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Ovarian cancer stem cells promote tumour immune privilege and invasion via CCL5 and regulatory T cells

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Summary

Emerging evidence indicates a link between the increased proportion of regulatory T cells (T_{regs}) and reduced survival in patients who have been diagnosed with cancer. Cancer stem cells (CSCs) have been indicated to play a vital role in tumour initiation, drug resistance and recurrence. However, the relationship between T_{regs} and CSCs remains largely unknown. Here, we sorted out ovarian cancer stem-like side population (SP) cells and $CD133^+$ cells to investigate the influence of ovarian CSCs on T_{regs}. Among the various immune-related molecules that we assessed, C-C motif chemokine ligand 5 (CCL5) was the most elevated in ovarian CSCs relative to that in the non-CSCs. The expression of its receptor, C-C motif chemokine receptor 5 (CCR5), was also increased on the surface of T_{regs} in ovarian cancer patients. This receptor-ligand expression profile indicated that ovarian CSCs recruit T_{regs} via CCL5–CCR5 interactions. We further assessed the expression of interleukin (IL)-10 in T_{regs} cultured with different cancer cells. T_{regs} cultured in conditioned medium (CM) from ovarian $CD133^+$ cells expressed a higher level of IL-10 than T_{regs} cultured in CM from $CD133^-$ cells, indicating that T_{regs} exert pronounced immune-inhibitory functions in CSC-rich environments. Furthermore, co-culture with ovarian cancer cell lines induced the expression of matrix metalloproteinase-9 (MMP9) in T_{regs} which, in turn, enhanced the degradation of the extracellular matrix and enabled the invasion of tumour cells, thereby facilitating tumour metastasis. For the first time, to our knowledge, our findings describe the relationship between ovarian CSCs and T_{regs}, and demonstrated that these two cell populations co-operate to promote tumour immune tolerance and enhance tumour progression.

Keywords: immune tolerance, invasion, ovarian cancer stem-like cells, regulatory T cells

Introduction

Ovarian cancer is the most lethal gynaecological malignancy, accounting for 50% of mortality [1]. Due to the lack of typical clinical symptoms in early stage disease, approximately 75% of patients are diagnosed at an advanced stage [2]. Because of the high recurrence rate, the median overall survival of ovarian cancer patients is still less than 65 months after treatment with surgery and chemotherapy [1]. The concept of cancer stem cells (CSCs) was first proposed due to the similarities between tumour cells and stem cells, with properties such as self-renewal, multi-lineage differentiation potential and capacity to produce organs [3,4]. CSCs have been defined as a rare subpopulation of cancer cells with the ability to form tumours, self-renew and undergo differentiation [5]. The existence of CSC was first described in acute myeloid leukaemia [6], breast cancer [7] and brain tumours [8]. Ovarian CSCs can be separated from tumour cells using surface protein markers, such as c-Kit [9], CD133 [10], CD24 [11], CD44 and CD117 [12], or via functional assays, such as by taking advantage of their ability to export the DNA-binding dye Hoechst 33342 [13,14]. CD133, a surface marker of neural stem cells [15], has been used recently to isolate CSCs from tumours with properties such as self-renewal, tumour

initiation and tumour reconstitution [16,17]. Side population (SP) cells are cells that can export Hoechst 33342. We and others have demonstrated previously that ovarian cancer SP cells exhibit enhanced tumour formation capacity, colony formation ability, chemoresistance and pluripotency [13,14]. With high levels of expression of stem cell markers, CSCs have been proposed to cause tumour chemoresistance and relapse [18]. Therefore, understanding the role of CSCs in tumour immunity is of great significance for cancer immunotherapy.

Regulatory T cells (T_{regs}) modulate the immune system negatively under both physiological and pathological conditions [19]. There are four mechanisms by which T_{regs} suppress responder T cells [20–24]. First, T_{regs} secrete inhibitory cytokines such as interleukin (IL)-35, IL-10 and transforming growth factor (TGF)-β to inhibit T cells directly. Secondly, T_{regs} in the tumour microenvironment produce granzyme B and perforin to suppress responder cells, in the manner of cytotoxic T cells. Thirdly, T_{regs} induce apoptosis in effector cells by metabolic deprivation of the cytokine IL-2, using the specific affinity of IL-2 towards CD25. Lastly, T_{regs} suppress dysfunctional dendritic cells (DCs) and T cells by directly binding with them through surface molecules such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and lymphocyte activation gene-3 (LAG-3).

Tumour-associated antigen (TAA)-specific T cells can infiltrate the tumour microenvironment [25], and these T cells can also be induced via tumour vaccines [26]. However, the immune system often fails to eliminate cancer cells. It has been demonstrated that tumour cells develop potent, overlapping mechanisms to foster immune privilege by including the infiltration of T_{regs} into the tumour microenvironment [27–30]. T_{regs} enriched in ovarian carcinoma lead to reduced survival [27] and this has also been observed in many other solid tumours, including melanomas, renal cancer, breast cancer, cervical cancer [31] and colorectal cancer [32]. T_{regs} infiltrate into tumour sites via various mechanisms, such as trafficking, differentiation, expansion and conversion [33]. C-C motif chemokine ligand 22 (CCL22) [27,34], CCL5 [30,35] and CCL28 [28] are responsible for trafficking, while TGF-β and DCs are associated with the activation of T_{regs} induction in the tumour microenvironment [33].

Currently, many therapeutic strategies targeting T_{regs}, including CD25-specific antibody, CTLA-4-specific antibody and IL-2 immunotoxin, are being developed [33,36]. However, antibodies targeting T_{regs} fail to induce tumour rejection by the immune system, and thus the mechanisms by which T_{regs} infiltrate into tumour sites need to be elucidated further. CSCs have been reported to inhibit T cell activation and induce T_{reg} recruitment from peripheral blood mononuclear cells (PBMCs) in melanoma and glioma patients [37]. However, only a few researchers have addressed the influence of ovarian CSCs on the recruitment and inhibitory function of T_{regs}. This study aimed to elucidate the effects of ovarian CSCs on T_{regs} *in vitro*. In this study, we compared the expression of a series of immunosuppression-associated molecules in CSCs and non-CSCs, and found that the expression of indoleamine 2,3-dioxygenase 1 (IDO1), CCL5 and C-X-C motif chemokine ligand 2 (CXCL2) was up-regulated in CSCs, with CCL5 being the most affected. The expression of C-C motif chemokine receptor 5 (CCR5), the receptor of CCL5, was increased in the T_{regs} isolated from ovarian cancer patients, enabling ovarian CSCs to recruit more T_{regs} through the CCL5–CCR5 interactions. We also found that T_{regs} incubated in conditioned medium (CM) from ovarian CSCs expressed higher levels of the inhibitory cytokines IL-10, indicating that T_{regs} in CSC-rich microenvironments display enhanced inhibitory properties. Furthermore, co-culture with ovarian cancer cell lines promoted the expression of matrix metalloproteinase-9 (MMP9) in T_{regs} which, in turn, enhanced the invasion of tumour cells. Altogether, for the first time, we demonstrated the effect of ovarian CSCs on the recruitment and inhibitory function of T_{regs} and found that ovarian cancer cells and T_{regs} co-operate to promote tumour progression.

Materials and methods

Ovarian cancer cells culture

The human ovarian cancer cell line highly invasive ovarian cancer cells (HEY) was acquired from the MD Anderson Cancer Center. The cell line HEY A8 was provided by Professor Gong Yang from Fudan University Shang Hai Cancer Center (FUSCC). These two cancer cell lines were cultured in RPMI-1640 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (GIBCO, Carlsbad, CA, USA). Another ovarian cancer cell line, HO-8910PM, was obtained from Shanghai Institute of Biochemistry and Cell Biology Cell Bank and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. All the cells were incubated at 37°C with 5% CO_2.

