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Human epithelial ovarian carcinoma cell-derived cytokines cooperatively induce activated $CD4^+CD25^-CD45RA^+$ naïve T cells to express forkhead box protein 3 and exhibit suppressive ability *in vitro*

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Regulatory T cells play an important role in tumor escape from host antitumor immunity. Increased frequencies of $CD4^+CD25^+$ regulatory T cells have been documented in the tumor sites, malignant effusions, and peripheral blood of patients with ovarian carcinoma. However, the mechanism involved remains unclear. In the present study, we collected high-purity human $CD4^+CD25^-CD45RA^+$ naïve T cells by microbead cell separation. These cells did not express FOXP3 by single-cell analysis, and few cells expressed FOXP3 when they were activated with anti-CD3/CD28 dual signal. However, more cells expressed FOXP3 when the supernatant of human epithelial ovarian carcinoma cell culture was added, yet not the supernatant of normal human ovarian surface epithelia cell culture. Neutralization assays revealed that neutralizing antibody against transforming growth factor β (TGF-β), interleukin-10, and interleukin-4 did not abrogate elevated FOXP3 expression induced by carcinoma cell culture supernatant, whereas neutralizing leukemia inhibitory factor (LIF) partially abrogated FOXP3 expression, but LIF alone could not increase FOXP3 expression in activated naïve T cells. Further, an *in vitro* coculture suppression assay showed that these cells could suppress the proliferation of autologous $CD4^+CD25^-CD45RA^-$ T cells. In summary, our findings show that ovarian carcinoma cells are able to induce expression of FOXP3 and exhibit suppressive ability in activated naïve T cells by producing soluble substances, and multiple cytokines involve in the induction of FOXP3 expression. (*Cancer Sci* 2009; 100: 2143–2151)

The existence of tumor-specific T-cell immune responses to human malignant tumors has been well documented. Tumor-specific cytotoxic T lymphocytes have been identified in the tumor-infiltrating lymphocytes isolated from ovarian carcinoma.[1,2] However, in most patients, tumor progression goes on in spite of tumor-specific immune responses.

Emerging evidence suggests that regulatory T cells play an important role in tumor escape from immunological control. The best-characterized regulatory T cells are $CD4^+CD25^+$ regulatory T cells that specifically express FOXP3. They are anergic and do not proliferate after T-cell receptor (TCR) stimulation *in vitro*. They inhibit the proliferation of $CD4^+$ and $CD8^+$ T cells after stimulation via their TCR in a cytokine-independent yet cell contact-dependent manner. Regulatory T cells play an important role in the maintenance of peripheral tolerance. They are vital to the peripheral immune regulation mechanism and protect against autoimmunity and transplant rejection. They also suppress antitumor immune response. Experimental tumor models have shown that removal of $CD25^+$ T cells changes the immune response to tumors both *in vitro* and *in vivo*.[3] Depletion of $CD25^+$ T cells in mice resulted in a lower incidence or slower growth of B16 melanoma.[4] Attenuation of regulatory T cells by engaging the glucocorticoid-induced tumor necrosis factor receptor family-related protein with its natural ligand in combination treatment with existing immune stimulation regimens augmented antitumor immunity and eradicated metastatic 4T1 tumors in mice.[5] Elimination of regulatory T cells *in vivo* using the recombinant interleukin (IL)-2 diphtheria toxin conjugate DAB_{389}IL-2 enhanced the magnitude of vaccine-mediated, tumor-specific T-cell responses in humans.[6] More importantly, studies showed that high regulatory T-cell frequencies are associated with poor prognosis in tumor patients,[7–11] whereas a high $CD8^+$/regulatory T cell ratio predicts favorable prognosis.[12,13]

Increased frequencies of $CD4^+CD25^+$ regulatory T cells have been documented in the tumor sites, malignant effusions, and peripheral blood of patients with ovarian carcinoma[8,14,15] and several other types of carcinoma.[9,11,15–18] However, the mechanism involved in elevating $CD4^+CD25^+$ regulatory T cell frequencies remains unclear. Specific recruitment to the tumor could bring increase the number of regulatory T cells in tumor sites, but not in the peripheral blood. Accumulated evidence has shown that regulatory T cells can also be induced in the peripheral condition, and might arise from antigen-experienced $CD4^+CD25^-$ T cells in the suppressive cytokine milieu[19–22] or after interaction with naturally occurring $CD4^+CD25^+$ T cells.[19] They may also arise from $CD4^+CD25^-$ T cells activated by low antigen dose[23,24] or by immature dendritic cells.[25,26] Studies have shown that tumor cells can convert $CD4^+CD25^-$ T cells into regulatory T cells by secreting suppressive cytokine[27] or affecting the phenotype of dendritic cells.[28] More recently, it was reported that tumor cells could convert $CD4^+CD25^-$ naïve T cells into regulatory T cells in the absence of thymus and proliferation.[29] However, most of these studies were conducted using mice. It is not clear whether a similar conversion can occur in humans. Several studies have shown that activated human $CD4^+CD25^-$ T cells express high levels of FOXP3, and the suppressive capacity *in vitro* varies.[30–34] One recent study showed that a proportion of the regulatory population was generated from rapidly dividing, highly differentiated memory $CD4^+$ T cells *in vivo*.[35] It is reasonable that the $CD4^+CD25^-CD45RA^-$ memory T cell pool might contain some precursors of regulatory T cells; hence, further isolation of highly purified $CD4^+CD25^-CD45RA^+$ naïve T cells in humans is needed as the target cells for conversion experiments *in vitro*.

