Plasmacytoid Dendritic Cells Promote Immunosuppression in Ovarian Cancer via ICOS Costimulation of Foxp3⁺ T-Regulatory Cells

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Abstract

Epithelial ovarian cancer (EOC) is the fifth most common cause of cancer death among women. Despite its immunogenicity, effective antitumor responses are limited, due in part, to the presence of forhead box protein 3-positive (Foxp3⁺) T regulatory (Treg) cells in the tumor microenvironment. However, the mechanisms that regulate the accumulation and the suppressive function of these Foxp3⁺ Treg cells are poorly understood. Here, we found that the majority of Foxp3⁺ Treg cells accumulating in the tumor microenvironment of EOCs belong to the subset of Foxp3⁺ Treg cells expressing inducible costimulator (ICOS). The expansion and the suppressive function of these cells were strictly dependent on ICOS-L costimulation provided by tumor plasmacytoid dendritic cells (pDC). Accordingly, ICOS⁺ Foxp3⁺ Treg cells were found to localize in close vicinity of tumor pDCs, and their number directly correlated with the numbers of pDCs in the tumors. Furthermore, pDCs and ICOS⁺ Foxp3⁺ Treg cells were found to be strong predictors for disease progression in patients with ovarian cancer, with ICOS⁺ Treg cell subset being a stronger predictor than total Foxp3⁺ Treg cells. These findings suggest an essential role for pDCs and ICOS-L in immunosuppression mediated by ICOS⁺ Foxp3⁺ Treg cells, leading to tumor progression in ovarian cancer. Cancer Res; 72(20); 5240–9. ©2012 AACR.

Introduction

Epithelial ovarian cancer (EOC) is the second most common gynecologic malignancy, which causes more death than any other gynecologic cancer in Europe and the United States. Because of subtle symptoms, most patients present with widespread intraperitoneal metastases that lead to the formation of malignant ascites. Although the majority of patients show remission after initial surgical debulking and chemotherapy, most patients succumb to recurrent disease. Despite additional treatment modalities, the overall 5-year survival rate of these patients remains lower than 25%, obviating the need for the development of new therapeutic modalities.

Over the past years, there is increasing evidence that EOC is an immunogenic tumor. Tumor-infiltrating T cells capable of killing autologous ovarian cancer cells have been identified and shown to recognize a restricted number of tumor antigens such as HER-2/neu and NY-ESO-1 on the tumor cells (1, 2). The presence of these cytotoxic T cells in the tumor microenvironment of EOCs has been associated with a favorable clinical outcome (3–5). More recently, the ratio between cytotoxic and T regulatory (Treg) cells within tumors was shown to be a more accurate prognostic factor for patient survival than cytotoxic T cells alone (4). Indeed, Treg cells infiltrating the tumors of patients with EOCs can suppress the function of tumor-infiltrating cytotoxic T cells (6) and were found to significantly correlate with poor clinical prognosis independently of tumor stage, surgical treatments, and other factors affecting survival (7). Furthermore, tumor expression of forhead box protein 3 (Foxp3), the master transcription factor of Treg cell development specifically expressed by naturally occurring Treg cells, was shown to be an independent prognostic factor for progression-free and overall survival in patients with ovarian cancer (8). These data indicate a central role of tumor-infiltrating naturally occurring Foxp3⁺ Treg cells in cancer progression, although the mechanisms that promote the intratumoral accumulation and function of these cells are unclear.