Sorting of cancer stem-like cells

Ovarian cancer cell lines HEY and HEY A8 were stained with specific antibodies against human CD133 [phycoerythrin (PE), mouse immunoglobulin (IgG); Miltenyi Biotec, Bergisch Gladbach, Germany] at a density of 1×10^7/10 μl on ice in the dark for 30 min and purified by fluorescence-activated cell sorting (FACS) (FACSAria III; BD Biosciences, San Jose, CA, USA). The SP cells which pumped out Hoechst 33342 dye were isolated by ultraviolet laser cytometry, as described previously [14]. Briefly, the ovarian cancer cell line HO8910-PM was stained with 5 μg Hoechst 33342 (Sigma, Hiroshima City, Japan) per 1×10^6 cells in DMEM with 2% FBS for 90 min at 37°C. Verapamil, which

inhibits Hoechst 33342 efflux by SP cells, was added as a control. Cells were sorted and collected by ultraviolet laser cytometry (Beckman Coulter, Brea, CA, USA).

Isolation and flow cytometric analysis of T_{regs}

T_{regs} were isolated by FACS, and membrane-bound or intracellular factors of T_{regs} were analysed by flow cytometer. PBMC were separated via gradient centrifugation using Ficoll from the peripheral blood of healthy volunteers. The PBMCs were labelled with mouse anti-human CD4-fluorescein isothiocyanate (FITC; Biolegend, San Diego, CA, USA), mouse anti-human CD25-PE (Biolegend), mouse anti-human CD127-allophycocyanin (APC) (Biolegend), mouse anti-human forkhead box protein 3 (FoxP3)-BV421 (Biolegend) and mouse anti-human CCR5-PE-CY7 (Biolegend). For intracellular FoxP3 staining, after staining cell surface markers, the cells were fixed and permeabilized on ice for 40 min and then labelled with anti-FoxP3 antibody. The $CD4^+CD25^+CD127^{-/low}$ T_{regs} were purified via FACS (FACSAria III; BD Biosciences), and the results were analysed with FlowJo software (Tree Star Inc., Ashland, OR, USA). The $CD4^+$ T cells and T_{regs} were maintained in RPMI-1640 with 10% FBS.

For intracellular IL-10 and TGF-β analysis, cells were fixed and permeabilized on ice for 40 min and then labelled with mouse anti-human IL-10-PE or TGF-β-APC (Biolegend) antibodies. To detect the expression of MMP9, permeabilized tumour cells were stained with rabbit anti-human MMP9 (ab52625; Abcam, Cambridge, UK) antibody, followed by Alexa Fluor 488-conjugated mouse anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA, USA) at 4°C. As T_{regs} were collected from different samples, the expression of the positive group was analysed relative to that of the control group in the same sample. To assess IDO1 expression in $CD133^-$ and $CD133^+$ ovarian cancer cells, Hey-A8 cells were harvested and stained with surface marker CD133-APC for 30 min, and fixed and permeabilized for 40 min on ice. Subsequently, these cells were stained with anti-IDO1 antibody (ab211017; Abcam). After incubation for 30 min at 4°C, the cells were washed twice and then labelled with Alexa Fluor 488-conjugated antibodies (Jackson ImmunoResearch). The cells were analysed with flow cytometry. All subjects gave informed consent, and the study was approved by the ethics committee of the Obstetrics and Gynecology Hospital of Fudan University.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRIzol reagent, according to the manufacturer's instructions, and then reverse-transcribed to cDNA using an RT kit (360A; Takara, Tokyo, Japan). The mRNA expression of interest gene was quantified using the Applied Biosystems real-time PCR system (Life Technologies, Camarillo, CA, USA) with SYBR Green, and the housekeeping gene β-actin served as the control. The relative expression was analysed using the comparative Ct method (ΔΔCt), and each experiment was repeated at least three times.

Enzyme-linked immunosorbent assay (ELISA)

Isolated $CD133^{+/-}$ cancer cells or SP/non-SP cells were cultured at 2×10^4/500 μl l for 48 h in 24-well plates, and the conditioned media were collected and stored at −80°C. Freshly isolated T_{regs} were cultured in $CD133^+$ and $CD133^-$ conditioned medium at a concentration of 5×10^5/ml for 24 h, and phorbol myristate acetate (PMA) (PeproTech, Rocky Hill, NJ, USA) with ionomycin (PeproTech) were added to activate T_{regs} for another 12 h. Supernatants collected from the T_{regs} cultured in $CD133^+$ and $CD133^-$ conditioned media were defined as T_{reg}^+- and T_{reg}^--conditioned media. CCL5 (DRN00B; R&D Systems, Minneapolis, MN, USA) and CXCL2 (DL-GROb-Hu, DLDEVELOP, CN) levels were measured in the CSC and non-CSC conditioned media; IL-10 (D1000B; R&D Systems) levels were measured in $T_{reg}^{+/-}$-conditioned media using the corresponding ELISA kit according to the manufacturers' instructions.

Cell proliferation assay

T_{regs} were precultured in 96-well plates at a density of 2×10^4/well for 24 h. Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan) solution was added to each well, and the reaction mixture was incubated for 5 h at 37°C. The $CD133^+$ and $CD133^-$ cells were grown in 96-well plates at a concentration of 4×10^3/100 μl for 24 h. Then, 10 μl CCK8 solution was added to each well for 2·5 h. The proliferation ability of the different cells was measured based on their optical density at 450 nm.

Chemotaxis assay of T_{regs}

Chemotaxis assay was performed in 24-well plates with permeable membranes (5-μm pore, Corning) (Fig. 1a,b). Mixtures containing recombinant human CCL5 (PeproTech) with 0·1% bovine serum albumin (BSA) (Sigma) in RPMI-1640, $CD133^+$-CM and $CD133^-$-CM or $CD133^+$-CM with different concentrations of anti-CCL5 antibodies (PeproTech) were placed into the lower chamber. The chemotaxis assays were performed with this set-up in triplicate and were repeated several times. The chemotaxis assay was performed using two methods. First, purified T_{regs} (100 μl, 1×10^5) suspended in RPMI containing 0·1% BSA were added to the upper chambers and incubated for 4 or 6 h at 37°C with 5% CO_2. The T_{regs} that had migrated to the lower chamber were counted with an inverted microscope [38,39] (Fig. 1a). For the second method, $CD4^+$ T cells (100 μl, 5×10^5) were plated in the top chamber, and the percentage of T_{regs} in the lower chamber among the $CD4^+$ T cells that had migrated to the lower chamber was analysed with a flow cytometer [28]. The chemotactic index was represented as the ratio of the number of migrated T_{regs}