In the present study, we collected high-purity $CD4^+CD25^-CD45RA^+$ naïve T cells by microbead cell separation. These cells did not contain any regulatory T cells as no FOXP3 was expressed by single-cell analysis. Only a few of these

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$CD4^+CD25^-CD45RA^+$ T cells expressed FOXP3 when they were activated with anti-CD3/CD28 dual-signal for 3 days. However, more cells expressed FOXP3 when the supernatant of human epithelial ovarian carcinoma cell culture was added, yet not with the addition of supernatant of normal human ovarian surface epithelia cell (OSE). Neutralization assays revealed that multiple cytokines could be involved in the induction of FOXP3, because none of the transforming growth factor β (TGF-β), IL-4, IL-10, or leukemia inhibitory factor (LIF) antibodies could completely abrogate the induction of FOXP3. Further, a coculture suppression assay *in vitro* showed that these cells could suppress the proliferation of autologous $CD4^+CD25^-CD45RA^-$ T cells. Our study explores a novel mechanism of elevated regulatory T cells in the microenvironment of ovarian carcinoma.

Methods

Cell culture and supernatant collection. SKOV3 and CaoV3 ovarian epithelial carcinoma cell lines (ATCC, NA, USA) were cultured in RPMI-1640 culture medium (Gibco, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, CA, USA) and 100 U/mL penicillin plus 100 μg/mL streptomycin (Gibco). Cells were washed twice with PBS when they grew to 80% confluence and were then kept in serum-free culture medium for an additional 48 h. Supernatant was collected and debris was removed by centrifugation and then filtration through a 0.22-μm filter.

Primary culture of human ovarian carcinoma.
Human ovarian carcinoma tissue was obtained with informed consent from five patients during surgery, whose pathological diagnosis and staging are shown in Table 1. The tissue was digested into single cells by 0.25% trypsin (Sigma, St Louis, USA), and cultured in Maccoy 5A (Gibco) with 15% FBS and 100 U/mL penicillin plus 100 μg/mL streptomycin. They were passaged with 0.25% trypsin when the cells became confluent. When cells grew to approximately 80% confluence, the medium was replaced with RPMI-1640 without FBS for an additional 48 h. The supernatants were collected as described above.

OSE culture.
Primary culture of OSE was established from a normal-looking ovary of a 37-year-old patient undergoing surgery for lieomyoma by gently scraping the ovarian surface with the blunt side of a scalpel with informed consent. The OSE fragments were cultured in 199–MCDB 105 (1 : 1) (Gibco) with 15% FBS and 100 U/mL penicillin plus 100 μg/mL streptomycin. The cells were left undisturbed for at least 4 days, and kept growing to confluence for approximately 8–15 days before being routinely passaged with 0.25% trypsin. Positive expression of both vimentin and keratin was used to identify OSE and exclude stromal cells by immunocytochemistry (data not shown). When cells grew to approximately 80% confluence, the medium was replaced with RPMI-1640 without FBS for an additional 48 h. Supernatant was collected and debris was removed by centrifugation and then filtration through a 0.22-μm filter.

Table 1. Clinical information from five patients with epithelial ovarian carcinoma for ovarian carcinoma cell primary culture

Patient	Age (years)	Pathological diagnosis	FIGO staging
P1	54	Serous adenocarcinoma	IIa
P2	67	Mucinous adenocarcinoma	IIIc
P3	37	Endometrioid adenocarcinoma	IIb
P4	61	Serous adenocarcinoma	IIIb
P5	46	Serous adenocarcinoma	IIIc

Isolation of human cells. Human peripheral blood was obtained from normal healthy donors by the Blood Center of Zhejiang Province of China with informed consent. Peripheral blood mononuclear cells (PBMC) were prepared by centrifugation over Ficoll-Hypaque gradients. Monocytes and macrophages were depleted through plastic adherence in a cell culture flask for 45 min at 37°C. T cells were isolated over the autoMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T cells were collected by negative selection with a human $CD4^+$ T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. These $CD4^+$ cells were then sorted by Depletes procedure with human CD25 microbeads (Miltenyi Biotec) for $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells. $CD4^+CD25^-$ T cells were then further isolated for $CD4^+CD25^-CD45RA^+$ T cells and $CD4^+CD25^-CD45RA^-$ T cells by CD45RA microbead (Miltenyi Biotec) using the POSSEL procedure. For high purity, approximately 1×10^7 $CD4^+CD25^-CD45RA^+$ T cells were isolated from $3–4 \times 10^8$ PBMC in general. The purity of all isolated populations was routinely controlled by flow cytometry with phycoerythrin (PE)-conjugated anti-CD25 (Miltenyi Biotec), FITC-conjugated anti-CD4 mAb (Caltag, CA, USA), allophycocyanin (APC)-conjugated anti-CD25 (eBioscience, CA, USA), and APC-conjugated anti-CD45RA mAb (eBioscience). The post-sort purity for $CD4^+$ T cells, $CD4^+CD25^-$ T cells, and $CD4^+CD25^-CD45RA^+$ T cells was >96% (Fig. 1A).

T-cell culture. All T-cell cultures were conducted in RPMI-1640 medium supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM) (Gibco), and 10% heat-inactivated FBS at 37°C in a humidified atmosphere containing 5% CO_2. The MACS sorted cells were activated *in vitro* with 2 μg/mL plate-bound anti-CD3 (eBioscience) and 2 μg/mL soluble anti-CD28 (eBioscience) in a 24-well culture plate (Corning, NJ, USA) at 1 000 000 cells/mL in the presence or absence of human recombinant (hr) IL-2 (2 ng/mL) (Cytolab) for 3 days. The culture plate was coated overnight at 4°C with 2 μg/mL anti-CD3. In neutralization experiments, 10 μg/mL of neutralizing anti-TGFβ (R&D Systems, MN, USA), anti-IL-10 (Biosource), anti-IL-4 (PEPROTECH, NJ, USA), anti-LIF (R&D Systems) antibody or a combination of neutralizing anti-TGFβ and anti-IL-10 antibody was used. In induction experiments, hrLIF (R&D Systems) or IL-6 (R&D Systems) at 10 ng/mL was used.

