



## Regulation of CD4<sup>+</sup>FOXP3<sup>+</sup> T cells by CCL20/CCR6 axis in early unexplained recurrent miscarriage patients

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**ABSTRACT.** Expression and function of CCR6/CCL20 in CD4<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells (Tregs) was investigated in unexplained recurrent miscarriage (URM) patients. Flow cytometry, reverse transcription-polymerase chain reaction, enzyme-linked immunosorbent assay, western blots, and Transwell migration assays were used to analyze the expression and function of regulatory T cells in peripheral blood (PB) and decidual samples of women with URM and of healthy controls. Proportions of CD4<sup>+</sup>FOXP3<sup>+</sup> T cells and CCR6<sup>+</sup>CD4<sup>+</sup>FOXP3<sup>+</sup> T cells were lower in URM patients than in healthy controls for both PB lymphocytes and decidual samples ( $P < 0.05$ ). Expression levels of *FOXP3* and *CCR6* mRNA were lower in URM patients than in control subjects for PB and decidual samples ( $P < 0.05$ ). CCL20 protein levels were lower in URM patients than in controls ( $P < 0.05$ ). An effect of Treg migration was significantly blocked (by 89.13%) using a neutralizing anti-CCL20 antibody *in vitro*. Furthermore, CCL20-stimulated Tregs exhibited a 3.21-fold increase in migration and this was blocked using a neutralizing anti-CCL20 antibody. IL-10 concentration in culture supernatants of

CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs of URM patients was significantly lower than that in controls. Anti-CCL20 antibody inhibited IL-10 and IL-4 expression but increased IFN- $\gamma$  and IL-17 levels when there was cell-cell contact between PB CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells. No difference was detected when cell-cell contact was prevented by a semi-permeable Transwell membrane. CCL20-CCR6 could drive immune activity of CD4<sup>+</sup>FOXP3<sup>+</sup> Tregs, followed by their migration to the feto-maternal microenvironment. These results elucidated the mechanism by which Tregs exert this suppressive effect.

**Key words:** CD4<sup>+</sup>FOXP3<sup>+</sup> regulatory T cell; Chemokine receptor; Chemokine; Unexplained recurrent miscarriage

## INTRODUCTION

Mammalian pregnancy is a very complicated physiological process. When the maternal-fetal interface mediating immune tolerance is dysfunctional, it can lead to adverse pregnancy outcomes. The most common result is spontaneous abortion (Terness et al., 2007). Unexplained recurrent miscarriage (URM) is defined as the loss of three or more consecutive pregnancies before the 20th week of gestation (Cobbold and Waldmann, 2013). This condition is thought to be caused by the allojection of the fetus by the mother (Maciolek et al., 2014).

CD4<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells (Tregs) are critical to the maintenance of peripheral tolerance (Hilbrands et al., 2013) and are a subgroup of CD4<sup>+</sup> T cells, characterized by the expression of a key transcription factor, forkhead box P3 (FOXP3) (Elhogy et al., 2009). Tregs can also suppress maternal alloresponses targeted against the fetus (Hirahara et al., 2006). To carry out their suppressive function, Tregs have to migrate to the target site, and this occurs by a multistep process (Veiga-Parga et al., 2013; Whiteside, 2014). The expression of chemokines and chemokine receptors represent another central component of this intricate network (Rosenberg et al., 2009).

CC chemokine receptor type 6 (CCR6) is a marker of certain dendritic cell, B-cell, and memory T-cell subsets (Zou, 2006; Arruvito et al., 2007), and its expression in Langerhans cells and differentiated monocytes is induced by IL-10 (Welsh-Bacic et al., 2011). Importantly, CCR6 is highly expressed in Tregs, which suppress immune responsiveness (Kleinewietfeld et al., 2005; Frick et al., 2013; Li et al., 2013). Chemokine (C-C motif) ligand 20 (CCL20) is a chemokine ligand known to attract CCR6<sup>+</sup>-expressing cells by inducing migration (Marsigliante et al., 2013) and binding to endothelial cells (Qin et al., 2013). Their role in inducing immune tolerance in pregnancy has drawn increased attention recently.