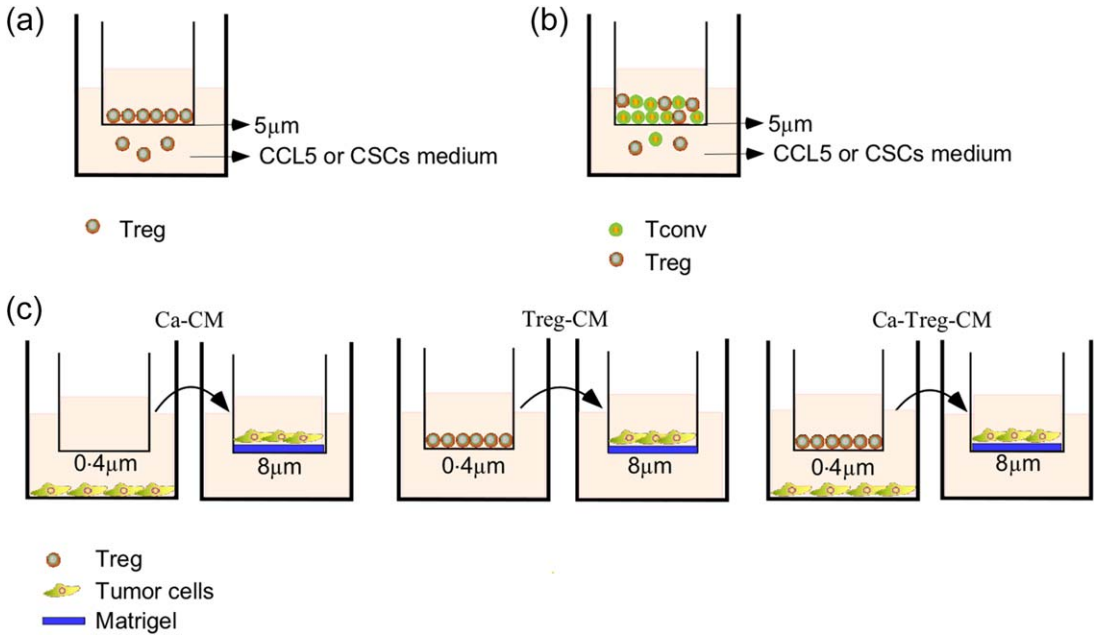

Fig. 1. Transwell chemotaxis assay, co-culture model, and invasion assay. (a,b) For chemotaxis assay, recombinant human C-C motif chemokine ligand 5 (CCL5), $CD133^+$-conditioned medium (CM), $CD133^-$-CM or $CD133^+$-CM with anti-CCL5 antibodies were added to the lower chambers of the chemotaxis apparatus with 5 μm pore-containing permeable membranes. Regulatory T cells (T_{regs}) (a) or $CD4^+$ T cells (b) isolated from peripheral blood mononuclear cells (PBMCs) were added to the upper chamber to migrate towards CCL5 or various types of CM. (c) For the co-culture experiments, T_{regs} were added to the upper chamber Transwell plates with 0·4 μm membrane, and ovarian carcinoma cells were cultured in the lower chamber. For invasion assay, Ca-CM, T_{reg}-CM and Ca-T_{reg}-CM were diluted 1:1 with complete medium and added to the lower chamber, while ovarian cancer cell lines HEY A8 or HO-8910PM were added the upper chamber to migrate towards the CM.

in the positive group relative to that in the control group [39] (Fig. 1b).

Invasion assay and co-culture model

For the invasion assay (Fig. 1c), cells in 24-well culture inserts (8 μm; Corning, New York, NY, USA) coated with diluted Matrigel (1 : 4, in serum-free medium; 40 μl/well) (BD Biosciences) were incubated at 37°C for 3h. For the co-culture model, ovarian carcinoma cells were co-cultured with 1×10^5 T_{regs} in 12-well plates, which were segregated with a 0·4-μm polycarbonate membrane to facilitate the exchange of the soluble factors but not cells, and the wells that contained cancer cells or T_{regs} alone were set as the controls. The CM of the co-culture cells (Ca-T_{reg}-CM), the control cancer cells (Ca-CM) or the control T_{regs} (T_{reg}-CM) was collected and diluted 1 : 1 with complete medium and added to the lower chamber of the invasion apparatus [40], while 3×10^4 of HEY A8 or 5×10^4 of HO-8910PM cells were placed into the upper chamber. After migration for 24 or 36 h at 37°C, the invaded cells were stained with crystal violet and observed under a microscope.

Statistical analysis

The data are presented as the mean ± standard deviation (s.d.). Real-time PCR and chemotaxis assays were performed in triplicate, and experiments were repeated at least three times. Comparisons were performed using the two-tailed Student's *t*-test or paired *t*-test. $P < 0{\cdot}05$ was considered significant.

Results

Characterization of immune-associated molecules in CSCs

CSCs have been thought to be responsible for drug resistance and recurrence in cancer. However, the relationship between ovarian CSCs and immune suppression in tumours remains largely unknown. Here, we isolated cancer stem-like SP cells and $CD133^+$ cells from ovarian carcinoma cell lines HO-8910PM, HEY and HEY A8 by FACS, as described previously [10,14] (Fig. 2a). To demonstrate the stemness-associated features of the sorted cells, the expression of pluripotency-related genes, such as Nanog homeobox (Nanog), octamer-binding transcription factor 4 (Oct4) and sex determining region Y box 2 (Sox2), was analysed by real-time PCR. Both $CD133^+$ cells and SP cells expressed elevated levels of pluripotent genes compared with $CD133^-$ cells and non-SP cells, respectively (Fig. 2b).

Tumour cells can interact with immune cells in the tumour microenvironment through the expression of immune-associated molecules, such as programmed cell

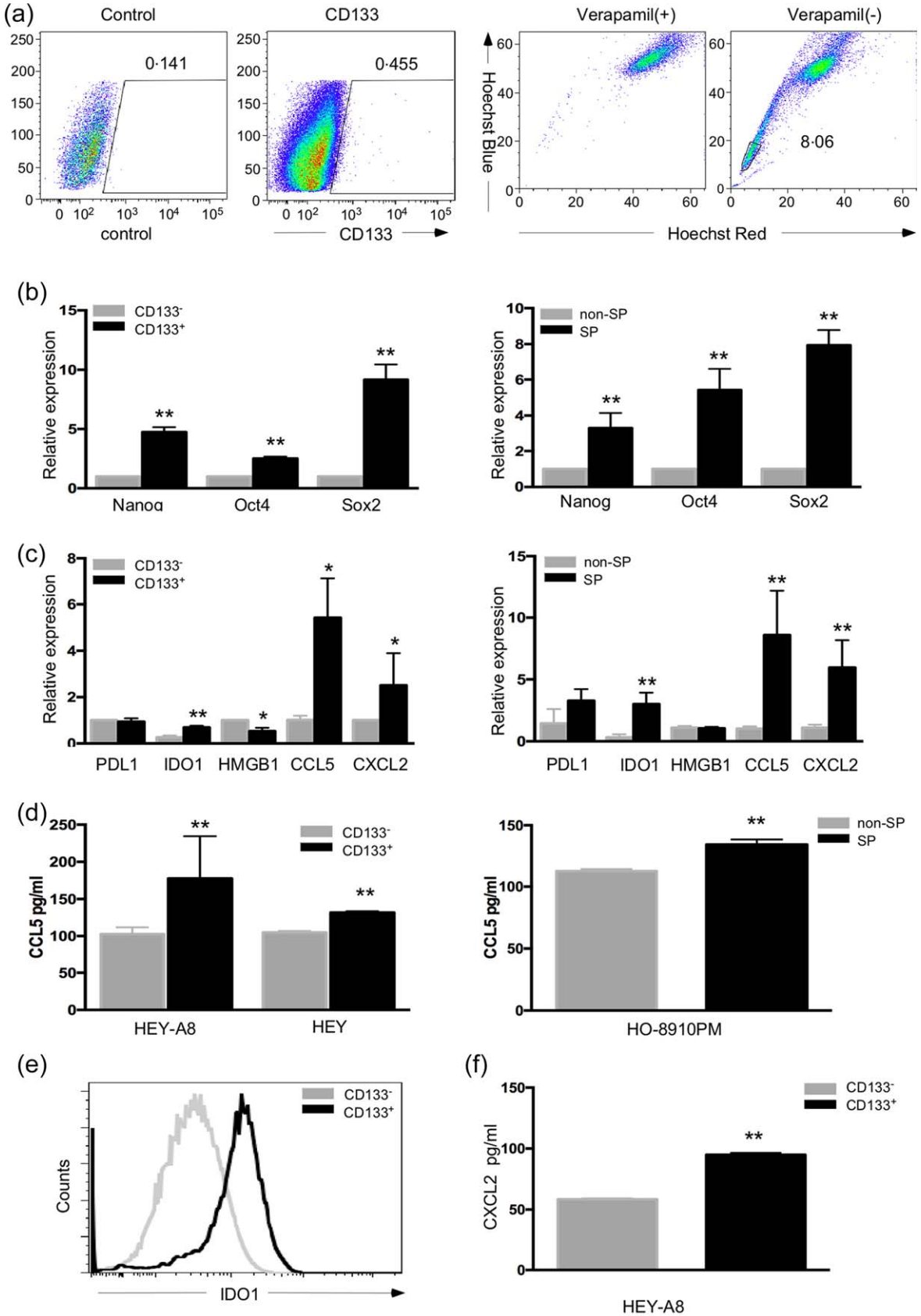
(a)
Control
CD133
Verapamil(+)
Verapamil(-)
0·141
0·455
8·06
control
CD133
Hoechst Red
Hoechst Blue
(b)
CD133-
CD133+
non-SP
SP
Relative expression
Nanog
Oct4
Sox2
(c)
PDL1
IDO1
HMGB1
CCL5
CXCL2
(d)
CCL5 pg/ml
HEY-A8
HEY
HO-8910PM
(e)
Counts
IDO1
(f)
CXCL2 pg/ml
HEY-A8