Flow cytometry. Flow cytometry was carried out on an EPICS XL (Beckman Coulter, CA, USA). Data were analyzed with the XL SYSTEM II, CA, USA. For staining of FOXP3, the cells were fixed and permeabilized using the eBioscience Fixation/Permeabilization kit (eBioscience) according to the manufacturer's protocol. The FOXP3 was stained with PCH101 PE and isotype control (eBioscience). Cell apoptosis was detected using the rh Annexin V-FITC Kit (Bendermedsystems, CA, USA) according to the manufacturer's protocol.

T-cell suppression assay *in vitro*. A coculture suppression assay *in vitro* was determined using a BrdU proliferation ELISA kit (Roche, IN, USA) according to the manufacturer's instructions. First, highly purified $CD4^+CD25^-CD45RA^+$ T cells were cultured in RPMI-1640 with 50% supernatant of SKOV3 cell culture for 3 days, then these cells were collected and isolated for $CD4^+CD25^+$ T cells (T_i) by CD25 microbead using the POSSEL procedure. Previously isolated autologous $CD4^+CD25^-CD45RA^-$ T cells (1×10^5/well) were cocultured with SKOV3-induced $CD4^+CD25^+$ T cells at different ratios (1 : 1, 0.5 : 1, 0.25 : 1, 0 : 1) for 72 h in 96-well flat-bottom plates (tissue culture grade, clear bottom) at 1×10^5/100 μL cell density, and the induced $CD4^+CD25^+$ T cells also were cultured solely in parallel. Cells were activated with 2 μg/mL soluble anti-CD3 and 2 μg/mL anti-CD28 in the presence of 2 ng/mL hrIL-2. During the final 20 h, 10 μL/100 μL BrdU labeling solution was added to the culture. The 450-nm absorbance of

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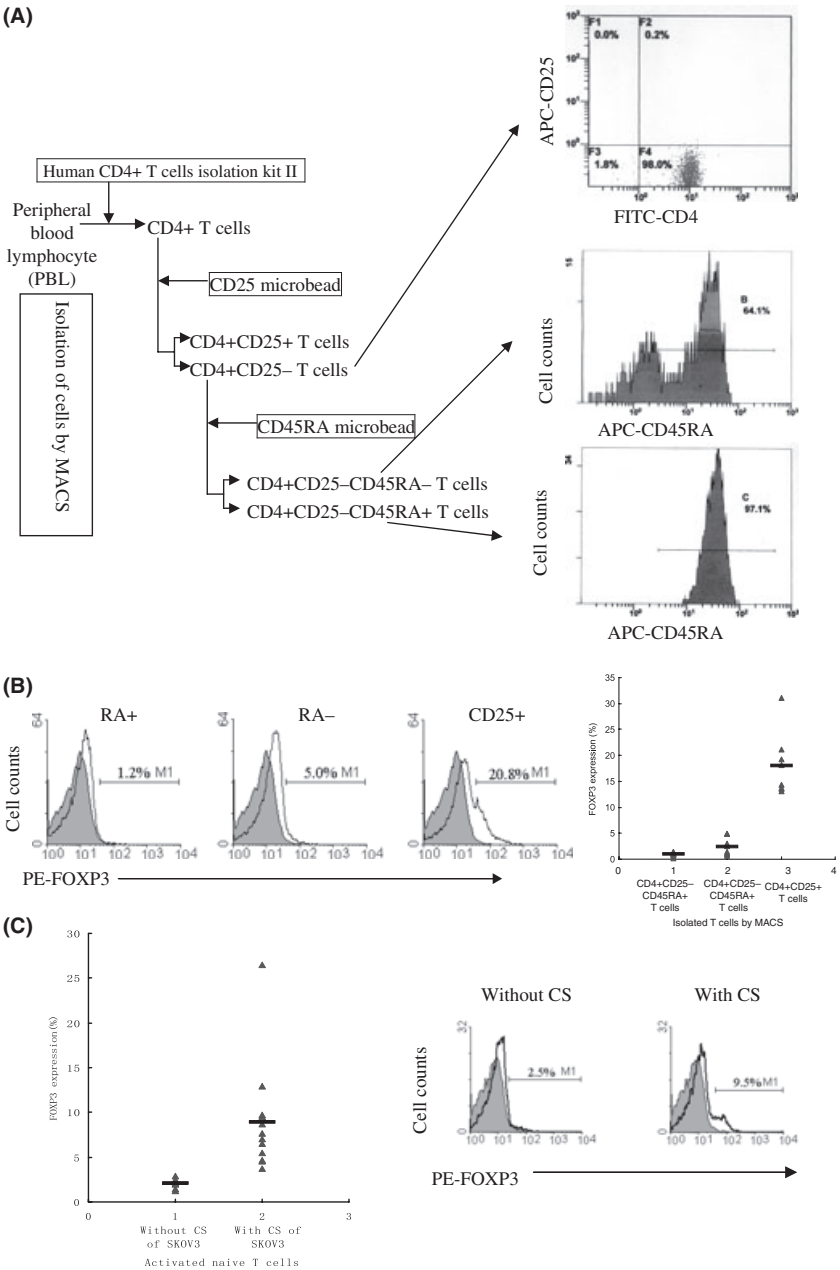

Fig. 1. Supernatant of SKOV3 cell culture increased the FOXP3 expression in activated naïve T cells. (A) $CD4^+CD25^-CD45RA^+$ naïve T cells were isolated by MACS (left), and the purity of isolated populations was controlled by flow cytometry (right). (B) FOXP3 expression in post-sorted $CD4^+CD25^-CD45RA^+$, $CD4^+CD25^-CD45RA^-$, and $CD4^+CD25^+$ T cells (left) by flow cytometry. The average percentage and range of FOXP3 expression in post-sorted $CD4^+CD25^-CD45RA^+$, $CD4^+CD25^-CD45RA^-$, and $CD4^+CD25^+$ T cells from at least eight independent experiments (right). (C) FOXP3 expression in activated naïve T cells treated with and without supernatant of SKOV3 cell culture (right) on day 3 by flow cytometry. The average percentage and range of FOXP3 expression in activated naïve T cells treated with and without culture supernatant (CS) of SKOV3 on day 3 from at least eight independent experiments (left).