This study is the first to report the regulation of CD4<sup>+</sup>FOXP3<sup>+</sup> T cells by the CCL20/CCR6 axis in URM.

## MATERIAL AND METHODS

### Subjects

All subjects were managed by the outpatient department of the Gynecology Specialist

Clinic at Renji Hospital (Shanghai, China) between December 2013 and May 2014. In this study, 20 women with URM and 20 healthy women at the early stages of pregnancy were included and peripheral blood and decidual samples were obtained from all subjects. The diagnosis of URM was made after excluding verifiable causes, such as chromosomal abnormalities, abnormalities of the uterus or cervix, endocrinal disorders, infections, metabolic diseases, congenital autoimmune disorders, and thrombophilia. Fetal heart activity was assessed using Doppler ultrasonography with a 6-MHz transducer at 7 and 9 weeks of gestation. The patients were advised to undergo an induced abortion when a fetal heartbeat was not detected in any of the examinations or when it disappeared after being detected previously. The mean age of patients with URM was  $29.6 \pm 2.5$  years. They had a mean of  $3.1 \pm 0.5$  miscarriages, at an average of  $50.1 \pm 9.5$  days of gestation. The mean age of the women in the control group was  $28.6 \pm 3.4$  years, and all of these women had one living child and no history of spontaneous abortion, ectopic pregnancy, or preterm delivery. Furthermore, in all women of the control group, fetal heart activity was identified during a week before the abortion, and elective terminations were conducted at  $52.8 \pm 14.8$  days of gestation, on average. This study was approved by the Ethics Review Board of Renji Hospital, and written consent was obtained from each participant.

### Flow cytometry

Mononuclear cells were isolated from the decidual samples by disaggregating the tissue, filtering it through a sterile net (100  $\mu\text{m}$ ; BD Biosciences, Heidelberg, Germany), and lysing the erythrocytes with RBC lysis buffer. The lymphocytes were then harvested by a discontinuous density gradient method. Peripheral blood mononuclear cells (PBMCs) were obtained following removal of the erythrocytes with RBC lysis buffer. Thereafter, the cells were washed, incubated with cell-surface staining monoclonal antibodies (FITC-conjugated antihuman CD4; BD Biosciences, San Jose, CA, USA; PE-conjugated antihuman CCR6, eBioscience, San Diego, CA, USA) for 30 min at 4°C in darkness, and then fixed in 1% paraformaldehyde for 40 min at 4°C in darkness. Staining for intracellular FOXP3 protein (BD Biosciences) was performed using a cell-fixation/cell-permeabilization kit (eBioscience), according to the manufacturer instructions. Isotype controls were used to exclude false-positive cells. Depending on the experiment, approximately  $10^5$  cells were analyzed using fluorescence-activated cell sorting (FACS-Calibur, BD Biosciences).

### RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

PBMCs (3 mL) were obtained following removal of the erythrocytes with RBC lysis buffer. Thereafter, the cells were resuspended with 1 mL TRIzol (Gibco, Life Technologies, Carlsbad, CA, USA). Frozen decidual tissues (50 mg) were treated with 1 mL TRIzol. RNA was then extracted with chloroform, precipitated with absolute ethanol, washed, and finally diluted in RNase-free water. RNA was quantified by estimating ultraviolet absorbance at 260 nm. For RT-PCR analyses, first-strand cDNA-synthesis was performed using the first-strand cDNA synthesis kit, according to the manufacturer recommendations. Primers used for RT-PCR analyses are shown in Table 1.