Fig. 2. C-C motif chemokine ligand 5 (CCL5) was elevated in ovarian cancer stem-like (SP) and $CD133^+$ cells. (a) Two methods were used to isolate cancer stem cells (CSCs) from ovarian cancer cell lines-$CD133^+$ CSCs were isolated from highly invasive ovarian cancer cells (HEY) A8 and HEY cells, and the Hoechst 33342-low SP cells were isolated from HO-8910PM cells. (b) The stem-like properties of the two groups of isolated CSCs were demonstrated by elevated expression of the pluripotency-related genes Nanog homeobox (Nanog), octamer-binding transcription factor 4 (Oct4) and sex determining region Y box 2 (Sox2). (c) Comparison of the immune-associated molecules programmed death-ligand 1 (PDL1), indoleamine 2,3-dioxygenase 1 (IDO1), high mobility group box 1 (HMGB1), CCL5 and chemokine (C-X-C motif) ligand 2 (CXCL2) in two kinds of ovarian CSCs and non-CSCs by polymerase chain reaction (PCR). (d) Supernatants from $CD133^{+/-}$ and SP/non-SP cells were collected and analysed for CCL5 expression by enzyme-linked immunosorbent assay (ELISA). (e) IDO1 expression was compared between $CD133^+$ cells and $CD133^-$ cells by fluorescence activated cell sorter (FACS). (f) CXCL2 protein expression was measured with ELISA in supernatants from $CD133^+$ and $CD133^-$ cells. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$

death ligand 1 (PD-L1), IDO1, high-mobility group box-1 protein (HMGB1), CCL5 and CXCL2. Among these molecules, PD-L1 and IDO1 can directly suppress T cells [41,42], HMGB1 and CCL5 can enhance T_{reg}-mediated immunosuppression [43,44] and CXCL2 can lead to immune tolerance by attracting myeloid-derived suppressor cells (MDSCs) to the tumour microenvironment [45]. To explore the role of ovarian CSCs in tumour immune evasion, these immunosuppression-associated molecules were assessed in CSCs and non-CSCs by real-time PCR (Fig. 2c). There was no difference in the expression of PDL1 between CSCs and non-CSCs. HMGB1 expression was lower in the $CD133^+$ cancer cells than that in the $CD133^-$ cells, while no difference was found between the SP and non-SP cells. IDO1, CCL5 and CXCL2 were elevated in the $CD133^+$ cancer cells and SP cells, and CCL5 exhibited the highest increase among these factors. The expression of CCL5 and CXCL2 in the supernatants of CSCs and non-CSCs was analysed further by ELISA. It was found that the CCL5 protein level was higher in the supernatant from $CD133^+$ cells than that in the supernatant from $CD133^-$ cells. Similar results were obtained for the supernatant from SP cells, indicating that the SP cells secrete a higher level of CCL5 than do the non-SP cells (Fig. 2d). Using flow cytometry, we found that IDO1 expression was higher in the $CD133^+$ cells than that in the $CD133^-$ cells (Fig. 2e). The level of CXCL2 was also higher in the supernatant from $CD133^+$ cells than that in the supernatant from $CD133^-$ cells (Fig. 2f).

T_{regs} from ovarian cancer patients expressed higher levels of CCR5 than T_{regs} from healthy controls

Cells expressing CCL5 and its receptor CCR5 were attracted via chemokine–receptor interactions [30,35]. It has been demonstrated that CCR5 is involved in the recruitment of T_{regs} in mice [35]. To determine the expression of CCR5 on T_{regs}, we isolated T_{regs} from ovarian cancer patients. Figure 3a shows the gating strategy and the identification of T_{regs}. First, the $CD4^+$ T cells were gated from the PBMCs. Subsequently, the $CD25^+CD127^{-/low}$ T_{regs}, representing a distinct fraction, were gated within the $CD4^+$ T cells. Then, a four-colour staining strategy was used to measure the intracellular FoxP3 expression in the $CD4^+CD25^+CD^{127-/low}$ T_{regs}, and we found that the majority of these T_{regs} expressed FoxP3. We then analysed CCR5 expression on T_{regs} from the PBMCs isolated from healthy volunteers ($n = 10$) and ovarian cancer patients ($n = 10$) with a flow cytometer (Fig. 3b,c). The level of CCR5 was increased in the T_{regs} from the ovarian cancer patients (P-T_{regs}) relative to that in the T_{regs} from healthy controls (H-T_{regs}). However, the conventional T cells (T_{conv}) from the healthy volunteers (H-T_{conv}) and ovarian cancer patients (P-T_{conv}) displayed a lower degree of CCR5 expression compared with the H-T_{regs} and P-$_{Tregs}$, respectively. Thus, these results suggested that T_{regs} of ovarian cancer patients displayed increased CCR5 expression, which might enable the ovarian CSCs to recruit T_{regs} preferentially to the tumour microenvironment through ligand–receptor interaction.

Next, the effect of CCL5 on the recruitment of T_{regs} was examined by two chemotaxis assays using culture plates with 5 μm pore-containing permeable membranes. For the first method, the $CD4^+CD25^+CD127^{-/low}$ T_{regs} sorted freshly from PBMCs were added to the upper chamber of the chemotaxis assay set-up to test their migration towards recombinant human CCL5, and the migrated T_{regs} in the lower chamber were counted with an inverted microscope. The chemotactic index of T_{regs} in the group with CCL5 was approximately two-fold higher than that in the group with blank control. Additionally, this migration of the T_{regs} towards CCL5 could be blocked by anti-CCL5 antibodies (Fig. 3d). For the second method, we added $CD4^+$ T cells sorted from PBMCs to the upper chamber to migrate towards CCL5 in the lower chamber, and the percentage of T_{regs} in the lower chamber was measured by flow cytometry. The percentage of T_{regs} in the lower chamber was higher in the CCL5 group than that in the control group, indicating that T_{regs} were the predominant population among the $CD4^+$ T cells that interact with CCL5 *in vitro* (Fig. 3e).

Ovarian CSCs recruited T_{regs} through chemokine CCL5

To determine the relationship between ovarian CSCs and T_{reg} recruitment via the CCL5–CCR5 axis, we performed a chemotaxis assay using two methods with CMs collected from different types of cancer cell populations. First, isolated T_{regs} were added to the upper chamber of the