Plasmacytoid dendritic cells (pDC) are a rare subset of circulating dendritic cells, which have also been found in the tumor microenvironment of many solid tumors, including EOCs, head and neck cancer, breast cancer, lung cancer, and skin tumors (9–13). Whereas pDCs are specialized effectors of antiviral immunity through their ability to produce high levels of type I IFNs upon activation, the role of pDCs in the tumor is less clear. Tumor pDCs appear to maintain their nonactivated...
immature state without production of type I IFNs. This may either reflect the lack of activation stimuli in the tumor microenvironment or the active suppression of pDC activation and IFN production by the tumor (9, 11, 14–18). Interestingly, the presence of immature pDCs in the tumor microenvironment is associated with a poor clinical outcome in patients with EOCs and breast cancer (10, 16), raising the possibility that pDCs play a role in establishing the tumor immunosuppression mediated by Foxp3+ Treg cells. This hypothesis is reinforced by the findings that pDCs drive Treg cell-mediated immunosuppression in murine models of asthma, transplantation, and oral tolerance (19–21). Interestingly, it has been shown that immature pDCs in EOCs have the ability to prime naive CD4 and CD8 T cells to produce interleukin (IL)-10 that inhibits tumor-specific cytotoxic T cells in vitro (14, 22). However, whether pDCs play a role in establishing the immunosuppression mediated by Foxp3+ Treg cells is unknown.

In this study, we found that the majority of Foxp3+ Treg cells accumulating in the tumor microenvironment of EOCs belong to the subset of Foxp3+ cells expressing inducible costimulator (ICOS). *Ex vivo*, the expansion and the suppressive function of these Foxp3+ ICOS+ Treg cells was strictly dependent on ICOS-ligand (ICOS-L) stimulation provided by tumor pDCs. *In vivo*, Foxp3+ ICOS+ Treg cells were found in close vicinity to pDCs, and their number significantly correlated with the numbers of pDCs in the tumor microenvironment. Furthermore, pDCs and ICOS+ Foxp3+ Treg cells but not ICOS–Foxp3+ Treg cells were found to be strong predictors for disease progression in patients with ovarian cancer. These data indicate an essential role for pDCs and ICOS-L in Foxp3+ Treg cell–mediated immunosuppression in EOCs, providing a potential new molecular target for therapeutic intervention.

Materials and Methods

Collection and processing of human samples

All studies were approved by the local Institutional Review Board for human research. For direct *ex vivo* flow cytometric and functional analyses, ascites and blood were collected from previously untreated patients with EOCs at the University of Texas MD Anderson Cancer Center (Houston, TX). Ascites were collected aseptically, and cells were harvested by centrifugation over a Ficoll-Hypaque density gradient. Peripheral blood was collected from patients with EOC or from buffy coats of healthy donors (Gulf Coast Regional Blood Center), and mononuclear cells were then obtained by Ficoll-Hypaque density centrifugation. For immunohistochemical analyses, tumor cryomaterial of 28 previously untreated patients with EOC was available from the MD Anderson Cancer Center Tissue Bank.

Flow cytometric analyses and cell sorting

For flow cytometry, cells were stained with monoclonal antibodies and analyzed on a FACSCalibur (BD Biosciences) as previously described (23). For cell sorting, DC subpopulations and Treg cell subsets were isolated from the buffy coat of healthy volunteers or from ascites and blood of patients with EOCs. ICOS+ and ICOS–Foxp3+ Treg cell subsets were identified as lineage– (BDCA2, CD11c, CD14, CD16, CD19, CD56, TCRγδ), CD4+, CD25+ cells and sorted on a FACSaria (BD Biosciences) as previously described (24). pDCs and conventional dendritic cells (cDC) were isolated as previously described (23). In brief, T, B, and natural killer (NK) cells, monocytes, and erythrocytes were depleted from blood mononuclear cells by using magnetic beads. The resulting cells were stained with anti-CD4, anti-CD11c, and a cocktail of fluorescein isothiocyanate (FITC)-conjugated anti-CD3, anti-CD14, anti-CD16, and anti-CD20 mAbs. cDCs were isolated as CD4+/−, CD11c+, lineage– cells and pDCs as CD4+, CD11c+, lineage–.

Immunohistochemistry and immunofluorescence

Tumor specimens were fixed in acetone and subsequently stained with anti-BDCA2 (Miltenyi Biotec), anti-Foxp3 (eBioscience), and/or anti-ICOS (Biolegend) antibodies or isotype control. For immunohistochemistry, signals were amplified by sequential incubation with biotinylated secondary antibodies and either an avidin peroxidase complex reagent or avidin phosphatase complex reagent (Vector Laboratories) before visualization of the immunoreactions. Secondary antibodies used for immunofluorescence were Alexa Fluor 488–labeled goat anti-mouse, Alexa Fluor 594–labeled goat anti-rat, and Alexa Fluor 647–conjugated streptavidin (all from Invitrogen).