each well was measured using contrast absorbance at 630 nm on an ELISA plate reader. The proliferation index (PI) was calculated using the equation

$$PI = A(T\ cells + Ti)\ -\ AT_i/(AT\ cells\ -\ Amedium),$$

where A(T cells + T_i) is the absorbance of autologous $CD4^+CD25^-CD45RA^-$ T cells cocultured with SKOV3-induced $CD4^+CD25^+$ T cells, AT_i is the absorbance of SKOV3-induced $CD4^+CD25^+$ T cells cultured alone, AT cells is the absorbance of autologous $CD4^+CD25^-CD45RA^-$ T cells cultured without induced $CD4^+CD25^+$ T cells, and Amedium is the absorbance of culture medium.

Results

Supernatant of ovarian carcinoma cell culture increased the FOXP3 expression in activated naïve T cells. The forkhead box transcription factor FoxP3 is specifically expressed in regulatory T cells. Several recent reports have further shown that expression of FoxP3 is sufficient to confer suppressive activity on naïve T cells.[36–40]

To investigate whether the supernatant of ovarian carcinoma cell culture increases the FOXP3 expression in activated naïve T cells, highly purified $CD4^+CD25^-CD45RA^+$ T cells were collected and cultured in RPMI-1640 with 50% supernatant of SKOV3 cell culture. The FOXP3 expression of activated T cells was determined by single-cell analysis using flow cytometry after 3 days. FOXP3 expression was observed in control $CD4^+CD25^+$ T cells as well as in activated naïve T cells cultured with supernatant of SKOV3 cell culture. In contrast, Foxp3 expression was absent in unstimulated $CD4^+CD25^-CD45RA^+$ T cells, and few cells expressed FOXP3 in activated $CD4^+CD25^-CD45RA^+$ naïve T cells without SKOV3 cell culture supernatant (Fig. 1B,C).

To further confirm that the supernatant of ovarian carcinoma cell culture could increase FOXP3 expression in activated naïve T cells, the cell culture supernatant from another ovarian carcinoma cell strain, CAOV3, was collected. We also established several primary cultures of human ovarian carcinoma (PCOC) cells (PCOC1, PCOC2, PCOC3, PCOC4, PCOC5) and collected the cell culture supernatant. As observed in the culture with supernatant of SKOV3 cell culture, the supernatant of CAOV3 and PCOC cell cultures also increased FOXP3 expression in activated naïve T cells (Fig. 2).

Supernatant of OSE cell culture did not increase FOXP3 expression in activated naïve T cells. Most human epithelial ovarian carcinomas arise from ovarian surface epithelial cells. To investigate whether the supernatant of OSE cell culture also induces FOXP3 expression in activated naïve T cells, we established a primary culture of OSE and collected the cell culture supernatant. Interestingly, the supernatant of OSE cell culture did not increase the FOXP3 expression in activated naïve T cells (Fig. 2), implying that human epithelial ovarian carcinoma cells acquire the capacity to induce regulatory T cells during tumor progression.

Neutralization of TGF-β and IL-10 did not influence the effect of SKOV3 cell culture supernatant to activated naïve T cells. Numerous studies have shown that regulatory T cells can be induced from naïve peripheral T cells with the suppressive cytokine milieu. Several cytokines, including TGF-β, IL-10, IL-4, and IL-13, are involved in the peripheral expansion of regulatory T cells. More recently, Victoria and colleagues observed the tumor conversion of naïve $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ regulatory T cells through the production of high levels of TGF-β in mice, and neutralization of TGF-β abrogated this conversion both *in vitro* and *in vivo*.[27] To investigate whether TGF-β in SKOV3 cell culture supernatant was required for the induction of FOXP3 expression, a neutralizing antibody against TGF-β was used. However, neutralization of TGF-β did not affect FOXP3 expression (Fig. 3).

We have found that ovarian carcinoma cells are able to synthesize and secrete IL-10.[41] It was reported that the IL-10 serum level was elevated in ovarian cancer patients compared with that in patients with benign ovarian tumors.[42] To further explore the mechanism by which SKOV3 cell culture supernatant induces FOXP3 expression, neutralizing antibody against IL-10 was used alone or in combination with anti-TGF-β. Similarly, neutralization of IL-10 also did not affect FOXP3 expression. Furthermore, when anti-IL-10 was combined with anti-TGF-β, the expression of FOXP3 even slightly increased (Fig. 3).

Neutralization of LIF partially counteracted the effect of SKOV3 cell culture supernatant to activated naïve T cells. Other cytokines could be involved in the induction of FOXP3 by supernatant of ovarian carcinoma cell culture. We have found that more than 20 types of cytokine were increased, yet approximately 20 types of cytokine were decreased in ovarian carcinoma cell culture supernatants compared with OSE cell supernatants in for 79 types of cytokine detected by protein microarray screening.[43] To further elucidate the cytokines involved, neutralizing antibodies against IL-4 and LIF were selected for neutralization assays, as the IL-4 levels in supernatants of SKOV3, CAOV3, and PCOC1 were increased more than two-fold, and LIF levels were increased more than two-fold in the cell culture supernatants of SKOV3 and the PCOC1