**Table 1.** Primers used for RT-PCR analyses.

FOXP3	Forward: 5'-AGTGCCTGTGTCCTCAATGGTC-3' Reverse: 5'-AGGGCCAGCATAGGTGCAAG-3'
CCR6	Forward: 5'-GGCAGTTACTCATGCCACCAA-3' Reverse: 5'-GGAGCAGCATCCACAGTTAAAG-3'
GAPDH	Forward: 5'-TGTGTCCGTCGTGGATCTGA-3' Reverse: 5'-TTGCTGTTGAAGTCGCAGGAG-3'

All PCR products were verified by sequencing. PCR was conducted using SYBR Green PCR Master Mix, according to the manufacturer instructions. Samples were run in triplicate. *CCR6* and *FOXP3* levels in each sample were normalized to *GAPDH* expression, and the relative change in mRNA levels are reported as fold-inductions as compared with an endogenous control using the  $2^{-\Delta\Delta C_t}$  method.

### Enzyme-linked immunosorbent assay (ELISA) for CCL20

Peripheral blood samples from patients with URM and from the controls were centrifuged at 10,000 g for 5 min at room temperature, and the serum was removed and stored at  $-80^{\circ}\text{C}$  until use. For the ELISA, the CCL20 protein levels were determined using commercially available ELISA kits (USCN Life Science Inc., Wuhan, China), according to the manufacturer instructions. Results are reported as pg/mL total protein.

### Western blots for CCL20

Proteins were extracted from frozen decidual samples by resuspending them in lysis buffer containing 500  $\mu\text{L}$  radioimmunoprecipitation assay buffer and 5  $\mu\text{L}$  phenylmethanesulfonyl fluoride for each 50-mg sample of tissue. The protein concentrations were determined using the bovine serum albumin protein assay (Pierce, Rockford, IL, USA). For western blot analysis, proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis and transferred to a nitro-cellulose membrane (Bio-Rad, Hercules, CA, USA) for 20 min at a constant voltage of 10 V. They were then incubated with the first antibody (rabbit polyclonal antibody against CCL20; Abcam, Cambridge, England) at  $4^{\circ}\text{C}$  overnight and then incubated with the biotinylated secondary antibody (goat anti-rabbit; DAKO, Hamburg, Germany) for 1 h at room temperature in darkness. The immunoreactive protein bands were analyzed with a Fotodyne Image Analyzer (Fotodyne, Inc., Hartland, WI, USA). Results are reported as densitometric values relative to those of the GAPDH protein, as estimated using the Image Quant software.

### Extraction and purity of peripheral blood lymphocytes (PBLs)

Approximately  $10^7$  PBLs were isolated by standard Ficoll-Hypaque density centrifugation.  $\text{CD4}^+$  T and  $\text{CD127}^{\text{dim}/-}$  cells were purified using MACS columns for the first selection step. The purity of the  $\text{CD4}^+\text{CD127}^{\text{dim}/-}$  T cells purified by this method was up to 90%, as confirmed by flow cytometric analysis. In the second step,  $\text{CD4}^+\text{CD25}^+\text{CD127}^{\text{dim}/-}$  T cells were directly labeled with CD25 microbeads and isolated by positive selection from

the pre-enriched CD4<sup>+</sup> T cell fraction. The purity of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> T cells was 85%. The lymphocytes were cultured in RPMI-1640 medium, supplemented with 1% penicillin/streptomycin, 1% glutamine, and 10% heat-inactivated fetal calf serum (Gibco BRL, Invitrogen).

### ***In vitro* migration assay**

Treg migration assays were performed on 24-well Transwell plates (Corning, Corning, NY, USA) with 5- $\mu$ m pores (Millipore, Darmstadt, Germany). A total of 10<sup>5</sup> Treg cells, a neutralizing antibody for CCL20 or goat immunoglobulin G, and Tregs restimulated with recombinant CCL20 for 12 h were placed in the upper wells of Transwell membranes containing 100 mL RPMI-1640 medium. Recombinant CCL20 or controls with 600 mL RPMI 1640 were placed in the lower chamber. Cells were allowed to migrate for 4 h at 37°C in an atmosphere containing 5% CO<sub>2</sub>. The number of Tregs that migrated to the lower chamber was then determined by using a cell counter (Vi-cell xR, Beckman Coulter, Brea, CA, USA). The chemotactic index was calculated as the ratio of the number of migrated cells in the test samples to that in the control samples.

