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Renal cell carcinoma may evade the immune system by converting $CD4^+Foxp3^-$ T cells into $CD4^+CD25^+Foxp3^+$ regulatory T cells: Role of tumor COX-2-derived PGE_2

JINFENG LI[1,3*], GUIWEN FENG[1*], JIA LIU[2*], RUIMING RONG[3], FEIFEI LUO[4], LIANG GUO[5], TONGYU ZHU[3], GUOMIN WANG[3] and YIWEI CHU[4]

[1]Kidney Transplantation Unit, The First Affiliated Hospital of Zhengzhou University; [2]Department of Internal Medicine, Henan Medical Workers College; [3]Department of Urology, Zhongshan Hospital, Shanghai Medical College, Fudan University; [4]Department of Immunology, and [5]State Key Laboratory of Genetic Engineering and Gene Research Center, Shanghai Medical College, Fudan University, Shanghai, P.R. China

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Abstract. Increased $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) predict poor prognosis in renal cell carcinoma (RCC). The aim of this study was to investigate the underlying causes of the aberrant accumulation of Tregs in RCC. pcDNA3.1-hCOX-2 and control pcDNA3.1 were transfected into the RCC cell line OS-RC-2. Under stimulation of anti-CD3/CD28 antibody and APC cells, isolated $CD4^+Foxp3^-$ T cells were co-cultured with transfected OS-RC-2 culture medium supernatants and different control supernatants, respectively, and 96 h later, the proportion of Tregs in each group was detected using FACS. The suppressive ability of naturally isolated Tregs and transformed Tregs was also analyzed using [^{3}H]-thymidine methods. The results showed that overexpression of COX-2 in OS-RC-2 cells led to higher expression of prostaglandin E_2 (PGE_2) in the culture medium supernatants. In addition, there was an apparent incremental increase in the percentage of Tregs in the $CD4^+Foxp3^-$ T cells cultured with the COX-2-overexpressing OS-RC-2 culture medium supernatants. Furthermore, transformed Tregs had the same suppressive ability as naturally isolated Tregs. In summary, transfected RCC cell line culture medium supernatants were capable of converting $CD4^+Foxp3^-$ T cells to Tregs by producing high levels of PGE_2, while COX-2 inhibitors reduced the proportion of transformed Tregs in a dose-dependent manner. Thus, COX-2 inhibitors may induce a local anti-tumor effect and, in turn, may contribute to the eradication of RCC by decreasing transformed Tregs.

Correspondence to: Dr Guomin Wang, Department of Urology, Zhongshan Hospital of Fudan University, 136 Yi Xue Yuan Road, Shanghai 200032, P.R. China
E-mail: guominwang99@126.com

Dr Yiwei Chu, Department of Immunology, Shanghai Medical College, Fudan University, 138 Yi Xue Yuan Road, Shanghai 200032, P.R. China
E-mail: ywchu@shmu.edu.cn

*Contributed equally

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Introduction

It is known that renal cell carcinoma (RCC) is relatively insensitive to cytotoxic agents and radiotherapy, and that the most promising agents used in the treatment of RCC are biological response modifiers, such as interleukin-2 and interferon-α (1). In addition, there are reports verifying spontaneous regression of pulmonary metastases from RCC after ablation of the primary tumor (2). These reports indicate the important role of the immune system in the formation and progression of RCC. $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) were initially described in terms of their ability to suppress autoimmune diseases in animal models (3,4). Increasing evidence indicates that Tregs also play an important role in cancer development and progression (5,6). It was reported that increased numbers of Tregs are not only associated with dismal prognosis in RCC, but also represent an independent predictor for overall and progression-free survival (7). Nevertheless, the underlying reasons for the aberrant accumulation of Tregs in RCC are unknown.

COX is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin E_2 (PGE_2). Two COX genes, COX-1 and COX-2, have been identified. COX-1 is constitutively expressed in many tissues and is involved in several physiologic functions, including cytoprotection of the stomach, vasodilation in the kidney and the production of a pro-aggregatory prostanoid, thromboxane, by the platelets. On the other hand, COX-2 is an inducible gene originally found to be induced by inflammation or by a variety of other stimuli, such as mitogens, cytokines and growth factors. Previous studies have stressed the potential role of COX-2 in carcinogenesis, and the induction of COX-2 has been reported in colorectal, gastric, breast, esophagus and lung carcinomas.

