a, P < 0.01; group e versus group a, P < 0.001; Fig. 5C, group c versus group a, P < 0.01; group e versus group a, P < 0.001). Meanwhile, the expression of CCL2 on DSCs also obviously elevated and then declined after sST2 treatment (Fig. 5B, group d versus group a, P < 0.001; group f versus group d, P < 0.001).

IL-33 up-regulates the expression of CCL2/CCR2 by activating NF-κB and ERK1/2 signal pathways in DSCs

To investigate the specific signaling pathways involved in the increased proliferation and invasion acceleration of DSCs induced by IL-33, we stimulated DSCs with 1 ng/ml IL-33 for indicated time. We monitored the phosphorylation of NF-κB (p65) and various MAP kinases (ERK1/2, JNK, P38), which could be induced by IL-33 stimulation with a peak of phosphorylation occurring at 30 min and decreased levels at 60 min. NF-κB phosphorylation could be blocked by sST2 treatment. In addition to NF-κB, IL-33 also induced activation of ERK1/2 with a peak of phosphorylation occurring at 5 min. Phosphorylation of this kinase could also be inhibited by sST2.

To further investigate the specific signaling pathways involved in the stimulation of CCL2/CCR2 expression in DSCs, we found that
signal transduction inhibitors on DSCs was examined. The results showed that BAY 11-7082 (inhibitor of NF-κB) and U0126 (inhibitor of ERK1/2) not only directly inhibited the CCL2/CCR2 expression of DSCs (Fig. 6B, group b versus group a, \( P < 0.001 \), group c versus group a, \( P < 0.001 \); Fig. 6C, group b versus group a, \( P < 0.001 \), group c versus group a, \( P < 0.001 \)), but also reversed the enhancement of CCL2/CCR2 expression of DSCs induced by IL-33 (Fig. 6B, group f versus group e, \( P < 0.001 \), group g versus group e, \( P < 0.001 \); Fig. 6C, group f versus group e, \( P < 0.001 \), group g versus group e, \( P < 0.001 \)).
Figure 2. rhIL-33 promotes the proliferation and invasiveness of DSCs. DSCs (1 × 10^4 cell/well, BrdU proliferation assay; 3 × 10^5 cells/well, cell cycle assay; 2 × 10^5 cells/well, invasion assay) were treated with rhIL-33 (0.01–10 ng/ml) with vehicle as control. Before stimulation with rhIL-33 (1 ng/ml), DSCs were preincubated with sST2 (100–400 ng/ml in a 2-fold dilution series) to block IL-33/ST2L signaling pathway. Thereafter, BrdU proliferation assay (A), invasion assay (B) and cell cycle flow cytometry (C) were conducted, respectively. The data shown represent independent experiments (mean ± SD), error bars depict the standard deviation of the sample mean. The images are from an experiment that is representative of three independently conducted experiments. The y-axis represents the percentage of cells. The x-axis, from the left, represents the cell cycle phases of G1 (green region), S (the region between G1 and G2) and G2 (blue region). ***P < 0.001, **P < 0.01.
group f versus group e, \( P < 0.001 \), group g versus group e, \( P < 0.001 \). Activation of JNK kinases but not p38 was also observed (Fig. 6A).

However, the JNK inhibitor, SP600125, did not affect IL-33-induced CCL2/CCR2 expression (Fig. 6B and C), suggesting that the IL-33-stimulated increase of CCL2/CCR2 expression might depend on NF-κB and ERK1/2 signal pathways.

