Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS co-stimulation of Foxp3+ T regulatory cells

Curdin Conrad¹,², Josh Gregorio¹, Yi-Hong Wang¹, Tomoki Ito¹, Stephan Meller¹, Shino Hanabuchi¹, Sonya Anderson³, Neely Atkinson⁴, Pedro T Ramirez³, Yong-Jun Liu¹, Ralph Freedman³, & Michel Gilliet¹,²

¹ Department of Immunology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030
² Current address: Department of Dermatology, University Hospital CHUV, Lausanne, Switzerland
³ Department of Gynecologic Oncology and Reproductive Medicine, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030
⁴ Department of Statistics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

RUNNING TITLE: pDCs driven Treg cell stimulation in ovarian cancer

KEY WORDS: Ovarian cancer, ICOS, T regulatory cells, plasmacytoid dendritic cells, immunosuppression

FINANCIAL SUPPORT: This work was funded by a PO1 grant from the NIH (CA128913), the American Cancer Society (RSG-06-173-01-LIB), the MDACC Ovarian Cancer SPORE, and the Gillson-Longenbaugh Foundation to MG

CORRESPONDENCE: Michel Gilliet, Department of Dermatology, University Hospital CHUV, Lausanne, Switzerland, T: ++41-21-314-0351, F: ++41-21-314-0382, michel.gilliet@chuv.ch

COMPETING INTERESTS: The authors declare that there are no conflicts of interest.

WORD COUNT: 4405; TOTAL NUMBER OF FIGURES: 6 (9 including supplemental data)
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 forkhead 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 EOC belong to the subset of Foxp3⁺ Treg cells expressing ICOS. The expansion and the suppressive function of these cells were strictly dependent on ICOS-L costimulation provided by tumor pDCs. 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 ovarian cancer patients, with ICOS⁺ Treg cell subset being a stronger predictor than total Foxp3⁺ Treg cells. These findings suggest an essential role for pDC and ICOS-L in immune suppression mediated by ICOS⁺ Foxp3⁺ Treg cells leading to tumor progression in ovarian cancer.
INTRODUCTION

Epithelial ovarian cancer (EOC) is the second most common gynecological malignancy, which causes more death than any other gynecological cancer in Europe and the United States. Due to 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 five-year survival 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 EOC has been associated with a favorable clinical outcome (3-5). More recently, the ratio between cytotoxic and T regulatory cells within tumors was shown to be a more accurate prognostic factor for patient survival than cytotoxic T cells alone (4). Indeed, T regulatory (Treg) cells infiltrating the tumors of EOC patients 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 forkhead 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...
pDCs driven Treg cell stimulation in ovarian cancer

ovarian cancer patients (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 EOC, 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 interferons (IFN) upon activation, the role of pDCs in the tumor is less clear. Tumor pDCs appear to maintain their non-activated 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 EOC and breast cancer patients (10, 16) raising the possibility that pDC 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 demonstrated that immature pDC in EOC have the ability to prime naïve CD4 and CD8 T cells to produce IL-10 that inhibits tumor-specific cytotoxic T cells in-vitro (14, 22). However, whether pDC 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 EOC belong to the subset of Foxp3⁺ cells expressing inducible co-stimulator (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 ovarian cancer patients. These data indicate an essential role for pDC and ICOS-L in Foxp3⁺ Treg cell-mediated immunosuppression in EOC, 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 cytometry and functional analyses, ascites and blood were collected from previously untreated patients with EOC at the University of Texas M.D. Anderson Cancer Center at Houston, Texas, USA. Ascites was collected aseptically and cells were harvested by centrifugation over a Ficoll–Hypaque density gradient. Peripheral blood was collected from EOC patients or from buffy coats of healthy donors (Gulf Coast Regional Blood Center, Texas) and mononuclear cells were then obtained by Ficoll–Hypaque density centrifugation. For immunohistochemistry analyses, tumor-cryomaterial of 28 previously untreated EOC patients was available from the M.D. Anderson Cancer Center tissue bank.