T-cell culture

Autologous ICOS+ and ICOS– Treg cells were cultured with or without pDCs and cDCs (DC/T-cell ratio of 1:2) in round-bottomed, 96-well culture plates for 4 days with our without of 50 μg/mL anti-ICOSL mAb (eBioscience) or a combination of 5 μg/mL anti-CD80 and 10 μg/mL anti-CD86 mAbs (R&D Systems) as previously described (24).

Analyses of T-cell cytokine production

Cultured ICOS+ and ICOS– Treg cells were collected and washed at 4 days of stimulation. For intracellular cytokine production, Treg cells were restimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) plus 2 μg/mL ionomycin for 6 hours. Brefeldin A (10 μg/mL) was added during the last 2 hours. The cells were stained with the combination of fluorochrome-labeled mAbs to Foxp3 (eBioscience), IL-10, and IFN-γ (BD Biosciences) with the Foxp3 Staining Kit (eBioscience) or Caltag FIX and PERM kit. Because of limited T-cell numbers obtained from malignant ascites, cytokine production analyses and suppressive function assays were conducted using cells isolated from healthy volunteers.

Suppressive function assay

We sorted 4 × 10⁶ CD4+ CD25RO− CD25− naive T cells as responders and mixed them with different numbers of cultured autologous ICOS+ and ICOS– Treg cells as suppressors and stimulated these cell types for 5 days with irradiated 2 × 10⁴ allogeneic T-cell–depleted peripheral blood mononuclear cells as stimulators. Cellular proliferation was assessed by [³H]thymidine incorporation as described (23).

Prediction of clinical outcome

We analyzed 23 patients with EOCs, from which both clinical and pathologic information were available (Supplementary Table S1). For these patients, tumor-infiltrating cell numbers...
of total Foxp3⁺ Treg cells, ICOS⁺ and ICOS⁻ Foxp3⁺ Treg cell subsets, and pDCs were quantified by histology in a blinded fashion and correlated with disease progression. Histologic quantification experiments represent the mean of 3 random high-power field (HPF; field with a 400-fold magnification).

**Statistical analyses**

Differences in cell numbers or percentages were determined by unpaired or paired t test, with \( P < 0.05 \) considered significant. For disease-free survival, data were censored at last follow-up for patients who were alive and disease-free at the time of analysis. Significances for cutoff point graphs were calculated using log-rank-tests, for proportional hazards models using likelihood ratio \( \chi^2 \) tests. These analyses were done using R software package, version 2.10.1.

**Results**

**pDCs and Treg cells accumulate in EOC**

To characterize the tumor microenvironment of ovarian cancer, we conducted flow cytometric analyses of malignant ascites from patients with EOCs. Malignant ascites are frequently associated with peritoneal metastases, and its cellular components directly reflect the cellular components in the tumor microenvironment (7). First, we sought to quantify the numbers of human DC subsets in the malignant ascites, pDCs were identified as lineage⁻, HLA-DR⁺, CD123⁺⁺ cells that co-express BDCA2, BDCA4, CD4, CD45RA but lack expression of CD11c (Fig. 1A). On the other hand, cDCs were identified as lineage⁻, HLA-DR⁺, CD123⁺ cells that lack BDCA2, BDCA4, CD4, CD45RA but express CD11c. When compared with peripheral blood, a significant increase in the percentages of pDCs was found in the tumor ascites (7.0-fold increase, \( n = 11 \)), indicating that pDCs accumulate in the tumor microenvironment (Fig. 1B). In contrast, there was a significant depletion of cDCs in the tumor ascites as compared with peripheral blood (Fig. 1C). Importantly, there was no difference in the percentages of both pDCs and cDCs in the peripheral blood between patients with EOC and healthy donors (Fig. 1B).