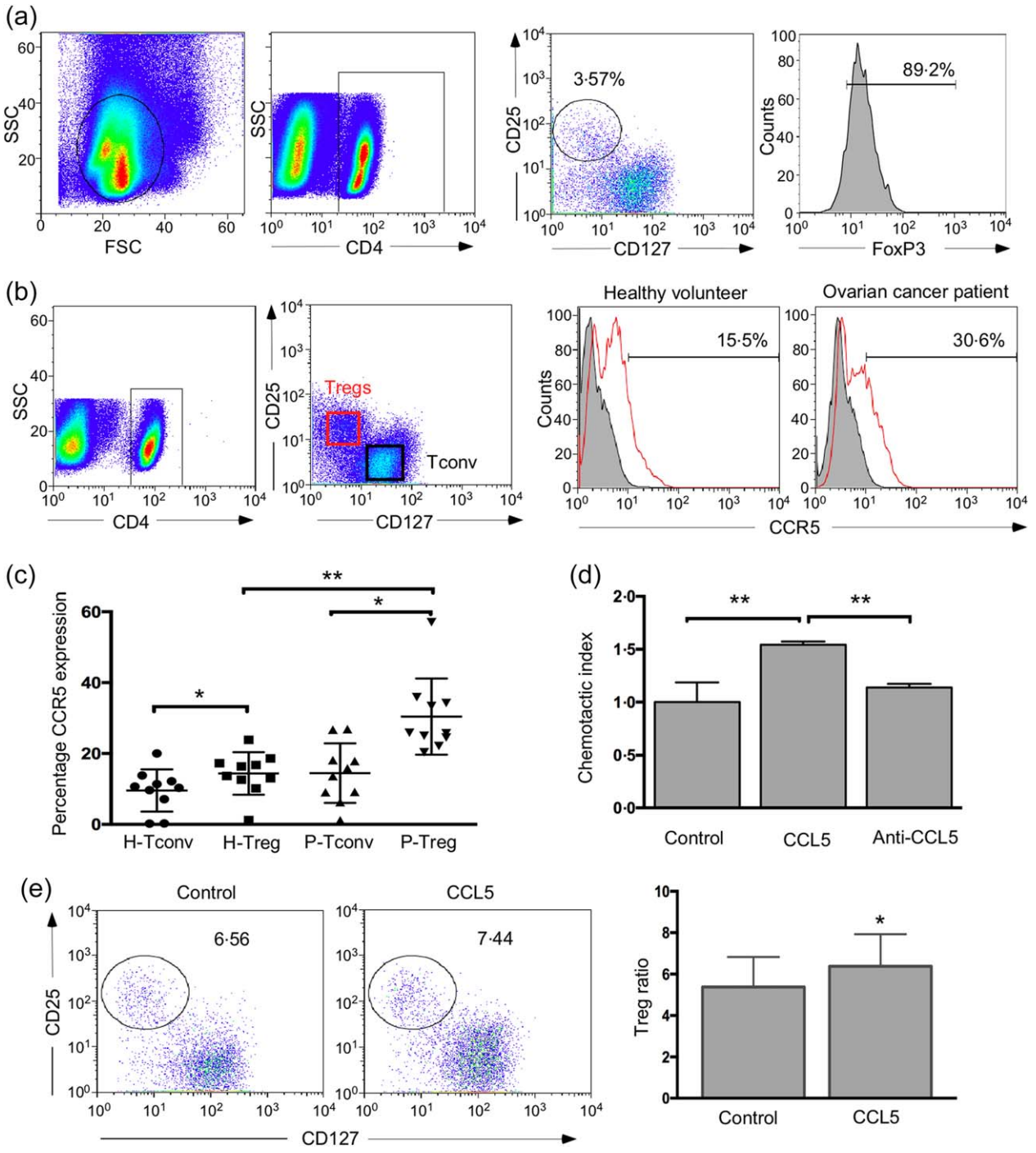

Fig. 3. C-C motif chemokine receptor 5 (CCR5) expression was increased on regulatory T cells (T_{regs}) from ovarian cancer patients. (a) Gating strategy of $CD4^+CD25^+CD127^{-/low}$ T_{regs} and fluorescence activated cell sorter (FACS) analysis of forkhead box protein 3 (FoxP3) expression in $CD4^+CD25^+CD127^{-/low}$ T_{regs}. T_{regs} were gated with CD25 and CD127 in $CD4^+$ T cell population. FoxP3 expression in the $CD4^+CD25^+CD127^{-/low}$ T_{regs} was measured by FACS. (b) CCR5 expression on the T_{regs} and conventional T cells (T_{conv}) sorted from peripheral blood mononuclear cells (PBMCs) of both ovarian cancer patients and healthy volunteers was analysed by flow cytometry. T_{regs} were isolated by gating on $CD4^+CD25^+CD127^{-/low}$ cells, and T_{conv} cells were gated as $CD4^+CD25^-$ cells. Histograms showing CCR5 levels on T_{regs} and T_{conv} from healthy volunteers and ovarian cancer patients. (c) Scatter-plots showing CCR5 levels on T_{regs} and T_{conv} from 10 pairs of healthy volunteers and ovarian cancer patients. P represents ovarian cancer patients, and H represents healthy volunteers. (d) Recombinant human C-C motif chemokine ligand 5 (CCL5) recruited T_{regs}, and CCL5-neutralization antibody could block this recruitment. The *y*-axis represents the chemotactic index. (e) Percentage of T_{regs} migrated towards CCL5 was analysed by flow cytometry ($n = 4$, paired *t*-test). $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$.

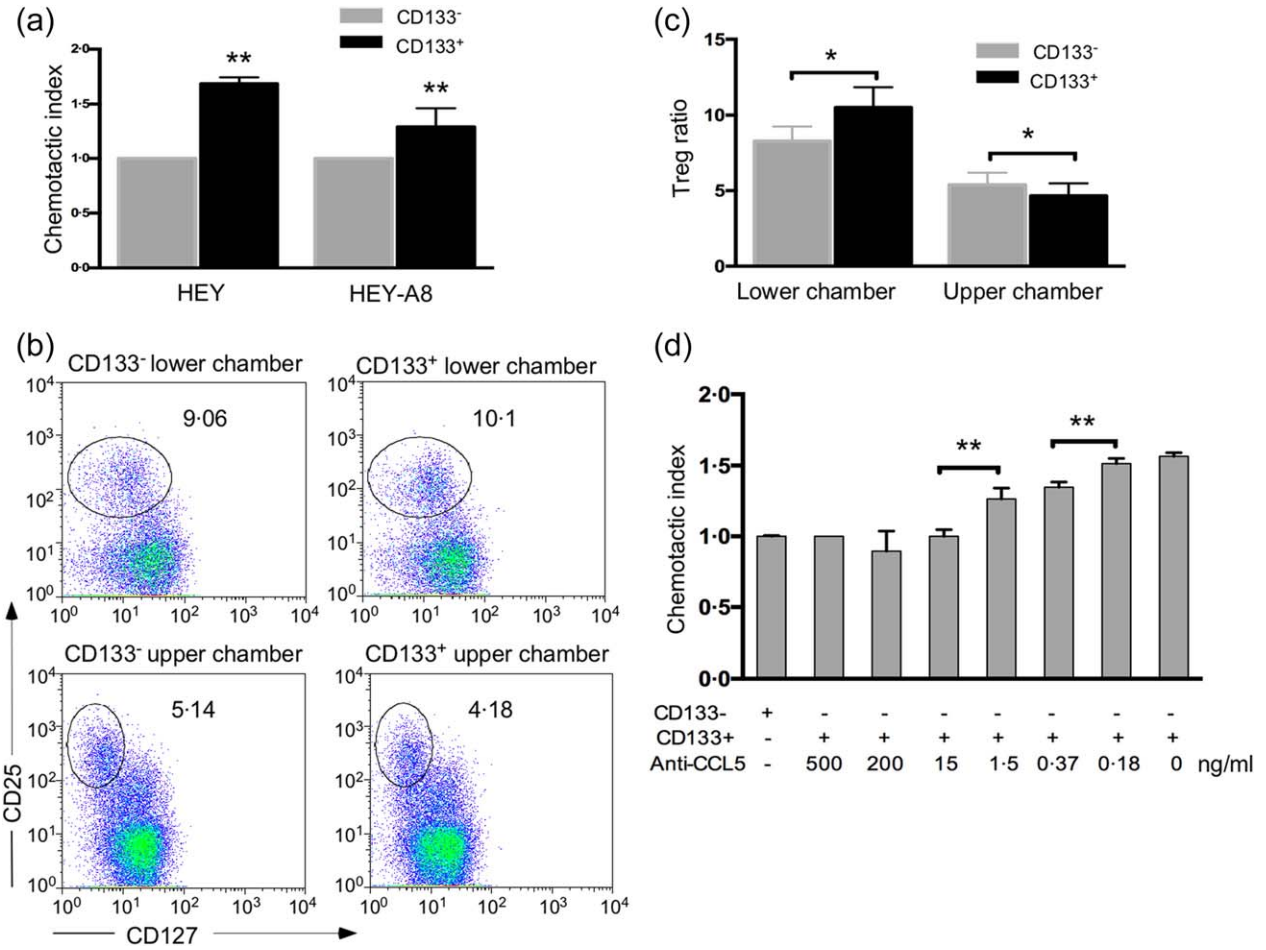

Fig. 4. Ovarian cancer stem cells (CSCs) recruited regulatory T cells (T_{regs}) through the chemokine C-C motif chemokine ligand 5 (CCL5). (a) $CD133^+$- conditioned medium (CM) recruited more T_{regs} than did $CD133^-$-CM, which were obtained using the cancer cell lines highly invasive ovarian cancer cells (HEY) and HEY A8, respectively. The number of T_{regs} that had migrated to the $CD133^-$-CM was set as the control for the chemotactic index in each group. (b) Fluorescence activated cell sorter (FACS) analysis was performed on T_{regs} in both lower and upper chamber in the $CD133^+$-CM and $CD133^-$-CM groups, after the $CD4^+$ T cells in the upper chambers had migrated toward the CM. (c) The ratio of T_{regs} in the lower and upper chambers of the $CD133^+$-CM and $CD133^-$-CM groups. (d) T_{reg} migration towards $CD133^+$-CM was abrogated by different concentrations of anti-CCL5 antibody. The group with 500 ng/ml anti-CCL5 antibody was used to define the reference chemotactic index in this panel. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$.