### **Treg *in vitro* suppression assays**

For direct suppression assays, 5 x 10<sup>4</sup> CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs and CD4<sup>+</sup>CD25<sup>-</sup> T cells (1:1) were cultured on 24-well plates coated with 10  $\mu$ g/mL mouse antihuman CD3 antibodies and 10  $\mu$ g/mL mouse antihuman CD28 antibodies (Gibco, Invitrogen). The cell culture medium contained RPMI-1640 supplemented with 1% penicillin/streptomycin, 1% glutamine, and 10% heat-inactivated fetal calf serum. A neutralizing antibody for CCL20 or goat immunoglobulin G was added. After incubation for 6 days at 37°C in an atmosphere containing 5% CO<sub>2</sub>, cell-free supernatants were collected, and the concentrations of IL-4, IFN- $\gamma$ , IL-10, and IL-17 in the supernatants were assessed using an ELISA kit (Biosource, Nivelles, Belgium).

### **Transwell experiments**

CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs were co-cultured with CD4<sup>+</sup>CD25<sup>-</sup> T cells, using a semi-permeable Transwell membrane (Nunc, Roskilde, Denmark). CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> T cells (5 x 10<sup>4</sup>) were added to the upper chambers, and isolated CD4<sup>+</sup>CD25<sup>-</sup> T cells (5 x 10<sup>4</sup>) were added to the lower chambers in an autologous setting. After incubation for 6 days at 37°C in an atmosphere containing 5% CO<sub>2</sub>, the concentrations of IL-4, IFN- $\gamma$ , IL-10, and IL-17 in the supernatants were assessed as described above.

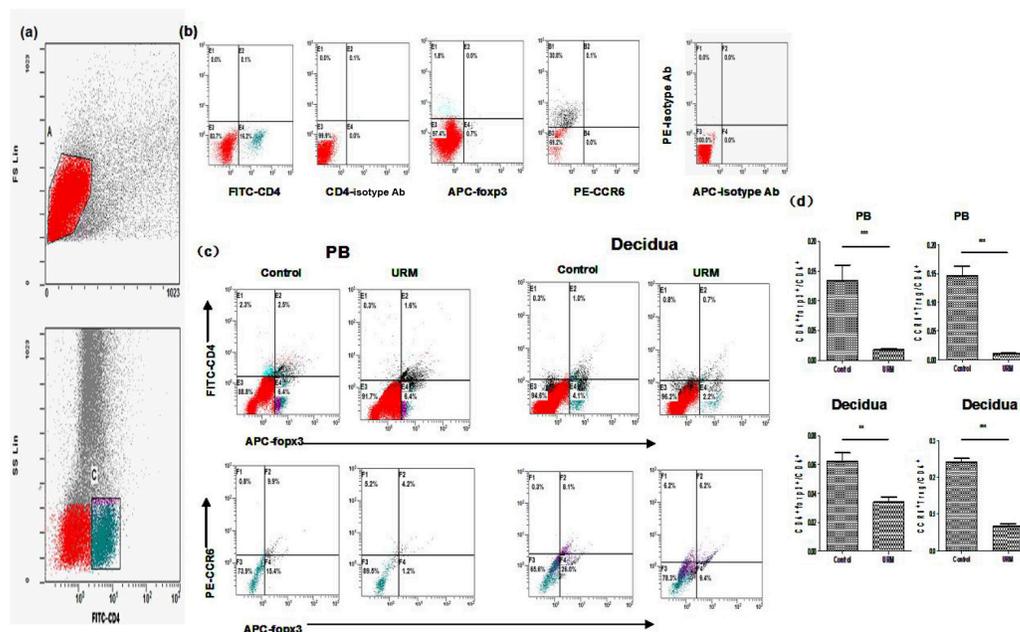
### **Statistical analysis**

All data are reported as means  $\pm$  SE of triplicate wells and repeated at least twice with consistent results. The Student *t*-test was used to compare the means of two independent groups. P values of less than 0.05 were considered to be statistically significant. All statistical analyses were performed on a personal computer with the statistical package GraphPad InStat (version 5.1; GraphPad Software, San Diego, CA, USA).