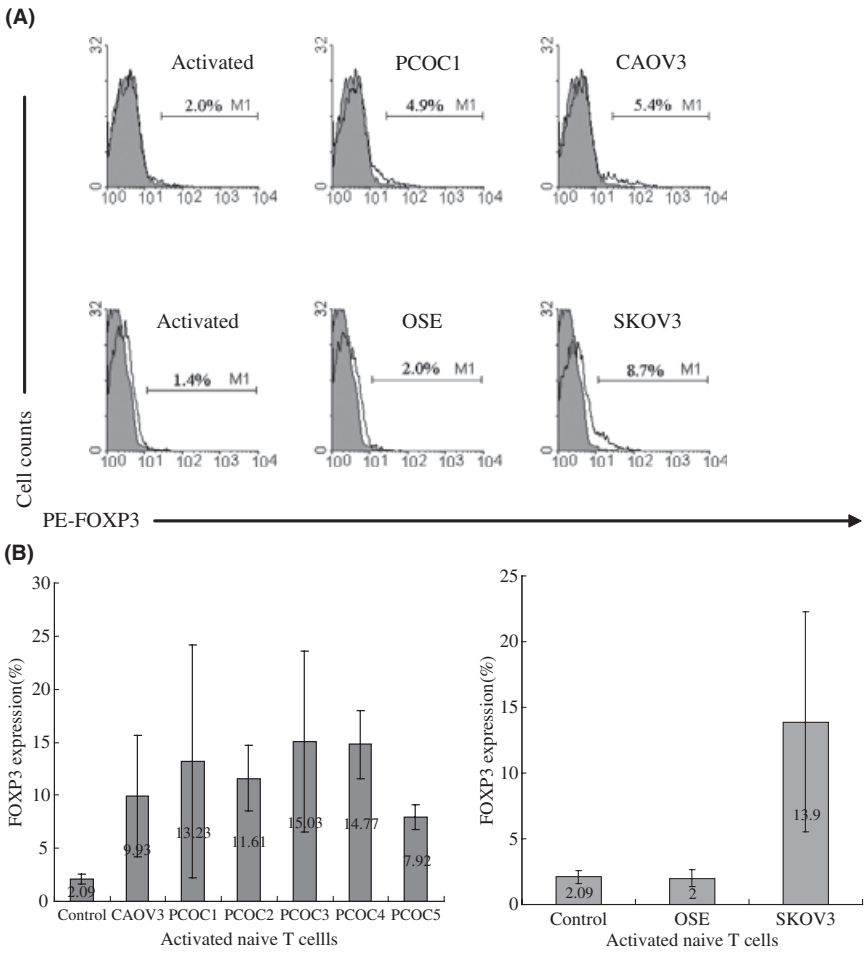

Fig. 2. Supernatants from CAOV3 and ovarian carcinoma cell culture revealed the same function as that from SKOV3, but supernatant from ovarian surface epithelia cells (OSE)did not. (A) FOXP3 expression in activated naïve T cells treated with culture supernatant (CS) of PCOC1, CAOV3, or OSE on day 3 by flow cytometry. One representative staining is shown for at least three independent experiments. (B) The average percentage and range of FOXP3 expression in activated naïve T cells treated with CS of five PCOC, CAOV3, or OSE on day 3 from at least three independent experiments.

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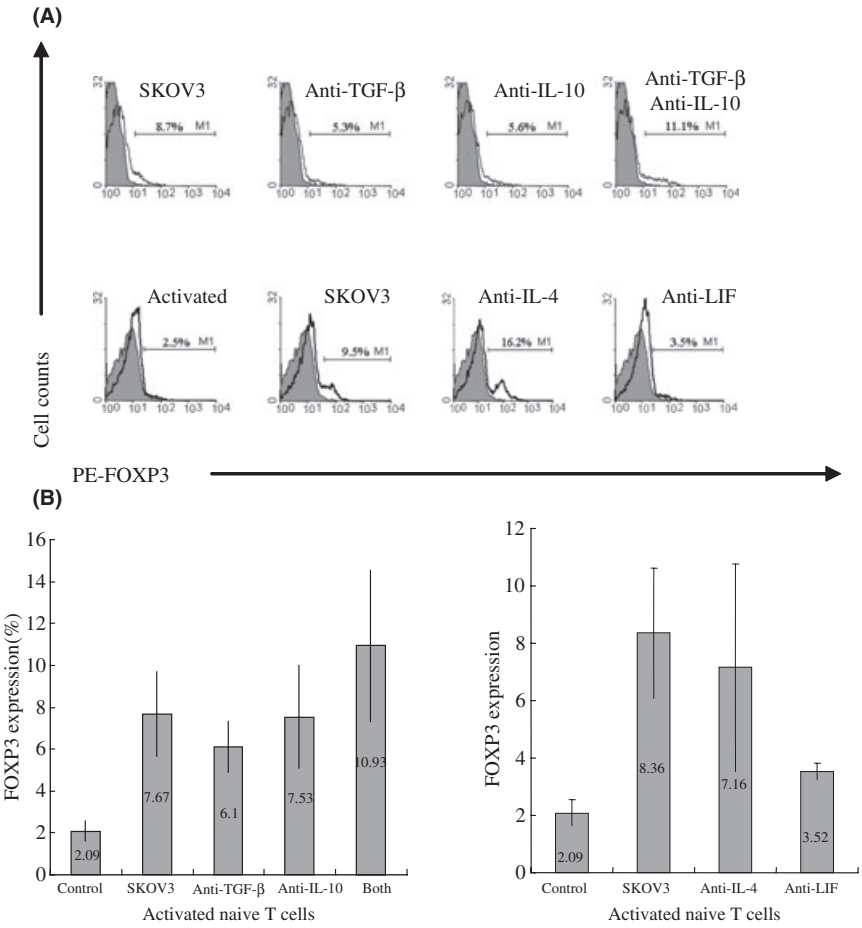

Fig. 3. Neutralization of transforming growth factor β (TGF-β), interleukin (IL)-10, or IL-4 did not abrogate elevated FOXP3 expression induced by SKOV3 cell culture supernatant, but neutralization of leukemia inhibitory factor (LIF) partially counteracted the effect of supernatant on naïve T cells. (A) FOXP3 expression in activated naïve T cells treated with culture supernatant (CS) of SKOV3 and neutralizing Ab against TGF-β, IL-10 (top), IL-4, and LIF (bottom) on day 3 by flow cytometry. One representative staining is shown for at least three independent experiments. (B) The average percentage and range of FOXP3 expression in activated naïve T cells treated with CS of SKOV3 and neutralizing Ab against TGF-β, IL-10 (left), IL-4, and LIF (right) on day 3 from at least three independent experiments.

and 1.8-fold in CAOV3 supernatant.[43] In addition, IL-4 was able to induce the development of $CD25^+CD4^+$ T cells with regulatory capacity in an Ag-specific manner from peripheral naïve $CD4^+$ T cells,[19] and LIF was associated with immune tolerance.[44,45] Unexpectedly, neutralization of IL-4 with 10 μg/mL anti-IL-4 also did not affect FOXP3 expression. Surprisingly, neutralizing LIF markedly decreased the FOXP3 expression, though it did not completely abrogate the effect of SKOV3 cell culture supernatant on activated naïve T cells (Fig. 3).