chemotactic apparatus, and the chemotactic index of T_{regs} in the $CD133^+$-CM group was found to be $(1{\cdot}7 \pm 0{\cdot}033)$- and $(1{\cdot}3 \pm 0{\cdot}077)$-fold higher than the chemotactic indices of T_{regs} in the $CD133^-$-CM groups using CM from HEY and HEY A8 cell lines, respectively, indicating that ovarian CSCs exert a greater chemotactic effect on T_{regs} than do non-CSCs (Fig. 4a). Secondly, to confirm the preferential recruitment of T_{regs} by $CD133^+$-CM, we added $CD4^+$ T cells into the upper compartments of the chemotaxis apparatus and assessed their migration towards $CD133^+$-CM and $CD133^-$-CM in the lower chamber. After 6 h, the $CD4^+CD25^+CD127^{-/low}$ T_{regs} in the lower and upper chambers were analysed via flow cytometry. The results showed that the $CD133^+$-CM recruited more T_{regs} to the lower chamber than did $CD133^-$-CM, while the percentage of T_{regs} in the upper chamber of the $CD133^+$ group was lower than that of the $CD133^-$ group (Fig. 4b,c). The percentage of T_{regs} in the lower chamber was higher than that in the upper chamber for both the $CD133^-$-CM and $CD133^+$-CM groups, indicating that the T_{regs} were attracted preferentially to the CM of $CD133^+$ and $CD133^-$ cells than the other $CD4^+$ T cells.

To identify whether CCL5 plays a role in the CSC-dependent preferential recruitment of T_{regs}, different concentrations of human CCL5-neutralizing antibodies were added to the $CD133^+$-CM to block the CCL5-related chemotaxis. The results showed that the recruitment of T_{regs} by $CD133^+$-CM could be abrogated completely when the concentration of anti-CCL5 antibody was higher than 15 ng/ml, and the blocking effect was dose-dependent below 15 ng/ml (Fig. 4d), suggesting that the recruitment of T_{regs} by $CD133^+$ cells was dependent upon CCL5. Taken

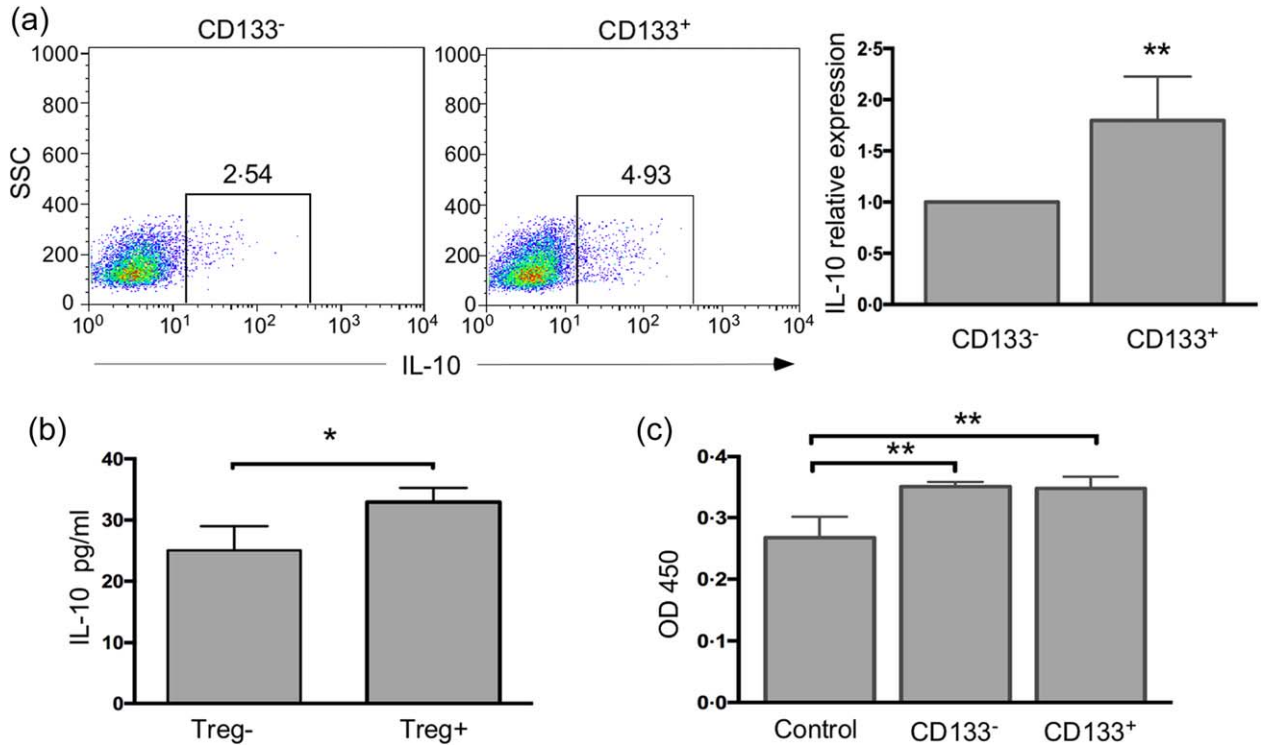

Fig. 5. Cancer stem cells (CSCs) promoted interleukin (IL)-10 expression and proliferation of regulatory T cells (T_{regs}). (a) Flow cytometric analysis of IL-10 expression in T_{regs} incubated with different conditioned medium (CM) obtained from $CD133^-$ cells and $CD133^+$ cells for 48 h. The data in the right histogram represent the relative expression of IL-10 in T_{regs} cultured in $CD133^+$-CM to that of T_{regs} cultured in $CD133^-$-CM, because the T_{regs} isolated from different samples had different baseline IL-10 expression. (b) IL-10 secretion by T_{regs} cultured in $CD133^+$-CM and $CD133^-$-CM was assessed by enzyme-linked immunosorbent assay (ELISA). (c) The optical density (OD)450 values from the cell counting kit 8 (CCK8) assay represent the proliferation of T_{regs} after incubation in control CM, $CD133^-$-CM and $CD133^+$-CM. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$.

together, a direct link between ovarian CSCs and T_{reg} recruitment through CCL5 was established in this study.

Ovarian CSCs promoted the inhibitory function and proliferation of T_{regs}

As T_{regs} can exert their function on effector T cells through inhibitory cytokines, such as IL-10 and TGF-β [24], the effect of CSCs on the function of T_{regs} was assessed by measuring IL-10 and TGF-β levels using flow cytometry and ELISA in T_{regs} that were incubated in $CD133^-$-CM and $CD133^+$-CM. $CD133^-$-CM and $CD133^+$-CM were collected, respectively, from isolated $CD133^-$ and $CD133^+$ cells that were cultured for 48 h. Treatment with $CD133^+$-CM increased the expression of IL-10 significantly, but not TGF-β (data not shown) in T_{regs} (Fig. 5a). Additionally, the level of IL-10 was higher in T_{reg}^+ CM than that in T_{reg}^- CM. IL-10 expression in T_{regs} incubated in the $CD133^+$-CM was 1·8 (± 0·2)-fold than that in T_{regs} incubated in $CD133^-$-CM. These results suggested that IL-10 was involved in the immunosuppressive function of T_{regs} induced by ovarian CSCs.