Similarly, immunohistochemistry results characterize COX-2 as being highly expressed in RCC (8,9). A previous report on lung cell carcinoma demonstrated the important role of COX-2-derived PGE_2 in the transformation of Tregs (10). In addition, our previous research also showed that peritumoral Tregs are positively correlated with intratumoral COX-2 expression in RCC (11).

In view of these findings, we hypothesized that COX-2-derived PGE_2 in RCC may play an important role in the development of cancer through the transformation of Tregs. In this study, we observed that COX-2-derived PGE_2 induced the transformation of $CD4^+Foxp3^-$ T cells to Tregs, and that COX-2 inhibitors specifically reduced the transformation process. Thus, this report highlights novel roles for COX-2 inhibitors, as they may sensitize RCC to immunotherapy.

Materials and methods

Materials. The human RCC cell line OS-RC-2 was purchased from the Cell Centre of the Chinese Academy of Science (Shanghai, China). Fetal bovine serum (FBS), RPMI-1640 medium and Lipofectamine™ 2000 reagent were from Invitrogen. Anti-COX-2 monoclonal antibody, NS-398 (a type of COX-2 inhibitor), PGE_2 and the PGE_2 ELISA kit were from Cayman Chemicals. Anti-CD3, anti-CD28 mAbs were from R&D Systems, FITC-conjugated anti-CD25 mAb, PE-conjugated anti-CD4 and FE-Cy5-conjugated Foxp3 were from BD. The human $CD4^+CD25^+$ regulatory T cell isolation kit was purchased from Miltenyi Biotec. Other reagents were commercially available in China. pSG5-COX-2 was kindly provided by Professor Richard J. Kulmacz (University of Texas, Houston, TX, USA).

Plasmid construction. The human COX-2 gene was amplified from cDNA of pSG5-COX-2 by polymerase chain reaction (PCR) using a forward primer (5'-ATAGAATTCATGCTC GCCCGCGCCCTGCT-3') engineered with a restriction enzyme site, *Xho*I, and a reverse primer (5'-CTAGGATCC CAGTTCAGTCGAACGTTCTT-3') engineered with a *Eco*RI site and then subcloned into plasmid pcDNA3.1. The correct insertion and orientation were confirmed by DNA sequencing.

Cell culture and transfection. The OS-RC-2 cell line was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37˚C in a 5% CO_2 humidified incubator. The OS-RC-2 cell line was transfected with pcDNA3.1-hCOX-2 using Lipofectamine™ 2000. Briefly, cells were plated in antibiotic-free RPMI-1640 in 60-mm^2 dishes. After 12 h (upon reaching 70-80% confluence), cells were transfected with pcDNA3.1-hCOX-2 in Lipofectamine 2000 reagent. Lipofectamine (15 μl) was diluted with 235 μl antibiotic-free RPMI-1640 and incubated at room temperature for 5 min. In a separate tube, 6 μg pcDNA3.1-hCOX-2 was diluted with 250 μl antibiotic-free RPMI-1640. Diluted Lipofectamine 2000 (250 μl) was added to the diluted pcDNA3.1-hCOX-2 (250 μl). Cells were washed with antibiotic-free RPMI-1640, and 3.1 ml antibiotic-free RPMI-1640 was added to each dish. pcDNA3.1-hCOX-2 and the Lipofectamine 2000 complex (500 μl) were added gently to the dish. After 6 h, 0.4 ml FBS was added to the dish without removing the transfection mix. In addition to the control medium (cells in RPMI-1640 supplemented with 10% FBS), cells were similarly transfected with the pcDNA3.1 vector. After 72 h, the COX-2 mRNA and protein expression of each group was confirmed by semiquantitative RT-PCR and Western blotting.

Semi-quantitative RT-PCR analysis. Extracted total RNA (1.5 μg) was used as a template for cDNA synthesis with a Takara RNA PCR kit and specific primers (COX-2 forward, 5'-TTCAAATGAGATTGTGGAAAAAT-3'; reverse, 5'-AGAT CATCTCTGCCTGAGTATCTT-3'; β-actin forward, 5'-CGA GCGGGAAATCGTGCGTGACATTAAGGAGA-3'; reverse, 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'). Amplification was carried out for 30 cycles under saturation, each at 94˚C for 5 min; 94˚C for 45 sec; 52˚C for 40 sec; and 72˚C for 40 sec, in a 50-μl reaction mixture. After amplification, 6 μl of each reaction mixture was analyzed by 1% agarose gel electrophoresis, and the bands were then visualized by ethidium bromide staining. The PCR products for COX-2 and β-actin were 305 and 449 bp, respectively. Relative mRNA levels were determined by comparing the PCR cycle threshold between cDNA of COX-2 and that of β-actin.