Next, we quantified other immune cell types including CD4⁺ T cells, CD8⁺ T cells, Foxp3⁺ Treg cells, B cells, and NK cells in the malignant ascites. Only Foxp3⁺ Treg cells were found to be significantly more abundant in the tumor ascites than in the peripheral blood (Fig. 2A and B). Strikingly, the accumulation of Foxp3⁺ Treg cells (depicted as tumor-to-peripheral blood ratio) was directly associated with an increase of pDC numbers, confirming the accumulation of pDC and Foxp3⁺ Treg cells in the malignant ascites. Large numbers of pDCs (Fig. 2C) and Foxp3⁺ Treg cells (Fig. 2D) were also found in the peritoneal metastases by immunohistochemical staining of tumor tissue.

**Preferential accumulation of ICOS⁺ Foxp3⁺ Treg cells in EOC**

Recently, 2 subsets of Foxp3⁺ Treg cells with distinct functional ability were identified on the basis of the expression

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**Figure 1.** Accumulation of pDCs in malignant ascites of patients with ovarian cancer. A, phenotypical analyses of pDCs and cDCs isolated from malignant ascites of patients with ovarian cancer. pDCs were identified as lineage⁻, HLA-DR⁺, CD123⁺⁺ cells that co-express BDCA2, BDCA4, CD4, CD45RA but lack expression of CD11c. cDCs were identified as lineage⁻, HLA-DR⁺, CD123⁺ cells that lack BDCA2, BDCA4, CD4, CD45RA but express CD11c. HLA, human leukocyte antigen. B, accumulation of pDCs in malignant ascites of ovarian cancer as compared with peripheral blood of patients (\( n = 11 \)) and healthy volunteers (\( n = 7 \)). G, decreased percentage of cDCs in malignant ascites as compared with peripheral blood of patients with ovarian cancer (\( n = 11 \)) and healthy volunteers (\( n = 7 \)). *, \( P < 0.005 \); **, \( P < 0.01 \). n.s., nonsignificant.

**Table 1.**

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**Figure 2.** A, Tumor ascites ovarian cancer. B, histological analyses of malignant ascites. C, functional activity were identified as lineage⁻, HLA-DR⁺, CD123⁺⁺ cells that co-express BDCA2, BDCA4, CD4, CD45RA but lack expression of CD11c. cDCs were identified as lineage⁻, HLA-DR⁺, CD123⁺ cells that lack BDCA2, BDCA4, CD4, CD45RA but express CD11c. HLA, human leukocyte antigen. B, accumulation of pDCs in malignant ascites of ovarian cancer as compared with peripheral blood of patients (\( n = 11 \)) and healthy volunteers (\( n = 7 \)). G, decreased percentage of cDCs in malignant ascites as compared with peripheral blood of patients with ovarian cancer (\( n = 11 \)) and healthy volunteers (\( n = 7 \)). *, \( P < 0.005 \); **, \( P < 0.01 \). n.s., nonsignificant.
of ICOS (24). The ICOS<sup>+</sup> Foxp3<sup>+</sup> Treg cell subset suppresses T-cell proliferation via IL-10–mediated suppression of antigen-presenting cells, whereas ICOS<sup>−</sup> Foxp3<sup>+</sup> Treg cell subset suppresses via TGF-β. To determine the contribution of these subsets in Foxp3<sup>+</sup> Treg cell–mediated immunosuppression in EOCs, single-cell suspensions from malignant ascites were stained for CD4, Foxp3, and ICOS. The percentages of ICOS<sup>+</sup> Foxp3<sup>+</sup> Treg cells among total CD4<sup>+</sup> T cells were significantly increased in tumor ascites compared with peripheral blood (Fig. 3A and D). The presence of ICOS<sup>+</sup> Foxp3<sup>+</sup> Treg cells within the tumor microenvironment was confirmed by immunohistochemical analysis of ovarian cancer tissue (Fig. 3B). The accumulation of ICOS<sup>+</sup> Foxp3<sup>+</sup> Treg cells (3.9-fold, n = 8, P = 0.0038) was highly significant (Fig. 3D) and disproportionally high in comparison to the accumulation of ICOS<sup>−</sup> Foxp3<sup>+</sup> Treg cells (1.5-fold, n = 8, P = 0.0485; Fig. 3E). Consequently, there was a significant increase in percentage of ICOS<sup>+</sup> Treg cells per total Foxp3<sup>+</sup> Treg cells in tumor ascites as compared with peripheral blood (Fig. 3A and C) and thus, a significant decrease in ICOS<sup>+</sup> Treg cells per total Foxp3<sup>+</sup> Treg cells (data not shown). Neither of the 2 Foxp3<sup>+</sup> Treg cell subsets showed any difference in peripheral blood of patients with cancer as compared with healthy donors (Fig. 3D). In addition, accumulation of ICOS<sup>+</sup> Foxp3<sup>+</sup> Treg cells in malignant ascites measured by flow cytometry was comparable with values within the cancer tissue measured by histologic analyses, indicating that the malignant ascites indeed reflect the tumor microenvironment (Fig. 3C). Taken together, there is a much more pronounced and significantly larger (P < 0.01) accumulation of ICOS<sup>+</sup> Foxp3<sup>+</sup> Treg cells in EOC than seen in ICOS<sup>−</sup> Treg cells.