T_{regs} have been proposed to be enriched in the tumour microenvironment via four mechanisms, including trafficking, differentiation, expansion and conversion [33]. To investigate whether CSCs can stimulate the expansion of T_{regs}, we pre-incubated the sorted T_{regs} in $CD133^-$-CM or $CD133^+$-CM in 96-well plates for 24 h, and the T_{regs} incubated in the RPMI (with 10% FBS) were used as controls. Then, CCK8 reagent was added to the cells, and the cells were incubated for another 5 h. Compared with the control medium, $CD133^-$-CM and $CD133^+$-CM both promoted proliferation in T_{regs} (Fig. 5c).

T_{regs} enhanced cancer cell invasion by increasing MMP9 expression

In our present study, T_{regs} have been demonstrated to be recruited to the tumour microenvironment partly by ovarian CSCs. To determine whether these T_{regs} affected tumour progression, we used co-culture experiments in which isolated T_{regs} were placed into the upper chamber, while ovarian cancer cells were in the lower chamber, segregated by a 0·4 μm polycarbonate membrane to facilitate the exchange of soluble factors but not of cells. We first focused on the expression of MMPs, and found no obvious change in the MMP9 expression in HEY cells in the presence of T_{regs};

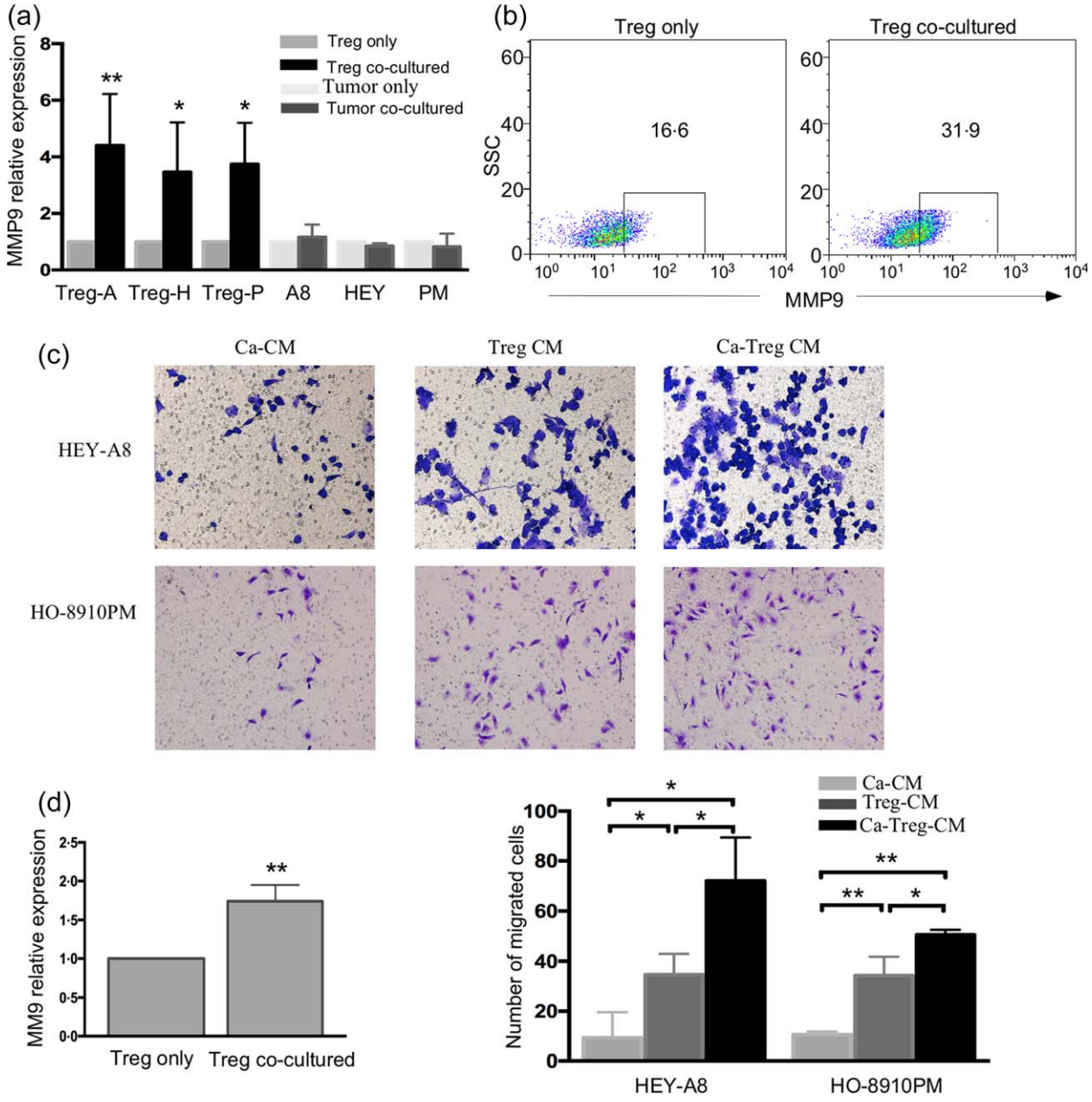

Fig. 6. Regulatory T cells (T_{regs}) enhanced the invasion of tumour cells through increased matrix metalloproteinase 9 (MMP9) expression by cancer cells. (a) MMP9 expression in the T_{regs} co-cultured with ovarian cancer cell lines was determined by real-time polymerase chain reaction (PCR). T_{reg}-A, T_{reg}-H and T_{reg}-P represent the T_{regs} that interacted with cancer cell lines highly invasive ovarian cancer cells (HEY) A8, HEY and HO-8910-PM, respectively. (b,d) Flow cytometric analysis of MMP9 expression in T_{regs} after they were co-cultured with cancer cells. The left panel in (d) represents the relative expression of MMP9 in the co-cultured T_{regs} to monocultured T_{regs}. (c,d) Crystal violet staining showed the Ca-T_{reg}-CM and T_{reg}-conditioned medium (CM) promoted tumour cells invasion *in vitro*. The right panel in (d) represents the number of cells that had invaded the membrane. $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$.

however, MMP9 expression was elevated in the T_{regs} after co-culturing (Fig. 6a). Similar results were found when other cell lines, including HEY A8(A8) and HO-8910PM (PM), T_{reg}-A, T_{reg}-H and T_{reg}-P represented T_{regs} that interacted with the cancer cell lines HEY A8, HEY and HO-8910-PM, respectively. To identify further the changes in MMP9 expression in T_{regs} after culturing them with cancer cells, indirect fluorescence immunoassay-based flow cytometry was used to evaluate MMP9 expression at the protein level, and the relative expression of MMP9 in the co-cultured T_{regs} was 1·74 (± 0·1)-fold higher than that of mono-cultured T_{regs} (Fig. 6b,d).

To investigate whether the increased MMP9 levels in the co-cultured T_{regs} could affect the invasion of cancer cells,