LIF alone did not increase FOXP3 expression in activated naïve T cells. There is no evidence to date that LIF is able to directly induce FOXP3 expression in activated naïve T cells or to induce $CD25^+CD4^+$ T cells to produce regulatory capacity from peripheral naïve $CD4^+$ T cells. To clarify whether LIF could induce FOXP3 expression in activated naïve T cells, 10 ng/mL LIF was added to the T cell culture medium. As shown in Figure 4, LIF alone could not increase FOXP3 expression in activated naïve T cells.

IL-6 did not inhibit FOXP3 expression in activated naïve T cells induced by SKOV3 cell culture supernatant. Our study had revealed that all neutralizing antibodies specific to the four cytokines mentioned above could not completely abrogated the effect of SKOV3 cell culture supernatant on activated naïve T cells. Hence, another approach to inhibit FOXP3 expression induced by ovarian carcinoma cell culture supernatant should be considered. In mice, it has been shown that IL-6 completely inhibits the generation of $Foxp3^+$ regulatory T cells induced by TGF-β. Instead, IL-6 and TGF-β together induce the differentiation of T(H)17 cells from naïve T cells, that is a subset of IL-17-producing T (T[H]17) cells distinct from T(H)1 or T(H)2 cells, which has been described and shown to have a crucial role in the induction of autoimmune tissue injury.[46] To investigate whether IL-6 could inhibit the expression of FOXP3 of human activated naïve T cells induced by SKOV3 cell culture supernatant, 10 ng/mL IL-6 was added to the culture medium. Unexpectedly, IL-6 also could not abrogate the effect of SKOV3 cell culture supernatant on activated naïve T cells (Fig. 5).

Isolated $CD4^+CD25^+$ T cells from naïve T cells cultured with SKOV3 cell culture supernatant suppress $CD4^+CD25^-$ T-cell proliferation. To examine the suppressive ability of induced $FOXP3^+$ T cells from activated naïve T cells cultured with SKOV3 cell culture supernatant, we isolated a $CD25^+$ population using the autoMACS Separator with CD25 microbeads to enrich the $FOXP3^+$ population. A coculture suppression assay *in vitro* was determined using a BrdU proliferation ELISA kit as described in the Methods. At the end of a 3-day culture, previously isolated autologous $CD4^+CD25^-CD45RA^-$ T cells were cocultured with various induced $CD25^+$ T-cell populations for another 3 days, then we measured the 450-nm absorbance of each well using a contrast absorbance 630 nm on an ELISA plate reader and calculated the PI with the equation mentioned in the Methods. As shown in Figure 6, the induced $CD25^+$ T cells showed a potent suppressive ability of $CD4^+CD25^-CD45RA^-$ T-cells proliferation in a dose-dependent manner. These data demonstrate that $CD4^+CD25^+$ T cells with suppressive ability can arise from $CD4^+CD25^-CD45RA^+$ naïve T cells cultured with SKOV3 cell culture supernatant, which should be associated with the expression of FOXP3.

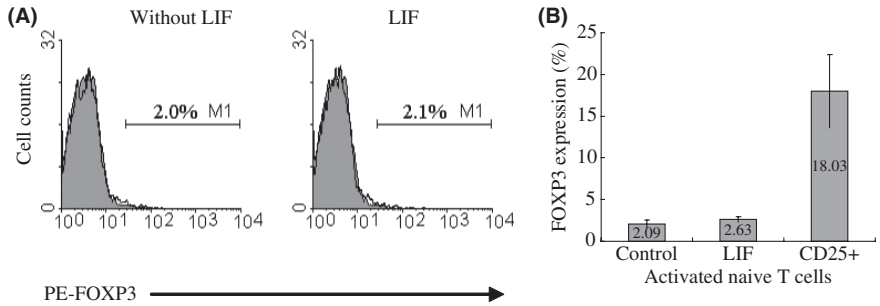

Fig. 4. Leukemia inhibitory factor (LIF) alone did not increase the level of FOXP3 expression in activated naïve T cells. (A) FOXP3 expression in activated naïve T cells treated with and without exogenous LIF on day 3 by flow cytometry. One representative staining is shown for four independent experiments. (B) The average percentage and range of FOXP3 expression in activated naïve T cells treated with and without exogenous LIF on day 3 from four independent experiments.