**pDCs stimulate ICOS<sup>+</sup> Foxp3<sup>+</sup> Treg cells via ICOS-L in EOC**

Survival and proliferation of ICOS<sup>+</sup> Foxp3<sup>+</sup> Treg cells is entirely dependent on ICOS stimulation and inhibited by concomitant CD28 stimulation (24). To identify the cell type responsible for the maintenance and expansion of ICOS<sup>+</sup> Treg cells in the EOC tumor microenvironment, we stained the cellular components of malignant ascites for ICOS-L, CD80, and CD86. We did not find any differences in expression of costimulatory molecules between pDCs isolated from malignant ascites and from peripheral blood of the same patient (Fig. 4A). This expression profile in patients with EOCs corresponded to...
the phenotype of resting pDCs in peripheral blood of healthy donors (23), thus indicating a maintained resting phenotype of pDCs within EOCs. Within tumor ascites, highest levels of ICOS-L were expressed on pDCs with a constitutive uniform expression of ICOS-L (Fig. 4B and C). cDCs, which were less frequent in tumor ascites, expressed intermediate ICOS-L levels. Monocytes are abundant in EOCs, but in line with previous publication (23), they did not show relevant ICOS-L expression (Fig. 4B and C). T cells, NK cells, and cancer cells did not express significant levels of ICOS-L either (Fig. 4B). In addition, pDCs were found to express low to undetectable levels of CD80 and CD86 (Fig. 4A and B), whereas costimulatory CD86 was abundant on both monocytes and cDCs. Thus, pDCs with their resting phenotype show the optimal constellation with high levels of ICOS-L and low CD86 expression required to stimulate survival and expansion of ICOS+ Foxp3 Treg cells (Fig. 4B). Intriguingly, using triple color confocal microscopy, pDCs were found to be in close vicinity of ICOS+ Foxp3 Treg cells within the tumor microenvironment (Fig. 4D). Accordingly, the percentage of pDCs in the tumor ascites was found to correlate with the percentage of ICOS+ Treg cells but not of the ICOS- subset (Fig. 4E and Supplementary Fig. S1). Neither did the percentage of cDCs correlate with the percentage of ICOS+ Treg cells in the tumor ascites (data not shown). Together, these data suggest that tumor pDCs expressing high levels of ICOS-L may costimulate the expansion of ICOS+ Foxp3 Treg cells in EOCs.