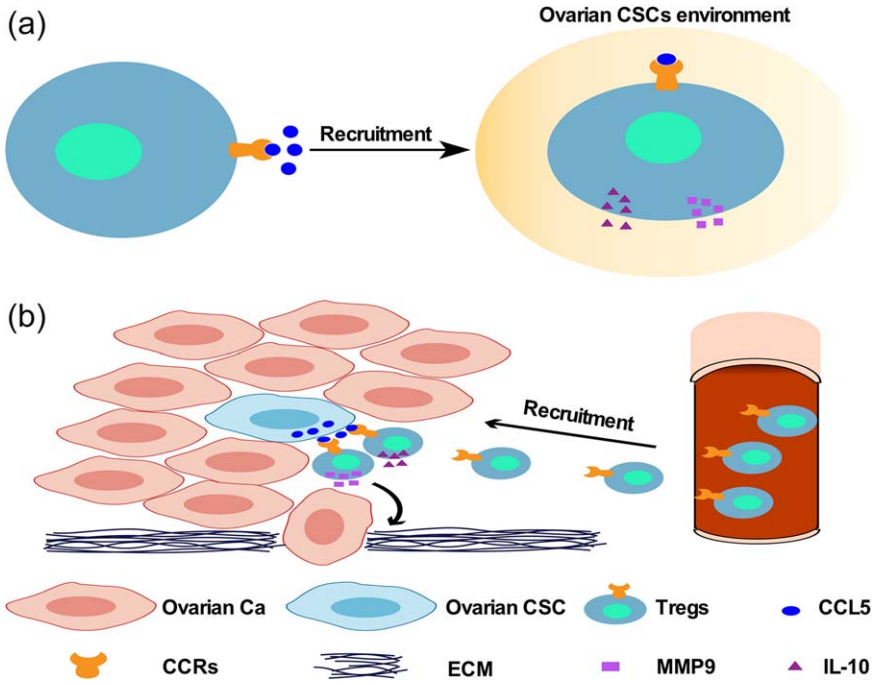

Fig. 7. Schematic representation of the relationship between cancer stem-like cells and regulatory T cells (T_{regs}). (a,b) Ovarian cancer stem cells (CSCs) affected immune tolerance by recruiting more T_{regs} to the tumour microenvironment than did non-CSCs, due to their high expression of C-C motif chemokine ligand 5 (CCL5) and the high expression of CCR5 on the T_{regs} of ovarian cancer patients. The T_{regs} infiltrated the microenvironment enriched in ovarian CSCs and secreted high levels of interleukin (IL)-10 to inhibit anti-tumour immunity. Ovarian cancer cells (Ca) promoted T_{regs} to express MMP9, which can degrade the extracellular matrix (ECM) and lead to the invasion of tumour cells.

we collected the conditioned medium from co-cultured cancer cells and T_{regs} (Ca-T_{reg}-CM) and conditioned medium from mono-cultured cancer cells (Ca-CM) to add to the lower chamber of the invasion apparatus, which was equipped with a 8-μm pore polycarbonate membrane and coated with Matrigel, and cancer cells were added to the upper chamber to migrate towards the CMs to test the influence of T_{regs} on the invasion of cancer cells. The data showed that compared with Ca-CM, Ca-T_{reg}-CM promoted the invasion of the cancer cell lines HEY A8 and HO-8910PM (Fig. 6c,d). To identify whether T_{regs} themselves induced the invasion of ovarian tumour cells, we collected medium from mono-cultured T_{regs} (T_{reg}-CM) to add to the lower chamber of the invasion apparatus and tested the invasion of cancer cells. The results showed that T_{regs}-CM enhanced the invasion of cancer cells to a much greater extent than did Ca-CM. Taken together, cancer cells up-regulated MMP9 expression in T_{regs} which, in turn, promoted the invasion of tumour cells via MMP9.

Discussion

Being at the top of the differentiation hierarchy, CSCs, which play an important role in tumorigenesis, may evade immune rejection preferentially, but the role of CSCs in tumour immune tolerance remains largely unclear. In the present study, we showed that both the $CD133^+$ cancer stem-like cells and the SP cells isolated from ovarian cancer cell lines displayed elevated expression of immunosuppression-associated molecules such as CCL5, CXCL2 and IDO1, which enabled the CSCs to evade immune response. Interestingly, some recent studies also indicated that the expression of tumour-associated antigens (TAA) is decreased in CSCs, and major histocompatibility complex (MHC) molecules and natural killer group 2, member D (NKG2D) ligand molecules are also reduced or absent in CSCs [37,46,47]. Other than the aberrant expression of immune-associated molecules, CSCs can also dampen the proliferation of T cells and induce the infiltration of T_{regs} in melanoma [37]. All these studies revealed the role of CSCs in promoting immune tolerance.

$CD4^+CD25^+CD127^{-/low}$ T_{regs}, a subpopulation of $CD4^+$ T cells, help to maintain immune homeostasis by inhibiting immune responses under physiological conditions [19]. Recently, emerging evidence indicated that the proportion of T_{regs} is increased in various types of solid tumour, which is associated with poor prognosis [31]. However, the effect of CSCs on the recruitment of T_{regs} remains unknown. We found that the $CD133^+$ cancer cells expressed high levels of CCL5, a molecule that has been reported previously to be expressed highly in ovarian CSCs to promote cancer invasion and migration by the up-regulation of MMP9 and induction of epithelial–mesenchymal transition (EMT) [10,48]. However, the relationship between CCL5 and T_{regs} recruitment has not been explored. The chemokines CCL5 and CCL22 have been reported to be responsible for T_{reg} recruitment [27,30,35,44]. In this study, we showed that CCL5 could attract T_{regs}, and $CD133^+$-CM recruited more T_{regs} than did $CD133^-$-CM, which could be blocked by antibodies against human CCL5, suggesting that the recruitment of T_{regs} by CSCs rely upon CCL5. Our results, combined with those from former reports, indicate that CCL5 expression in ovarian carcinoma CSCs has a

profound effect on both T_{regs} recruitment and tumour metastasis, and anti-CCL5 antibodies might thus provide a novel strategy for immunotherapy in ovarian cancer patients.

T_{regs} have been reported to display higher levels of CCR5, a receptor of CCL5, than effector T cells. CCR5 is responsible for the migration of T_{regs} in murine pancreatic cancer and in fungal infections [35,49]. However, the expression of CCR5 on T_{regs} isolated from ovarian cancer patients was not understood clearly. Here, we found that T_{regs} expressed more CCR5 than did the effector T cells, which was consistent with previous reports. CCR5 expression on T_{regs} isolated from ovarian cancer patients was significantly higher than that of T_{regs} isolated from healthy volunteers. Furthermore, CCL5 has been reported to be elevated in ovarian cancer patients [50], which might account for the accumulation of T_{regs} in the tumour microenvironment of ovarian cancer patients.

Inhibitory cytokines, such as IL-10 and TGF-β, are attributable for the inhibitory function of T_{regs}, but it is unclear whether ovarian CSCs are involved in affecting the secretion of these inhibitory cytokines by T_{regs}. CSCs in melanoma have been demonstrated to promote PBMCs to secrete IL-10 in a B7.2-dependent manner [37]. In this study, we showed that $CD133^{+}$-CM increased IL-10 expression significantly by T_{regs} *in vitro*, but had no effect on TGF-β secretion, suggesting that cancer stem cells probably promoted immune privilege partly by the induction of IL-10 secretion in T_{regs}.

T_{regs} inhibit immune response by inducing apoptosis in T cells and deregulating antigen-presenting cell dysfunction in the tumour microenvironment [33]. However, whether T_{regs} recruited by CSCs can affect tumour cells directly is still unclear. Previous studies have suggested that tumour-infiltrating immune cells, such as myeloid immune suppressor cells, neutrophils, mast cells and macrophages, secrete MMP9 to promote tumour survival, metastasis, angiogenesis and intravasation [51–55]. Tumour-specific T cells were reported to promote tumour cells to express MMP9 [40], and MMP9 was correlated with the proportion of T_{regs} in laryngeal cancer [56]. Here, we provided evidence that co-culture with tumour cells induced MMP9 expression in T_{regs}. Our results also showed that Ca-T_{reg}-CM promoted a higher degree of invasion in tumour cells compared with Ca-CM and T_{reg}-CM, which might be attributed to the increased MMP9 expression in T_{regs}. However, the effect of tumour cells on MMP9 expression in T_{regs} should be elucidated further with clinical cancer tissues to investigate whether the same phenomemon is involved in the tumour microenvironment *in vivo*.

In summary (Fig. 7), our data provide evidence for the first time that ovarian cancer stem-like cells express higher levels of immunosuppression-associated molecules, such as CCL5, CXCL2 and IDO1, and recruit T_{regs} through CCL5. We also show that T_{regs} from ovarian cancer patients express high levels of CCR5, the receptor for CCL5, which facilitates the recruitment of T_{regs} by ovarian cancer stem-like cells. Cancer stem-like cells can also increase IL-10 expression in T_{regs}. In exchange, T_{regs} promote the invasion of ovarian cancer cells through MMP9, the expression of which can be enhanced by co-culture of T_{regs} with cancer cells.

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Disclosure

The authors indicate no potential conflicts of interest.

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