Discussion

FOXP3 is the most specific marker yet for regulatory T cells. In mice, expression of FoxP3 is strictly correlated with regulatory activity, ectopic expression of a transgene encoding Foxp3 or a retroviral vector encoding Foxp3 in isolated $CD4^+CD25^-$ T cells arouses acquisition of suppressor properties,[36–39] and induced ablation of a loxP-flanked Foxp3 allele in mature regulatory T cells results in the loss of their suppressive function *in vivo* and acquisition of the ability to produce IL-2 and T helper type 1 cytokines.[39] Similar to the mouse system, ectopic expression of FOXP3 in human $CD4^+CD25^-$ T cells[47,48] or human leukemic $CD4^+$ Jurkat-T cells[49] also results in acquisition of suppressor properties, and genetic defects in FOXP3 cause IPEX, an X-linked autoimmune/inflammatory syndrome.[50,51] However, several studies *in vitro* have shown that activated T cells in humans do not display suppressive function despite high FOXP3 expression,[33,34] even cells that are activated in the presence of TGF-β,[52] although more similar studies have reported that human T cells with regulatory function are differentiated from both the naïve and memory $CD4^+$ T-cell pools, which is correlated with FOXP3 expression.[22,30,31,53–55] Differences in culture conditions and activation procedure of T cells could explain the notable inconsistency in the results among different research groups. Remarkably, most cultures without generation of regulatory T cells despite high FOXP3 expression have 5 days or longer anti-CD3/CD28 dual-signal stimulation,[33,34,52] whereas generation cultures of regulatory T cells have irradiated (3000 cGy) allogeneic T cell-depleted PBMC stimulation or less 3 days anti-CD3/CD28 dual-signal stimulation.[22,30,31,53,54] In

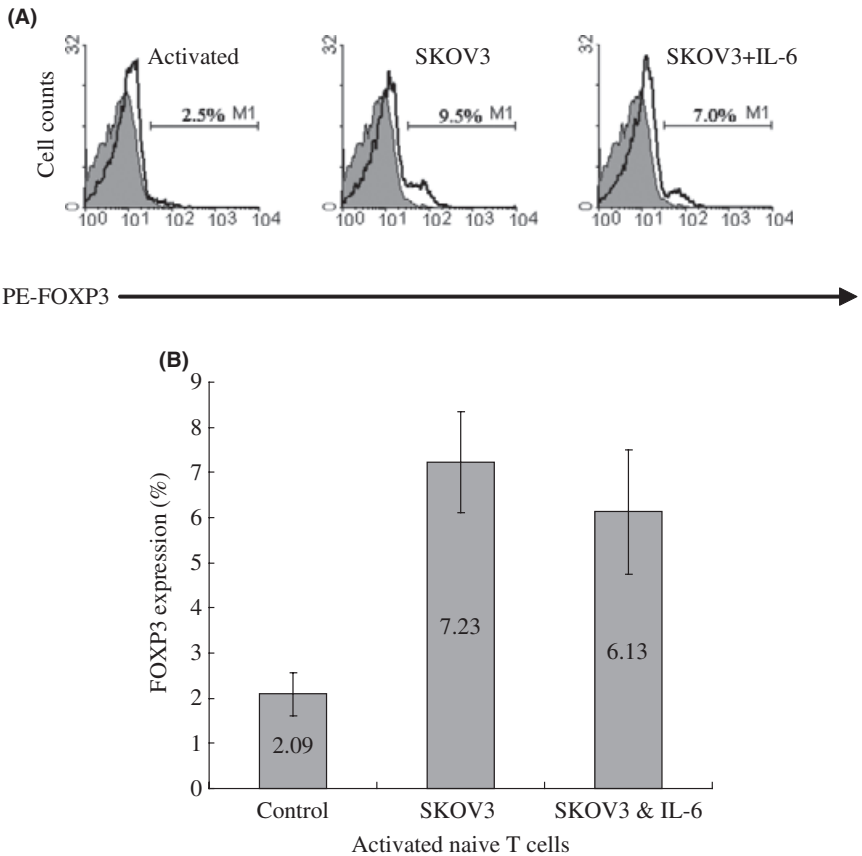

Fig. 5. Interleukin (IL)-6 did not inhibit FOXP3 expression in activated naïve T cells induced by SKOV3 culture supernatant. (A) FOXP3 expression in activated naïve T cells treated with culture supernatant (CS) of $SKOV3^{+/-}$ IL-6 on day 3 by flow cytometry. One representative staining is shown for four independent experiments. (B) The average percentage and range of FOXP3 expression activated in naïve T cells treated with CS of $SKOV3^{+/-}$ IL-6 on day 3 from four independent experiments.

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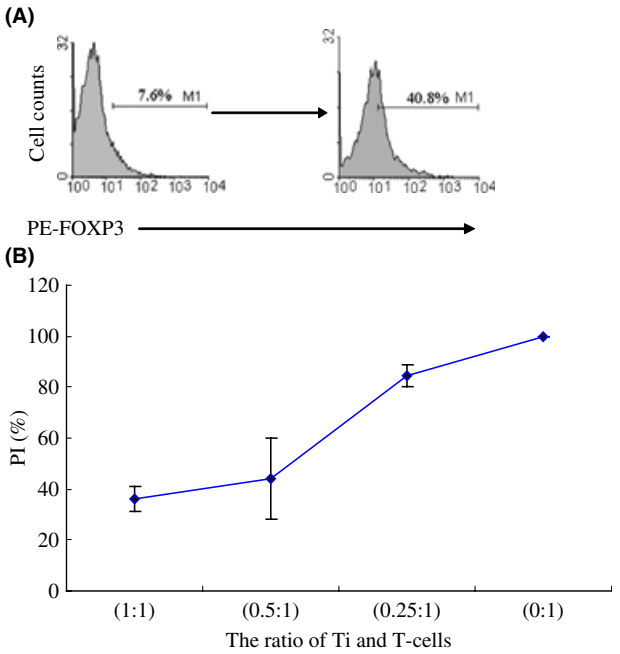

Fig. 6. Isolated $CD4^+CD25^+$ T cells from naïve T cells cultured with SKOV3 cell culture supernatant suppress $CD4^+CD25^-$ T cell proliferation. (A) $CD4^+CD25^+$ T cells were isolated by MACS from activated naïve T cells cultured with SKOV3 cell culture supernatant, and FOXP3 expression was detected by flow cytometry before (left) and after (right) sorting. (B) Autologous $CD4^+CD25^-CD45RA^-$ T cells were cocultured with induced $CD25^+$ T cell populations at diffierent ratios (1 : 1, 0.5 : 1, 0.25 : 1, 0 : 1) and the proliferation index (PI) was calculated. The average PI of three independent experiments is shown. Error bars are SD of triplicate samples. Ti, induced CD4+ CD25+ T cells.