**IL-33/ST2 expression decreases in DSCs from RPL**

To further explore the beneficial role of IL-33 in pregnancy, we compared the expression of IL-33/ST2 in DSCs from NP versus RPL. As shown in Fig. 7A, moderate nuclear and cytoplasmic IL-33/ST2 stainings were observed in the stromal cell compartment while the DSCs of RPL show weaker immunoreactivity than the normal DSCs. Furthermore,
Effects of IL-33/ST2 pathway on decidual stromal cells

Figure 4 (Continued)
Figure 5  The increased proliferation and invasiveness of DSCs induced by IL-33 are dependent on CCL2/CCR2 interaction. DSCs (1 × 10⁴ cell/well, BrdU proliferation assay; 3 × 10⁵ cells/well, cell cycle assay; 2 × 10⁵ cells/well, invasion assay) were treated with rhIL-33 (1 ng/ml), α-CCL2 (1 μg/ml), or CCR2 blocker RS102895 (100 ng/ml) with vehicle as control. After stimulation with rhIL-33 (1 ng/ml), the cells were treated with CCL2 neutralizing antibody (1 μg/ml) or RS102895 (100 ng/ml) for another 48 h. Thereafter, BrdU proliferation assay (A), cell cycle (B) and invasion assay (C) were conducted to analyze proliferation and invasiveness of DSCs, respectively. Error bars depict the standard deviation of the sample mean. The images are from an experiment that is representative of three independently conducted experiments. The x-axis, from the left, represents the cell cycle phases of G1 (green region), S (the region between G1 and G2) and G2 (blue region). *P < 0.05, **P < 0.01, ***P < 0.001.
Effects of IL-33/ST2 pathway on decidual stromal cells

A

B

C
qRT–PCR and western blot were performed on DSCs isolates from healthy controls (n = 6) and DSCs from RPL patients (n = 6). IL-33/ST2 expression was decreased in DSCs from spontaneous abortion at both gene (Fig. 7B) and protein levels (Fig. 7C).

**Discussion**

Despite more than a century of intensive study, the mechanism of successful pregnancy remains unclear. A large body of research suggests that DSCs play an important role in embryo implantation and placenta formation due to their pleiotropic functions such as facilitating invasion of trophoblasts and producing factors conductive to Th2 bias (Wang and Dey, 2006; Grewal et al., 2008; Bazer et al., 2009). As shown in Fig. 8, the key finding of the present report is that IL-33 can stimulate CCL2/CCR2 expression through activating NF-κB and ERK1/2 pathways, and further promote the proliferation, invasion of DSCs. In addition, we found that the proliferation and invasion related downstream molecules, such as PCNA, survivin, titin and MMP2 elevated in response to IL-33. Our findings, taken together with the recent reports from Granne et al. (2011), Kaitu‘U-Lino et al. (2012) and Salker et al. (2012), suggest that IL-33 is of particular importance in early placentation and that its dysregulation may contribute to the pathogenesis of diseases such as miscarriage and pre-eclampsia.

IL-33 is a versatile molecule that exerts important functions in various cell types (Mirchandani et al., 2012). In addition to its known role in asthma, cardiovascular disease and arthritis, there is convincing evidence that IL-33 functions as a mitogen in eosinophils, fibrocytes, endothelial cells and its proliferation promotion effect on mouse mammary adenocarcinoma can even facilitate tumor progression (Cho et al., 2009; Chow et al., 2010; Bianchetti et al., 2012; Jovanovic et al., 2012). Besides, IL-33 can also promote the migration of TH2 lymphocytes, fibrocytes and endothelial cells, and aggravate the invasion and metastasis of breast tumor (Komai-Koma et al., 2007; Choi et al., 2009; Bianchetti et al., 2012; Milovanovic et al., 2012). Despite this, it is unclear whether IL-33 plays a role in regulating the growth and invasion of DSCs, and the exact molecular mechanism of the response to IL-33 has yet to be investigated.