Western blotting. Samples containing 60 μg of protein were denatured by incubation at 100˚C for 8 min, subjected to 10% (w/v) denatured sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After regular blocking and washing, the membrane was incubated with a mouse anti-human COX-2 antibody at a concentration of 1 μg/ml at 4˚C overnight. After incubation with a horseradish peroxidase-conjugated secondary antibody (goat anti-mouse for COX-2) at a concentration of 0.2 μg/ml at room temperature for 1 h, protein was detected by enhanced chemiluminescence using ECL Plus Western blotting detection reagents. To confirm that equal amounts of protein were loaded in each lane and transferred efficiently, the bound antibody was stripped off the membranes with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM mercaptoethanol and 2% SDS), and the membranes were reprobed with a mouse anti-human GAPDH antibody at a concentration of 0.2 μg/ml, followed by incubation with a secondary antibody and chemiluminescence detection as described above.

PGE_2 detection by ELISA. To examine PGE_2 production in OS-RC-2 parent cells and transfectants, $3x10^5$ cells were seeded into each well of a 35-mm^2 dish and cultured overnight. The medium was refreshed with serum-free RPMI-1640 the following day, and cells were cultured for another 24 h. In addition, 12.5, 25, 50 and 100 μM NS-398 was added to the supernatants, respectively. Cell-free culture medium (CM) were prepared by collecting supernatants and using centrifugation to remove cell debris. The PGE_2 concentration was determined using a PGE_2 ELISA kit according to the manufacturer's instructions. The prepared cell-free CM supernatants were also stored at -70˚C for the following culture experiment. The different levels of PGE_2 in the transfected OS-RC-2 cell line supernatants and control groups were analyzed by ELISA.

Isolation of CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells. Anticoagulated buffy coat was obtained with informed consent from healthy adult volunteers. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation. Subsequently, PBMCs were indirectly labeled with the biotin-antibody cocktail and anti-biotin microbeads, and $CD4^+$ T cells were separated by negative selection. Next, $CD4^+$ T cells were directly labeled with CD25 microbeads and, subsequently, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were separated. The purity of the isolated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells was >90%, as determined by FACS.

Culture of CD4⁺CD25⁻ with different culture medium supernatants. Freshly isolated $CD4^+CD25^-$ ($8x10^5$) T cells were cultured either in complete T-cell medium (RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 10 mM non-essential amino acids, 1 mM sodium pyruvate and 4 mM L-glutamine) or in CM supernatants collected from different tumor cell CM, in addition to MMC-treated APC ($1.6x10^6$) in the presence of 2 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 antibodies for 96 h. $CD4^+CD25^-$ T cells cultured in T-cell medium were used as a negative control, while $CD4^+CD25^-$ T cells in T-cell medium (containing 39 μM PGE_2) were used as a positive control. In addition, 100 μM NS-398 was added before collection of the CM supernatants in the COX-2-overexpressing OS-RC-2 cells. Foxp3 protein expression was then analyzed by FACS according to the manufacturer's instructions.

Treg cell suppression assay. Freshly isolated $CD4^+CD25^-$ T cells ($2x10^7$) were cultured with COX-2-overexpressing OS-RC-2 CM supernatants for 96 h, and transformed Tregs were subsequently separated using MACS, as previously described. Next, freshly isolated $CD4^+CD25^-$ T cells were cultured with transformed Tregs, freshly isolated Tregs or control complete T-cell culture medium in the presence of 2 μg/ml anti-CD3 and anti-CD28 antibodies for 72 h in a 96-well U-bottom plate. During the last 18 h, 0.5 μCi/well [^{3}H]-thymidine was added to the culture. Cells were harvested and counted in a scintillation counter.

Statistical analysis. Data are shown as the means ± SD. Statistical analysis of the data was performed using the two-tailed independent Student's t-test with SPSS 12.0 software (SPSS, Chicago, IL, USA); $p<0.05$ was considered statistically significant.