## RESULTS

Decreased frequency of CCR6<sup>+</sup>CD4<sup>+</sup>FOXP3<sup>+</sup> T cells in patients with URM

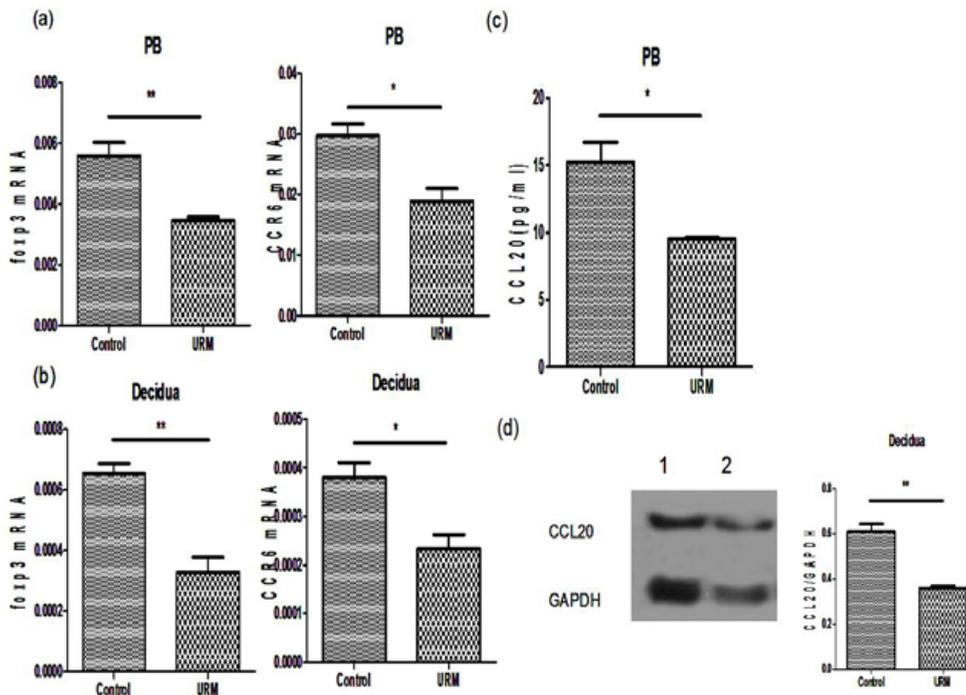
Using flow cytometry, we evaluated the expression of CCR6 in CD4<sup>+</sup>FOXP3<sup>+</sup> Tregs in the PB and decidual samples of the two groups (Figure 1a-c). We detected a lower proportion of CD4<sup>+</sup>FOXP3<sup>+</sup> T cells and CCR6<sup>+</sup>CD4<sup>+</sup>FOXP3<sup>+</sup> T cells among the PBLs of patients with URM than those of healthy control subjects ( $13.4 \pm 2.6$  vs  $1.76 \pm 0.93\%$ ,  $14.6 \pm 1.6$  vs  $1.01 \pm 0.9\%$ , respectively;  $P < 0.001$ ; Figure 1d). The proportions of CD4<sup>+</sup>FOXP3<sup>+</sup> T cells and CCR6<sup>+</sup>CD4<sup>+</sup>FOXP3<sup>+</sup> T cells in the decidual samples of patients with URM were also lower than those of healthy control subjects ( $6.2 \pm 0.6$  vs  $3.4 \pm 0.4\%$ ,  $24.2 \pm 1.0$  vs  $6.8 \pm 0.6\%$ , respectively;  $P = 0.0016$  and  $P < 0.001$ , respectively; Figure 1d).