In this study, we first demonstrated that DSCs constitutively express IL-33, and immunohistochemistry revealed immunoreactive IL-33/ST2 staining in DSCs, consistent with previous study (Salker et al., 2012). It has been shown that IL-33 is an endogenous ‘danger’ signal or ‘alarmin’ (Haraldsen et al., 2009). Its nuclear localization combined with the lack of a signal sequence for export lead to the notion that IL-33 is released mainly upon tissue injury or infection (Palmer and Gabay, 2011). However, the presence of IL-33 in human decidua is not unexpected as recent studies show that fibroblasts under mechanical strain and cultured human smooth muscle cells in vitro can secrete IL-33 in the absence of necrosis (Schmitz et al., 2005; Kakkar et al., 2012). It is therefore not incredible that the profound change in cell shape and actin dynamics upon decidualization trigger nuclear export and secretion of IL-33 via a non-classic mechanism. And IL-33 expression in DSCs may also result from the contractile phenotype biomechanically induced by culture of the cells on plastic which is inevitable because of the need of experiment. In contrast, in a recent publication, Fock et al. (2013) described that no IL-33 secretion could be detected in DSCs by ELISA; an alternative hypothesis to resolve this inconsistency is that more cell number and less medium we used in the culture system enriched the concentration of IL-33 to a higher lever which was above the limit of detection.

Next we probed into the effect of IL-33 on the biological behaviors of DSCs, we disclosed in the present study that IL-33 reinforced the proliferation and invasiveness of DSCs in vitro, and the increased mitotic and invasive ability of DSCs could be reversed by sST2 (the decoy receptor of IL-33), suggesting that IL-33 was capable of modulating the proliferation and invasion of DSCs via IL-33/ST2 axis. This conjecture was further confirmed by cell cycle assay, which unambiguously unveiled that enhanced proliferation index (PI = (5% + G2%) / (G1% + S% + G2%)) of DSCs induced by IL-33 could be abolished by sST2.

To clarify the downstream effective molecules of IL-33 in regulating the proliferation and invasion of DSCs, we further found that IL-33 can promote PCNA, survivin, titin and MMP2 mRNA expression, which further the evidence that IL-33 affects proliferation and invasiveness of DSCs. PCNA and survivin can reflect the state of cell proliferation while MMP2 contributes to invasion of cells (Ray and Stetler-Stevenson, 1994; Stoimenov and Helleday, 2009; Coumar et al., 2013). Titin, also known as connectin, is reported to be related to the invasiveness of cytotrophoblast (Du et al., 2007). Given that these four molecules also elevated in CCL2-treated endometrial stromal cells (Li et al., 2012), we speculate whether there might be a link between IL-33 and CCL2.

Accumulating evidence indicates that IL-33 can induce increased cytokine and chemokine production. For example, mast cells, epithelial cells, endothelial cells and murine fibroblasts can all produce CCL2 in response to IL-33 in vitro (Moulin et al., 2007; Funakoshi-Tago et al., 2008; Yamagi et al., 2010). Sure enough, in this study, we observed that the expression of CCL2 and CCR2 increased along with increasing IL-33 concentration and then declined after adding sST2.

The decidual microenvironment is dominated by a complicated cytokine—chemokine network (Dimitriadis et al., 2005). Our previous studies have found integrating roles of multiple chemokines, such as CXCL12/ CXCR4 and CCL2/CCR2, participate in the mitosis and invasion of DSCs (Liet al., 2012; Ren et al., 2012). Taking into account the role of IL-33 in the regulation of DSCs proliferation and invasion, we wonder whether IL-33/ST2 signal may indirectly regulate DSCs behaviors through regulating CCL2/CCR2 expression. As we expected, after blocking CCL2 signaling by CCL2 neutralizing antibody and CCR2 blocker, the proliferation of DSCs decreased, the stimulatory effect of...
IL-33 on DSCs proliferation was strikingly inhibited as well. And the invasiveness of DSCs varied in a similar manner in response to CCL2 neutralizing antibody and CCR2 blocker. Our previous study has revealed that CCL2 contributes to Th2 polarization at the feto-maternal interface, which may contribute to the generation of micromilieu optimal for the acceptance of the
semiallogenic fetus (He et al., 2012). It is also documented that CCL2 is a potent chemoattractant for macrophages which play a critical role in sustaining the development and the function of corpora lutea in early pregnancy (Melgarejo et al., 2009; Care et al., 2013). Moreover, IL-33 can also amplify the development of alternative activation macrophages (M2 macrophages) or promote the shift of M1 macrophages to M2 lineage which plays a part in type 2 immune response during tissue wound healing and airway inflammation (Kurowska-Stolarska et al., 2009; Yin et al., 2013). Therefore, based on these reports, together with our finding, we propose that IL-33 may also involve in maternal–fetal immunoregulation via the downstream CCL2 and contribute to pregnancy maintenance which has yet to be verified.