Flow cytometry 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 EOC patients. ICOS⁺ and ICOS⁻ Foxp3⁺ Treg cell subsets were identified as lineage⁻ (BDCA2, CD11c, CD14, CD16, CD19, CD56, TCRγδ), CD4⁺, CD25hi⁺ cells and sorted on a FACSAria (BD Biosciences) as previously described (24). pDCs and cDCs were isolated as previously described (23). In brief, T, B, and 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 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 specimen 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 50μg/ml anti-ICOSL mAb (eBioscience) or a combination of 5μg/ml anti-CD80 and 10μg/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 50ng/ml PMA plus 2μg/ml ionomycin for 6 hours. 10μg/ml brefeldin A 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 Bioscience) with the foxp3 staining kit (eBioscience) or Caltag FIX and PERM kit. Due to limited T cell numbers obtained from malignant ascites, cytokine production analyses and suppressive function assays were performed using cells isolated from healthy volunteers.

**Suppressive function assay:** We sorted 4×10^4^ CD4^+^ CD45RO^-^ 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 2×10^4^ allogeneic T cell-depleted PBMCs as stimulators. Cellular proliferation was assessed by [3H]thymidine incorporation as described (23).

**Prediction of clinical outcome:** We analysed from 23 EOC patients, from which both clinical and pathological information were available (Suppl. Table 1). 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 three random high power fields (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 cut-point graphs were calculated using log-rank-tests, for proportional hazards model using likelihood ratio chi-square tests. These analyses were done using R software package, version 2.10.1.
RESULTS

pDCs driven Treg cell stimulation in ovarian cancer

To characterize the tumor microenvironment of ovarian cancer, we performed flow cytometry analyses of malignant ascites from EOC patients. Malignant ascites is 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, conventional dendritic cells (cDC) were identified as lineage⁻, HLA-DR⁺, CD123⁻ cells that lack BDCA2, BDCA4, CD4, CD45RA, but express CD11c. When compared to peripheral blood, a significant increase in the percentages of pDCs was found in the tumor ascites (7.0-fold increase, n=11), indicating that pDC accumulate in the tumor microenvironment (Fig. 1B). By contrast, there was a significant depletion of cDCs in the tumor ascites as compared to peripheral blood (Fig. 1C). Importantly there was no difference in the percentages of both pDCs and cDCs in the peripheral blood between EOC patients 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 compared to the peripheral blood (Fig. 2A and 2B). 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⁺
pDCs driven Treg cell stimulation in ovarian cancer

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, two subsets of Foxp3⁺ Treg cells with distinct functional ability were identified based on the expression of ICOS (24). The ICOS⁺ Foxp3⁺ Treg cell subset suppresses T cell proliferation via IL-10-mediated suppression of antigen-presenting cells, while ICOS⁻ Foxp3⁺ Treg cell subset suppresses via TGF-beta. To determine the contribution of these subsets in Foxp3⁺ Treg cell-mediated immunosuppression in EOC, single cell suspensions from malignant ascites were stained for CD4, Foxp3, and ICOS. The percentages of ICOS⁺ Foxp3⁺ Treg cells among total CD4⁺ T cells were significantly increased in tumor ascites compared to peripheral blood (Fig. 3A and 3D). The presence of ICOS⁺ Foxp3⁺ Treg cells within the tumor microenvironment was confirmed by immunohistochemical analysis of ovarian cancer tissue (Fig. 3B). The accumulation of ICOS⁺ Foxp3⁺ Treg cells (3.9-fold, n=8, p=0.0038) was highly significant (Fig 3D), and disproportionately high in comparison to the accumulation of ICOS⁻ Foxp3⁺ Treg cells (1.5-fold, n=8, p=0.0485) (Fig. 3E). Consequently, there was a significant increase in percentage of ICOS⁺ Treg cells per total Foxp3⁺ Treg cells in tumor ascites as compared to peripheral blood (Fig. 3A and 3C) and thus, a significant decrease in ICOS⁻ Treg cells per total Foxp3⁺ Treg cells (*data not shown*). Neither of the two Foxp3⁺ Treg cell subsets showed any difference in peripheral blood of cancer patients as compared to healthy donors (Fig 3D). Additionally, accumulation of ICOS⁺ Foxp3⁺ Treg cells in malignant ascites measured by flow cytometry was comparable to values within the cancer tissue measured by histological analyzes indicating that the malignant ascites
pDCs driven Treg cell stimulation in ovarian cancer

indeed reflects the tumor microenvironment (Fig. 3C). Taken together, there is much more pronounced and significantly larger (p<0.01) accumulation of ICOS⁺ Foxp3⁺ Treg cells in EOC than seen in ICOS⁻ Treg cells.