**Figure 1.** Expression of CD4<sup>+</sup>FOXP3<sup>+</sup> T cells and CCR6<sup>+</sup>CD4<sup>+</sup>FOXP3<sup>+</sup> T cells in the peripheral blood (PB) and decidual samples of patients with unexplained recurrent miscarriage (URM). Representative images showing the staining results for a fluorescence-activated cell sorting (FACS) analysis for PB lymphocytes (PBLs) and decidual samples from one patient with URM and a healthy woman in the early stage of pregnancy (control). (a) Lymphocytes and CD4<sup>+</sup> lymphocytes. (b) Cell-surface and intracellular stain. (c) Percentage of CD4<sup>+</sup>FOXP3<sup>+</sup> T cells expressing CCR6 in the PBLs and decidual tissues isolated from URM patients (N = 20) and control women (N = 20). (d) Bars represent median values (B and C). Values are reported as means and SE. \*\* $P < 0.01$  vs control. \*\*\* $P < 0.001$  vs control.

We also observed that the expression levels of *FOXP3* and *CCR6* mRNA were lower in patients with URM than in the control subjects for PB samples ( $0.0056 \pm 0.00042$  vs  $0.0035 \pm 0.00013$ ,  $P = 0.0086$ ;  $0.029 \pm 0.002$  vs  $0.019 \pm 0.0021$ ,  $P = 0.0215$ , Figure 2a) and decidual samples ( $0.00065 \pm 0.000036$  vs  $0.00033 \pm 0.000049$ ,  $P = 0.006$ ;  $0.00038 \pm 0.000032$  vs  $0.00023 \pm 0.000028$ ,  $P = 0.0269$ , respectively; Figure 2b). CCL20 protein levels were assessed

by ELISA and western blots. The CCL20 levels were lower in patients with URM than in the control subjects ( $15.25 \pm 1.47$  vs  $9.54 \pm 0.10$ ,  $P = 0.0178$ ;  $0.61 \pm 0.04$  vs  $0.36 \pm 0.0084$ ,  $P = 0.0025$ ; Figure 2c and d).



**Figure 2.** Expression of *FOXP3* and *CCR6* mRNA and CCL20 protein levels in the peripheral blood (PB) and decidual samples of patients with unexplained recurrent miscarriage (URM). Representative images showing the gene and protein expression results for PB lymphocytes (PBLs) and decidual samples from one patient with URM and a healthy woman in the early stage of pregnancy (control). (a) Gene expression levels in PBLs. (b) Gene expression levels in the decidual samples. (c) CCL20 protein levels in PBLs. (d) CCL20 protein levels in the decidual samples (1 = control; 2 = URM). \* $P < 0.05$  vs control. \*\* $P < 0.01$  vs control.

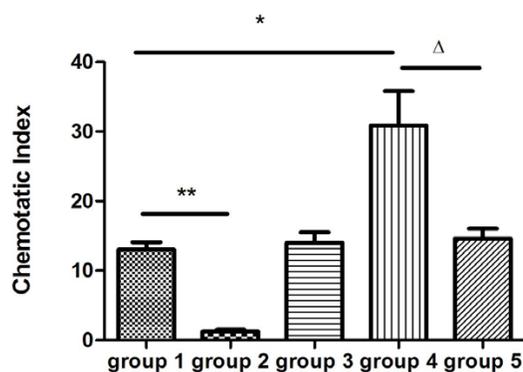
### Migration of Tregs was mediated by CCR6 and CCL20 interactions

As shown in Figure 3, Treg migration was significantly blocked (by 89.13%) using a neutralizing anti-CCL20 antibody ( $11.5 \pm 2.5$  vs  $1.25 \pm 0.25$ ,  $P = 0.0015$ ) *in vitro* and CCL20-stimulated Tregs exhibited 3.21-fold higher cell migration than medium-stimulated controls ( $36.88 \pm 4.94$  vs  $11.5 \pm 2.5$ ,  $P = 0.03$ ). Furthermore, neutralizing anti-CCL20 antibody also decreased migration of cells stimulated with CCL20 for 12 h by 60.41% ( $36.88 \pm 4.94$  vs  $14.6 \pm 1.44$ ,  $P = 0.028$ ).