addition, in one study although the authors considered that activated human $CD4^+CD25^-$ T cells transiently expressed FOXP3 but did not obtain suppressive ability, these T cells from three of nine donors displayed suppressive ability *in vitro*.[34] We also found that activated $CD4^+CD25^-CD45RA^-$ T cells expressed high levels of FOXP3, and their suppressive capacity *in vitro* varied. Interestingly, activated $CD4^+CD25^-CD45RA^-$ T cells without suppressive ability despite high FOXP3 expression were correlated with a high cell apoptosis rate by flow cytometry (unpublished data, Zhao XF, Ye F, Chen LL, Lu WG, Xie X, 2008). It was conceivable that 5-day or longer activated $CD4^+CD25^-$ T cells without suppressive ability were the result of a high cell death rate that partially resulted from activation-induced cell death. As a result, we affirmed that FOXP3 was competent as a specific marker in our assays.

It is well known that partial antigen-touched naïve T cells will develop into memory T cells, and these memory T cells could quickly differentiate into helper or effector T cells upon re-exposure to their cognate antigen. However, the development of antigen-touched naïve regulatory T cells remains largely unknown. Neither do we know whether the antigen-touched naïve regulatory T cells could develop into memory T cells, nor whether the possible memory regulatory T cells express FOXP3 in rest state. As mentioned above, activated $CD4^+CD25^-$ T cells *in vitro* express a high level of FOXP3,[30–34] and human $CD4^+CD25^{hi}$-$Foxp3^+$ regulatory T cells are derived by rapid turnover of memory populations *in vivo*.[35] There should be a regulatory population that is included in memory populations, which does not express FOXP3 in rest state, quickly expresses FOXP3 once exposed to its cognate antigen, and then acquires suppressive ability. Therefore, we further isolated $CD4^+CD25^-$ T cells for $CD4^+CD25^-CD45RA^+$ naïve T cells. Interestingly, isolated $CD4^+CD25^-CD45RA^+$ naïve T cells did not express FOXP3, and few cells expressed FOXP3 when activated with anti-CD3/CD28 dual-signal stimulation for 3 days in the absence of suppressive cytokines *in vitro*. The expression of FOXP3 was obviously increased when the supernatant of ovarian carcinoma cell culture was added into the culture system. However, the supernatant of OSE cell culture did not increase the expression of FOXP3. Therefore, we consider that supernatant of ovarian carcinoma cell culture may induce naïve T cells to differentiate into regulatory T cells at the initial stage when these cells are activated, and thus may inhibit the induction of effective antitumor immunity in ovarian carcinoma patients.

Several mechanisms should be involved in the tumor-induced expansion of regulatory T cells. Cytokines derived from tumor cells or local tumor-infiltrating immune cells play a significant role. However, up to the present, most of those conversion phenomena were observed in mice. Valzasina *et al.* reported that tumor cells could convert $CD4^+CD25^-$ naïve T cells into regulatory T cells in the absence of thymus and proliferation using an undifferentiated colon carcinoma of BALB/c mice.[29] Liu *et al.* reported that mouse prostate tumor TRAMP-C2 cells and mouse renal cell carcinoma RENCA cells could convert $CD4^+CD25^-$ T cells into regulatory T cells by secreting TGF-β.[27] Several studies showed that TGF-β, IL-10, and IL-4 were able to induce the development of $CD4^+CD25^+$ T cells with regulatory capacity.[19–22,53] The present study indicated that supernatant of ovarian carcinoma cell culture induced the expression of FOXP3 in activated naïve T cells, its effective components probably were the ovarian carcinoma cell-derived soluble substances including various kinds of cytokines. Thus, we selected TGF-β, IL-10, IL-4, and LIF, which were elevated in cell culture supernatants and tissues of ovarian cancer, for further study. Unexpectedly, none of the neutralizing Abs specific to these cytokines could completely abrogate the induction of FOXP3 by ovarian cancer cells, although LIF neutralizing Ab markedly decreased the expression of FOXP3 induced by supernatant of ovarian carcinoma cell culture in our study. But LIF alone nevertheless could not increase the FOXP3 expression of activated naïve T cells. Indeed, there should be a complicated interaction among these cytokines derived from ovarian carcinoma cells, which cooperatively induces the expression of FOXP3 in activated naïve T cells and differentiates these cells into regulatory T cells.

It has been reported that LIF is associated with immune tolerance.[44,45] However, the mechanism involved remains elusive. Remarkably, the transmembrane protein gp130 is a shared component of the receptor complexes for IL-6 and LIF, and IL-6 together with TGF-β induces the differentiation of pathogenic T(H)17 cells from naïve T cells in mice.[46] Further research is needed to elucidate the potential mechanism of LIF for induction of FOXP3.

Coculture suppression assays are needed to confirm that the FOXP3 expression induced by supernatant of ovarian carcinoma cell culture correlates with regulatory T cell activity. In our study, <10% of the induced naïve T cells expressed FOXP3 in most of our assays, and FOXP3 was not a suitable marker for viable cell isolation. So we isolated $CD25^+$ populations using the autoMACS Separator with CD25 microbeads to enrich the $FOXP3^+$ population. Interestingly, the isolated $CD25^+$ T cells showed a potent suppressive ability of $CD4^+CD25^-CD45RA^-$ T cell proliferation in a dose-dependent manner.

Our data show that ovarian carcinoma cells are able to induce the expression of FOXP3 and exhibit suppressive ability in activated naïve T cells by producing soluble substances, which may participate in ovarian carcinoma-induced immune tolerance. Multiple cytokines are involved in the induction of FOXP3

expression, and therapy with neutralization Ab specific to single cytokines may not be effective. Our findings reveal a novel mechanism of immune tolerance induced by ovarian carcinoma cells, suggesting that multiple interventions should be considered for facilitating efficacious antitumor immune responses in patients with ovarian cancer.

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