Results

COX-2 expression in OS-RC-2 parent cells and transfectants. We first examined the expression of COX-2 in the human RCC cell line OS-RC-2 and transfectants by semi-quantitative RT-PCR and Western blotting. Three groups were formed: the control, pcDNA3.1 and pcDNA3.1-hCOX-2 groups. As shown in Fig. 1, COX-2 mRNA in the above three groups was 0.4739±0.02377, 0.4325±0.02045 and 0.7922±0.07139, respectively. Compared to the pcDNA3.1 and control groups, the pcDNA3.1-hCOX-2 group had higher COX-2 mRNA expression ($p<0.05$), while COX-2 mRNA expression did not differ significantly between the pcDNA3.1 and control group ($p>0.05$). As shown in Fig. 1, COX-2 protein in the above three groups was 0.4533±0.05883, 0.4321±0.03362 and 0.8593±0.1332, respectively. Compared to the pcDNA3.1 and control groups, the pcDNA3.1-hCOX-2 group had higher COX-2 protein expression ($p<0.05$), while COX-2 protein expression did not differ significantly between the pcDNA3.1 and control group ($p>0.05$).

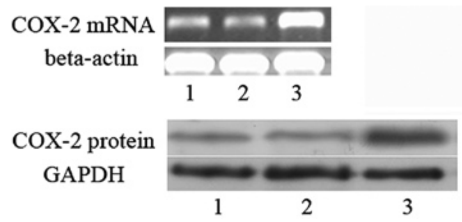

Figure 1. RT-PCR and Western blotting revealing increased COX-2 expression after pcDNA3.1-hCOX-2 transfection. 1, control group; 2, pcDNA3.1 group; 3, pcDNA3.1-hCOX-2 group.

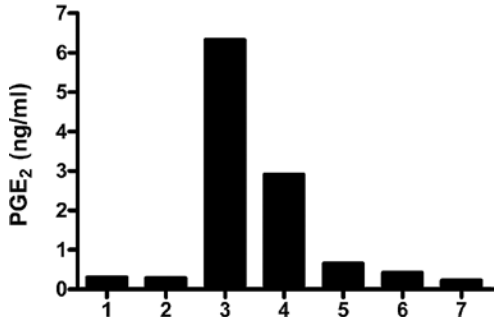

Figure 2. PGE_2 production in the culture medium supernatants after the different treatments. 1, control group; 2, pcDNA3.1 group; 3, pcDNA3.1-hCOX-2 group; 4, pcDNA3.1-hCOX-2 + 12.5 μM NS-398; 5, pcDNA3.1-hCOX-2 + 25 μM NS-398; 6, pcDNA3.1-hCOX-2 + 50 μM NS-398; 7, pcDNA3.1-hCOX-2 + 100 μM NS-398.

Assessment of PGE₂ production in parent cells and transfectants. To assess the synthesis of PGE_2 in OS-RC-2 cells and transfectants, the production of PGE_2 was measured by ELISA. As shown in Fig. 2, the PGE_2 levels in the three groups were 0.3030±0.03246, 0.2817±0.03181 and 6.330±1.181 ng/ml, respectively. Compared to the pcDNA3.1 and control groups, the pcDNA3.1-hCOX-2 group had higher PGE_2 production ($p<0.05$), while PGE_2 production did not differ significantly between the pcDNA3.1 and control group ($p>0.05$). After the addition of NS-398 at 12.5, 25, 50 and 100 μM, respectively, to the pcDNA3.1-hCOX-2 transfection group, the PGE_2 levels were 2.906±0.5892, 0.6484±0.09880, 0.4189±0.06513 and 0.2221±0.04094, respectively.

CD4⁺CD25⁻ T cells cultured with different tumor cell culture medium supernatants express Foxp3. PBMCs were obtained by the density gradient centrifugation method. Subsequently, $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs were isolated using MACS and the purity of the isolated cells was analyzed by FACS. As shown in Fig. 3, MACS steadily separated the $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs, and the purity of the two groups was >90%, respectively. The Foxp3 protein

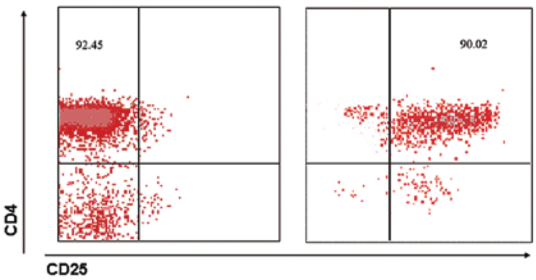

Figure 3. Representative FACS plots of isolated CD4+CD25- T cells and CD4+CD25+ T cells. Left panel, CD4+CD25- T cells; right panel, CD4+CD25+ T cells.