To test this hypothesis, purified pDCs were used to stimulate autologous ICOS+ and ICOS- Foxp3 Treg cells. Because of limited cell numbers obtained from tumor ascites, we first carried out experiments using cells isolated from peripheral blood of healthy volunteers. Whereas ICOS+ Treg cells underwent rapid apoptosis in culture, their viability was maintained and cell numbers increased in the presence of pDCs (Supplementary Fig. S2A). This process was completely dependent on ICOS costimulation as it was abolished by neutralizing anti-ICOS-L antibodies. In contrast, viability and expansion of ICOS- Foxp3 Treg cells did not depend on pDCs and their expression of ICOS-L (Supplementary Fig. S2A). In contrast to pDCs, cDCs expressing intermediate levels of ICOS-L and high levels of costimulatory molecules (Fig. 4B and C) did not show any effect on viability and expansion of ICOS+ Treg cells (data not shown and previously published; ref. 24). Addition of cDCs to the culture led to a pronounced proliferation of ICOS+ Treg

Figure 3. (A) Mainly ICOS+ Foxp3+ Treg cells accumulate in ovarian cancer. A, representative fluorescence-activated cell-sorting (FACS) analysis of ICOS and Foxp3 expression on CD4+ T cells isolated from malignant ascites and peripheral blood of the same patient. B, double-color immunohistochemical staining of tumor microenvironment for Foxp3 (red) and ICOS (blue). ICOS+ Foxp3+ Treg cells appear double positive for Foxp3 (arrowheads) and ICOS- Foxp3- Treg cells appear double positive for ICOS and Foxp3 (arrows). C, among total CD4+ Foxp3+ Treg cells, the ICOS+ subset shows an increased percentage in malignant ascites as compared with peripheral blood. FACS analyses were confirmed by histologic quantifications. D and E, increased percentage of ICOS+ (D) and ICOS- (E) Foxp3+ Treg cells per total CD4+ T cells in malignant ascites as compared with peripheral blood. No difference could be seen between peripheral blood of patients with ovarian cancer (n = 8) and healthy volunteers (n = 11). **, P < 0.005; ††, P < 0.0001; †††, P < 0.0005.
cells instead (24). It has been shown that ICOS$^+$ Foxp3$^+$ Treg cells exert their immunosuppressive function via IL-10–dependent inhibition of dendritic cell function (24). To test whether ICOS-L costimulation by pDCs would also promote the suppressive function of ICOS$^+$ Foxp3$^+$ Treg cells, we first conducted intracellular cytokine staining for IL-10 and IFN-γ. ICOS$^+$ Treg cells cultured with autologous pDCs producing significantly more IL-10 (Fig. 5A). The ability of pDCs to promote IL-10 production in Treg cells was dependent on ICOS costimulation as it was blocked by anti-ICOS-L antibodies, whereas anti-CD80/CD86 did not have any effect. In contrast to IL-10, IFN-γ production was unaffected by blockage of ICOS costimulation (Fig. 5A). Next, we tested the effect of pDCs on immunosuppressive capacity of ICOS$^+$ Treg cells. ICOS$^+$ Treg cells cultured with pDCs were found to efficiently suppress a primary mixed lymphocytic reaction in a ratio-dependent manner (Fig. 5B). By adding anti-ICOS-L antibodies to the culture, this suppression was completely blocked to levels of unstimulated ICOS$^+$ Treg cells (data not shown) or ICOS$^+$ Treg cells with cDCs (Fig. 5B), which accordingly did neither promote immunosuppressive functions of ICOS$^+$ Treg cells nor their viability. Taken together, these data indicate that
pDCs promote viability, expansion, and immunosuppressive functions of ICOS⁺ Foxp3⁺ Treg cells via ICOS-L costimulation. Next, we confirmed these findings using cells isolated from malignant ascites of patients with EOCs. Indeed, Treg cell subsets isolated from tumor ascites of patients with EOCs showed similar behavior as Treg cells from peripheral blood of healthy donors (Fig. 5C and Supplementary Fig. S2). ICOS⁺ and ICOS⁻ tumor Treg cells were cocultured with autologous blood pDCs. Viability of ICOS⁺ but not of ICOS⁻ tumor Treg cells were dependent on pDCs and ICOS costimulation (Fig. 5C). To further confirm that tumor pDCs have similar capacities, blood Treg cells were cultured with pDCs isolated from both peripheral blood and tumor ascites from the same patients with EOCs. pDCs from both compartments increased ICOS⁺ Treg cell numbers significantly, a process that was blocked by anti-ICOS-L antibodies (Fig. 5D).