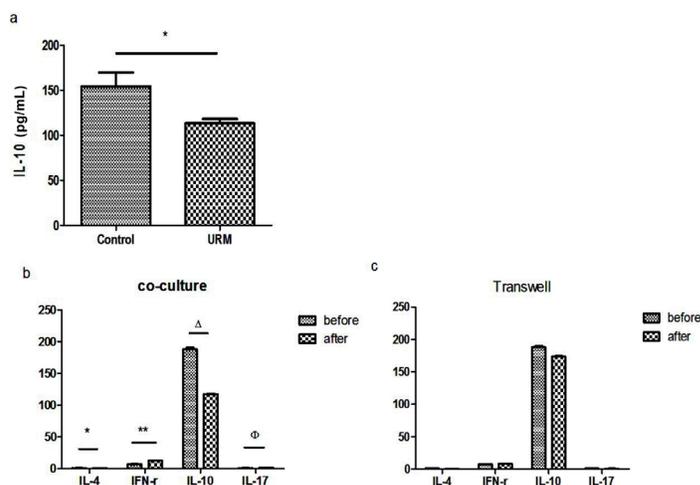
### Anti-CCL20 antibody inhibited the expression of IL-10 and IL-4 but increased IFN- $\gamma$ and IL-17 levels in co-cultures, resulting in a TH1/Th2, Treg/TH17 imbalance

The IL-10 concentration in the culture supernatants of the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs was significantly lower in patients with URM than in healthy controls ( $154.4 \pm 15.17$  vs  $113 \pm 5.19$ ,  $P = 0.021$ ; Figure 4a). As shown in Figure 4b, the anti-CCL20 antibody inhibited

the expression of IL-10 ( $188.1 \pm 2.52$  vs  $116.5 \pm 1.77$ ,  $P = 0.02$ ) and IL-4 ( $1.19 \pm 0.13$  vs  $0.64 \pm 0.34$ ,  $P = 0.015$ ) but increased IFN-r ( $7.29 \pm 0.22$  vs  $12.91 \pm 0.32$ ,  $P = 0.0047$ ) and IL-17 levels ( $1.21 \pm 0.95$  vs  $1.54 \pm 0.58$ ,  $P = 0.0475$ ) for cell-cell contact between PB CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells (1:1) with a neutralizing antibody for CCL20 or isotype control monoclonal antibody (mAb), resulting in a TH1/Th2, Treg/TH17 imbalance. However, there was no difference between these cytokine levels when cell-cell contact was prevented by a semi-permeable Transwell membrane ( $P > 0.05$ ; Figure 4c).



**Figure 3.** Migration of Tregs was mediated by CCR6 and CCL20 interactions. The chemotactic index (CI) was calculated as the ratio of the number of cells that migrated in the test samples to that in the control samples. Group 1, Transwell chemotaxis assay for Tregs in the presence of CCL20; Group 2, a neutralizing antibody to CCL20 was added to the upper chambers to examine the response; Group 3, isotype-matched immunoglobulin G was added to the upper chambers; Group 4, Tregs restimulated with recombinant CCL20 for 12 h were placed on the upper wells; Group 5, Tregs restimulated with recombinant CCL20 for 12 h were placed on the upper wells and then treated with a neutralizing antibody against CCL20. \*,  $\Delta P < 0.05$  vs control. \*\* $P < 0.01$  vs control.