The activity of IL-33 as a signaling molecule is known to be mediated by multiple pathways, including ERK1/2, JNK, p38 and NF-κB pathways. The concomitant activation of multiple signaling pathways by IL-33 was observed in certain types of cells (Kurowska-Stolarska et al., 2008; Silver et al., 2010; Andrade et al., 2011). For instance, in eosinophils, the concomitant activation of ERK1/2 and NF-κB is associated with IL-33-mediated production of CCL2 (Chow et al., 2010). Little, however, is known about the signaling pathways triggered by IL-33 in DSCs and their contribution to chemokine production. In the present study, we ascertained that phosphorylation of ERK1/2, JNK and NF-κB increased after treatment with IL-33 while no substantial p-p38 increase downstream of IL-33. Moreover, IL-33-induced CCL2/CCR2 expression was suppressed by the inhibitors of ERK1/2(U0126), NF-κB (BAY 11-7082) but not JNK (SP600125). The critical role of NF-κB in the cytokine gene transcription has been suggested in previous studies (Khoroooshi et al., 2008), and mounting evidence has revealed that the ERK cascade could regulate proliferation, differentiation, cytokine production of various cells (Duan and Wong, 2006; Li et al., 2011). Therefore, our study demonstrated that ERK1/2 and NF-κB pathways were involved in modulating IL-33-mediated CCL2/CCR2 up-regulation.

To further explore the beneficial role of IL-33 in pregnancy, we compared the expression of IL-33/ST2 in DSCs from NP with spontaneous abortion. Decreased IL-33/ST2 expression was observed in DSCs from spontaneous abortion at both gene and protein levels. This observation would be in line with the description of a lower implantation rate of transferred embryos owing to IL-33 knockdown in decidualized endometrial stromal cells (Salker et al., 2012). However, a clinical study measuring the circulating serum level of IL-33/sST2 in pregnant women demonstrated an elevated IL-33 level at 6 weeks’ gestation in women with live pregnancies destined to fail (Kaitu’U-Lino et al., 2012). Given the limitation that the RPL cohort were samples taken from miscarriage patients with non-viable fetus and tissues, the reason for this inconsistency might be that the observed lower expression of IL-33/ST2 in our study is a consequence of spontaneous abortion while the elevated IL-33 in clinical study may be a compensatory response to attempting to rescue the pregnancy.

To sum up, we have identified IL-33 as a novel factor influencing the functional role of DSCs, which may modulate their proliferation and invasion effects through up-regulation of CCL2/CCR2 production via NF-κB and MAPK/ERK1/2. Though the relationship of inter-individual variability in IL-33/ST2 levels to pregnancy outcome awaits further studies, our data provide information in searching for effective therapeutic approaches to treat miscarriage and some pregnancy complications.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

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**Authors’ roles**

W.-T.H. conducted all experiments and prepared the figures and the manuscript. M.-Q.L. assisted with cell culture and data analysis. W.L. aided in immunohistochemistry and ELISA assay. L.-P.J. performed patients, obtained specimens and generated clinical data. D.-J.L. reviewed the study arrangement and paper. X.-Y.Z. was in charge of the study design, overseeing the completion of the study, editing and finalizing of the manuscript.

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**Conflict of interest**

None declared.

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