**ICOS⁺ Foxp3⁺ Treg cells and pDCs predict disease progression in EOC patients**

Because Treg cell–driven immunosuppression has been linked to disease progression in EOCs and because we found a preferential expansion of the ICOS⁺ Treg cell subset, we sought to investigate whether ICOS⁺ Treg cells would be a more accurate predictor of clinical outcome than total Foxp3⁺ Treg cell numbers. Indeed, in our study population (Supplementary Table S1), we found that the ICOS⁺ Foxp3⁺ Treg cell subset but not the total Foxp3⁺ Treg cell numbers can predict disease progression using the Cox proportional hazard model.
indicate that ICOS\textsuperscript{+} Treg cells but not ICOS\textsuperscript{−} Treg cells predict a poor clinical outcome and that ICOS\textsuperscript{+} Treg cells are a better predictor of disease progression than total Foxp3\textsuperscript{+} Treg cells.

Because pDCs stimulate ICOS\textsuperscript{+} Foxp3\textsuperscript{+} Treg cell–mediated immunosuppression, we next sought to assess whether the tumor pDC numbers would also correlate with clinical outcome in patients with EOCs. Using a Cox proportional hazard model, numbers of pDCs were found to be a significant predictor of disease progression ($P = 0.013$; Fig. 6A). Furthermore, patients with more or equal than 10 pDCs per HPF ("high pDC" group) were found to have a significantly reduced progression-free survival compared with patients with less than 10 pDCs per HPF ("low pDC" group; Fig. 6C). The "high pDC" patients experienced a 12.4-fold higher progression hazard as compared with "low pDC" patients (95% CI, 2.2–70.2). Thus, our data identify the presence of ICOS\textsuperscript{+} Treg cells and pDCs in the tumor microenvironment as predictor of poor clinical outcome in patients with EOCs.

Discussion

There is increasing evidence for a role for Treg cells in the development and progression of cancer. In EOCs, Treg cells contribute to tumor growth cells by inhibiting tumor-specific immunity (7). Indeed, large numbers of Foxp3\textsuperscript{+} Treg cells were found to infiltrate the tumor microenvironment of EOCs (6, 7) and were associated with poor clinical outcome (7, 25). Recent evidence suggests that Foxp3\textsuperscript{+} Treg cells are attracted into the tumor microenvironment of EOCs via CCL28 produced by hypoxic tumor cells (26, 27). However, how Treg cells are stimulated and maintained in the tumor microenvironment of EOCs is unknown.

Our study identifies a predominant role of a subset of Foxp3\textsuperscript{+} Treg cells in EOCs. This subset constitutively expresses ICOS and belongs to the thymic-derived naturally occurring Foxp3\textsuperscript{+} Treg cells (24). We now show that these ICOS\textsuperscript{+} Foxp3\textsuperscript{+} Treg cells accumulate in the tumor of patients with EOCs and represent a stronger predictor of disease progression than the total numbers of Foxp3\textsuperscript{+} Treg cells. Thus, our data expand and help clarify the concept of Treg cell–mediated immunosuppression in EOCs by showing the predominant role of ICOS\textsuperscript{+} Foxp3\textsuperscript{+} Treg cells.

Survival, proliferation, and function of ICOS\textsuperscript{+} Foxp3\textsuperscript{+} Treg cells are strictly dependent on ICOS costimulation. A contribution of ICOS costimulation in Treg cell–dependent immunosuppression had been previously shown in mouse models of asthma (28), diabetes (29), autoimmune encephalomyelitis (30), and mucosal tolerance (31). Our data now show that ICOS costimulation may also promote tumor immunosuppression and progression, suggesting that ICOS could represent a new potential target for therapeutic intervention in EOCs.