**Figure 4.** Inhibition of CCL20 expression resulted in an imbalance in cytokines of TH1/Th2 and Treg/TH17 cells. (a) IL-10 levels in the PB samples of women with normal pregnancies and those with URM. (b) Results of enzyme-linked immunosorbent assay (ELISA) to assess the concentrations of IL-4, IFN-r, IL-10, and IL-17 in culture supernatants when there was cell-cell contact. (c) ELISA results to assess the concentrations of IL-4, IFN-r, IL-10, and IL-17 in culture supernatants in the Transwell. Values are reported as means  $\pm$  SE and all experiments were repeated three times. \*,  $\Delta$ ,  $\Phi P < 0.05$  vs control. \*\* $P < 0.01$  vs control.

## DISCUSSION

Differential expression of chemokine receptors directs the migration of leukocytes to distinct tissues and microenvironments. The majority of human CD4<sup>+</sup>FOXP3<sup>+</sup> Tregs express CCR6, and *in vitro* Treg migration is regulated in a CCR6-dependent manner (Acosta-Rodriguez et al., 2007). In this study, we demonstrated that expression of the CCR6-CCL20 axis was much lower in both the PB and decidual samples of patients with URM than of normal pregnant controls at the gene and protein levels.

We thus focused on the function of CCR6-CCL20 directional chemotaxis. To determine CCR6 function in Tregs, their migratory responses to CCL20 were examined using a Transwell assay (Wallace et al., 2011). CCL20 neutralizing mAb treatment significantly reduced migration of Tregs, and recombinant CCL20 stimulated Treg migration. Furthermore, a neutralizing anti-CCL20 antibody also decreased the migration of cells stimulated with CCL20 for 12 h. Importantly, the CCR6-CCL20 axis might play an essential role in the recruitment of Tregs to the fetoplacental environment.

The discovery of the Th1/Th2, Treg/Th17 balance significantly influenced the outcome of pregnancies (Hosokawa et al., 2014). Th1/TH2 and Treg/Th17 cells are two lymphocyte subsets with opposing effects. In a normal pregnancy, TH2 and Tregs prevent the generation of an immune response against fetal tissue and decreases in these cell types are associated with abortion. In contrast, TH1 and Th17 cells promote inflammatory responses, autoimmunity, and transplant rejection in humans and an increase in Th1 and Th17 cells, which is related to decreased Th2 and Tregs, has been observed in URM patients (Dieu-Nosjean et al., 2001).

The functions of Tregs are mainly mediated by cell-cell contact or by the production of anti-inflammatory cytokines such as IL-10 (Sahin et al., 2013). In our study, the number of Tregs that produced IL-10 was significantly lower in the PB of patients with URM than in the PB of control subjects. Similarly, Tregs isolated from the joints of rheumatoid arthritis patients inhibited IL-17 secretion from effector T cells (Ishida et al., 2006). In this study, after the administration of anti-CCL20 mAb, CD4<sup>+</sup>CD25<sup>+</sup> Tregs suppressed IL-4 and IL-10 expression in CD4<sup>+</sup>CD25<sup>-</sup> T cells and cell-cell contact was necessary for the former cells to exert their inhibitory effects. IFN- $\gamma$  and IL-17 expression increased by cell-cell contact. However, cytokines did not exhibit significant differences among the Transwell cultures.

In conclusion, we found that CCR6-CCL20 expression levels in the PB and decidual of patients with URM were decreased significantly at the early embryonic period. This period comprises the first 8 weeks of the development of the conceptus after fertilization. The CCR6-CCL20 axis could draw Treg migration. IL-10 production in Tregs was decreased in URM. The administration of anti-CCL20 mAb inhibited IL-10 and IL-4 expression, but increased IFN- $\gamma$  and IL-17 levels in CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cell co-cultures by cell-cell contact, resulting in an imbalance of TH1/Th2 and Treg/TH17. These findings suggested that Tregs have the ability to maintain a tolerant maternal-fetal microenvironment during early pregnancy and that the decreased suppressive ability of Tregs may play a role in the pathogenesis of URM. We expect that treatment measures directed at achieving a balance between Th1/Th2 and Treg/Th17 cells may be a promising option for URM.

## Conflicts of interest

The authors declare no conflict of interest.

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