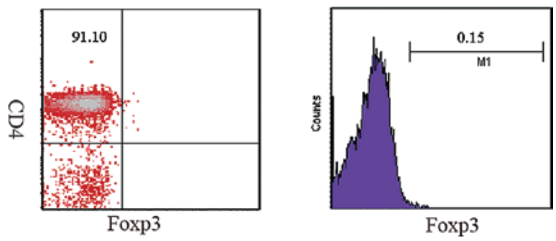

Figure 4. FACS analysis of Foxp3 protein expression in isolated CD4+CD25- T cells.

expression of CD4+CD25- T cells was also analyzed by FACS. As shown in Fig. 4, >90% CD4+CD25- T cells exhibited no Foxp3 protein expression. The Foxp3 MFI showed a similar tendency. Transfected OS-RC-2 CM supernatants clearly induced the transformation of CD4+Foxp3- T cells to CD4+Foxp3+ Tregs. As shown in Fig. 5, the proportion of CD4+Foxp3+ Tregs reached 30.00±2.618% in the transfection group after a 96-h co-culture, while upon the addition of 100 μM NS-398 beforehand in the transfection group, the proportion of CD4+Foxp3+ Tregs was only 7.990±1.227%.

Transformed Tregs cultured with COX-2-overexpressing OS-RC-2 CM supernatants exhibited suppressive activity. To examine their suppressive effect on CD4+CD25- T-cell proliferation, suppression assays were performed using different Tregs: CD4+CD25- T-cell ratios in an *in vitro* functional assay using [^{3}H]-thymidine methods. As shown in Fig. 4, freshly isolated CD4+CD25- T cells were cultured with natural Tregs or tranformed Tregs at different ratios. The results demonstrated that CD4+CD25- T cells cultured with transformed Tregs did not proliferate as compared with those cultured with natural Tregs. Notably, CD4+CD25- T cells cultured with CM derived from T-cell medium proliferated vigorously when stimulated with anti-CD3 and anti-CD28 antibodies. These results strongly suggest that transformed Tregs demonstrate a potent suppressive ability similar to that of naturally derived Tregs.