Several findings suggest that within the tumor microenvironment, effective activation and expansion of ICOS\textsuperscript{+} Foxp3\textsuperscript{+} Treg cells is triggered by a tumor-infiltrating pDCs. First, within the tumor, pDCs were found to accumulate in the tumor microenvironment and express the highest levels of ICOS-L along with low levels of CD80 and CD86. This appears
to be the optimal constellation for an efficient stimulation of ICOS+ Foxp3+ Treg cells. Second, coculture of pDCs with autologous ICOS+ Foxp3+ Treg cells induced their expansion, IL-10 production and the suppressive function via ICOS co-stimulation. Third, tumor-infiltrating pDCs were found in the close vicinity to ICOS+ Foxp3+ Treg cells. Thus, our data suggest that tumor-infiltrating pDCs set up an immunosuppressive tumor microenvironment through the expansion of the ICOS+ Foxp3+ Treg cell subset and identify a new mechanism of IL-10-mediated immunosuppression in EOCs. This adds to the previously described IL-10-mediated immunosuppression by tumor-associated monocytes/macrophages (32).

Although the ability of pDCs to stimulate ICOS+ Foxp3+ Treg cells requires MHC-TCR interactions, the nature of the antigen presented by pDCs is currently unknown. It is generally accepted that capacity of pDCs to internalize antigens by phagocytosis is very limited and it has been proposed that they may rather present endogenous antigens. However, we cannot exclude that pDCs may present tumor antigens to ICOS+ Foxp3+ Treg cells, in particular because we observed a stronger expansion of ICOS+ Treg cells when stimulated with tumor pDCs than with blood pDCs despite having similar maturation phenotype. Interestingly, a recent study has shown that pDCs are able to uptake exogenous antigens via specific receptors (33).

Several mechanisms for pDC recruitment into peripheral tissues have been proposed. pDCs selectively express chemokine-like receptor 1 (CMKL1), which directs pDC migration through its agonist chemerin (34). CXCR4-ligand CXCL12 (SDF-1) alone or in combination with CXCR3-ligands CXCL10 (IP-10), CXCL11 (ITAC), and CXCL9 (Mig) induced during inflammatory responses represent additional pDC chemotactants (35). Interestingly, EOC tumor cells have been shown to produce large amounts of SDF-1, which may directly recruit pDCs into tumors (14).

Taken together, our findings provide new targets for therapeutic intervention to block tumor immunosuppression in EOCs. One strategy would be to block ICOS-L/ICOS co-stimulation to reduce expansion and function of ICOS+ Treg cells in the tumor microenvironment. Another approach would be targeting pDC recruitment into the tumor. Intriguingly, inhibitors of CXCL12-CXCR4 have shown to reduce tumor growth in mice models for EOCs (36, 37). Alternatively, resting pDCs could be activated with TLR ligands to produce type I IFNs to mount a strong antitumor immune response. Resting pDCs isolated from malignant ascites of patients with EOCs were indeed capable of producing large amounts of type I IFNs upon ex vivo stimulation with CpG, a TLR9 agonist (data not shown), and the efficacy of this approach has already been shown in several cancers (13, 38–42).

In conclusion, we identified a new mechanism how pDCs drive immunosuppression in EOCs mediated through ICOS+ Foxp3+ Treg cells in an ICOS-L-dependent manner. This mechanism might be relevant for other malignancies as well, as the accumulation of ICOS+ Treg cells in tumors has been described for melanoma (39) and breast cancer (44).

Disclosure of Potential Conflicts of Interest

Y. J. Liu is on the consultant/advisory board for Roche. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: C. Conrad, M. Gilliet
Development of methodology: C. Conrad, Y.-H. Wang, T. Ito, Y.-J. Liu, M. Gilliet
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Conrad, J. Gregorio, Y.-H. Wang, S. Meller, R. Freedman, M. Gilliet
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Conrad, J. Gregorio, N. Atkinson, M. Gilliet
Writing, review, and/or revision of the manuscript: C. Conrad, R. Freedman, M. Gilliet
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Conrad, S. Hanabuchi, M. Gilliet
Study supervision: C. Conrad, P. T. Ramirez, M. Gilliet
Consented patients: S. Anderson, M. Gilliet

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