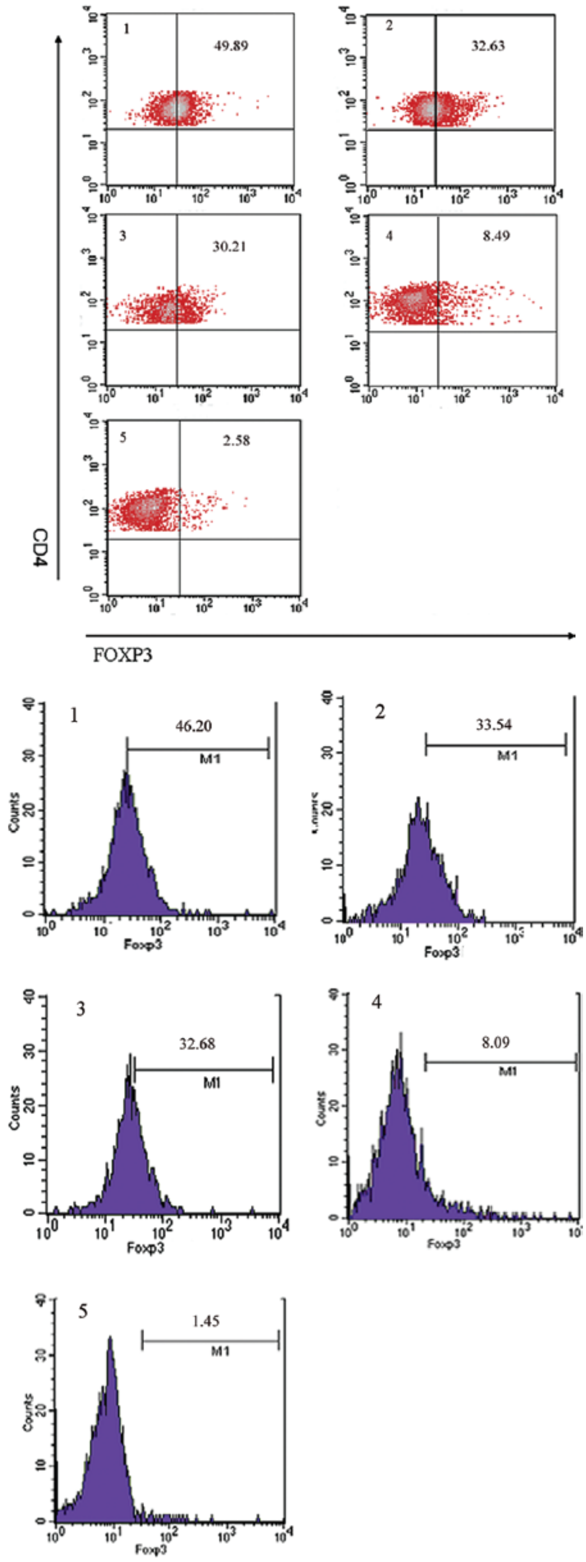

Figure 5. Transfected OS-RC-2 cells convert CD4+Foxp3- T cells into CD4+Foxp3+ T cells. Isolated CD4+Foxp3- T cells were co-cultured with the transfected OS-RC-2 cell line and different control groups for 96 h. The protein levels of Foxp3 were measured by intracellular staining and analyzed by FACS. 1, PGE_2 group (39 μMol/l); 2, transfection group; 3, transfection + 100 μMol/l IgG; 4, transfection + 100 μMol/l NS-398; 5, control group.

Discussion

Extensive research has verified that the proportion of Tregs significantly increases in patients with different types of cancer, including RCC, compared to healthy donors (7,12). An increased number of Tregs has been confirmed to be responsible for defective antitumor immunity of the host and poor prognosis in RCC patients (7). In addition, interleukin-2 immunotherapy resulted in a significant decrease in Tregs in RCC

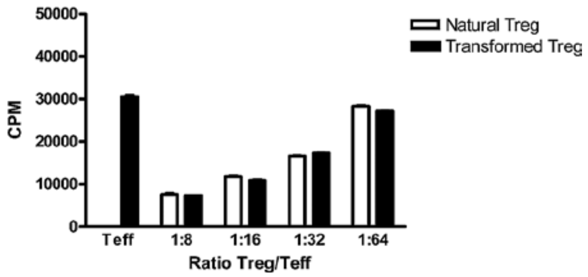

Figure 6. Inhibitory effect of naturally derived Tregs and transformed Tregs on the proliferation of $CD4^+CD25^-$ T cells.

patients achieving an objective clinical response (13), and elimination of Tregs followed by vaccination dramatically improved the stimulation of tumor-specific T-cell responses compared to vaccination alone (14). There are at least two subsets of Treg cells. One subset, also known as naturally occurring Treg cells, develops during the normal process of T-cell maturation in the thymus. The other subset develops as a consequence of the activation of mature T cells under particular conditions in the periphery (15,16). The negative regulatory role of Tregs has been amply demonstrated, suggesting that the presence of Tregs in the tumor microenvironment promotes tumor progression by inhibiting antitumor immunity (17,18). However, it is unknown whether they are naturally occurring Tregs that are recruited to the tumor sites or whether they arrive at tumor sites originally as $CD4^+$ Th cells and are later converted to Tregs in the tumor microenvironment. Since the presence of Tregs in tumor sites is associated with poor prognosis in RCC patients (11), elucidating the origin and mechanism of increased Tregs in RCC patients would have extensive clinical applications.

Our previous study demonstrated that peritumoral Tregs were positively correlated with intratumoral COX-2 expression in RCC patients. To further explore whether COX-2-derived PGE_2 converts $CD4^+CD25^-$ T cells into Tregs, freshly isolated $CD4^+CD25^-$ T cells were stimulated with anti-CD3, CD28 antibody and APC cells for 96 h in the presence of different RCC CM supernatants. We found that the COX-2-overexpressing OS-RC-2 cell line transformed $CD4^+CD25^-$ T cells to Tregs, while when NS-398 was added beforehand, the effect of tumor CM supernatants on the generation of Tregs was abrogated. This further proves that COX-2-derived PGE_2 is a key factor in the conversion of $CD4^+CD25^-$ T cells to Tregs. Moreover, a co-culture experiment revealed that transformed Tregs also suppressed the proliferation of $CD4^+CD25^-$ T cells.

In summary, COX-2-derived PGE_2 plays an important role in the process of converting $CD4^+CD25^-$ T cells into Tregs. This may be one of the underlying reasons for a higher proportion of Tregs existing in RCC patients as compared to normal donors. COX-2 inhibition might contribute to eradicating RCC by inhibiting the transformation of Tregs. Thus, clinical application of COX-2 inhibitors may benefit patients with high intratumoral COX-2 immunostaining.

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