**pDC stimulate ICOS⁺ Foxp3⁺ Treg cells via ICOS-L in EOC**

Survival and proliferation of ICOS⁺ Foxp3⁺ 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⁺ 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 EOC patients corresponded to the phenotype of resting pDCs in peripheral blood of healthy donors (23), thus indicating a maintained resting phenotype of pDCs within EOC. Within tumor ascites, highest levels of ICOS-L were expressed on pDCs with a constitutive uniform expression of ICOS-L (Fig. 4B and 4C). cDCs, which were less frequent in tumor ascites, expressed intermediate ICOS-L levels. Monocytes are abundant in EOC, but in line with previous publication (23), they did not show relevant ICOS-L expression (Fig. 4B and 4C). 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 4B), while 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 Suppl. Fig. 1). 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 EOC.

To test this hypothesis, purified pDCs were used to stimulate autologous ICOS\(^+\) and ICOS\(^-\) Foxp3\(^+\) Treg cells. Due to limited cell numbers obtained from tumor ascites, we first performed 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 (Suppl. Fig. 2A). This process was completely dependent on ICOS costimulation as it was abolished by neutralizing anti-ICOS-L antibodies. By contrast, viability and expansion of ICOS\(^-\) Foxp3\(^+\) Treg cells did not depend on pDCs and their expression of ICOS-L (Suppl. Fig. 2A). In contrast to pDCs, cDCs expressing intermediate levels of ICOS-L and high levels of costimulatory molecules (Fig. 4B and 4C) did not show any effect on viability and expansion of ICOS\(^+\) Treg cells (data not shown and previously published (24)). Addition of cDCs to the culture led to a pronounced proliferation of ICOS\(^-\) Treg 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 performed intracellular cytokine staining for IL-10 and IFN-\(\gamma\). ICOS\(^+\) Treg cells cultured with
autologous pDCs produced significantly more IL-10 (Fig. 5A). The ability of pDCs to promote IL-10 production in Treg cells was dependent of 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 co-stimulation (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 EOC patients. Indeed, Treg cell subsets isolated from tumor ascites of EOC patients showed similar behavior as Treg cells from peripheral blood of healthy donors (Fig. 5C and Suppl. Fig. 2). ICOS⁺ and ICOS⁻ tumor Treg cells were co-cultured with autologous blood pDCs. Viability of ICOS⁺ but not of ICOS⁻ tumor Treg cells were dependant on pDCs and ICOS co-stimulation (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 EOC patient. pDCs from both compartments increased ICOS⁺ Treg cell numbers significantly, a process which was blocked by anti-ICOS-L antibodies (Fig. 5D).

ICOS⁺ Foxp3⁺ Treg cell and pDCs predict disease progression in EOC patients
Because Treg cell-driven immunosuppression has been linked to disease progression in EOC 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 (Suppl. Table 1), 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 (p=0.029 vs p=0.291, Fig. 6A). Additionally, to visualize progression free survival differences in a Kaplan-Meier curve, we divided EOC patients into two groups. We found patients with equal or more than 13 ICOS⁺ Foxp3⁺ Treg cells per high power field (HPF) (corresponding to the 75% percentile of the mean count in all patients) showed a significantly reduced progression-free survival as compared to patients with less than 13 ICOS⁺ Foxp3⁺ Treg cells per HPF (Fig. 6B). Patients in the “high ICOS⁺ Treg cell” group experienced a 7.5-fold higher progression hazard as compared with the “low-medium ICOS⁺ Treg cell” group” (95% confidence interval, 1.2–45.1). By contrast, ICOS⁻ Foxp3⁺ Treg cells did not correlate with disease progression using the cox hazard model (p=0.481, Fig. 6A). Thus, our data indicate that ICOS⁺ Treg cells but not ICOS⁻ Treg cells predict a poor clinical outcome and that ICOS⁺ Treg cells are a better predictor of disease progression than total Foxp3⁺ Treg cells.

Because pDCs stimulate ICOS⁺ Foxp3⁺ Treg cell-mediated immunosuppression, we next sought to assess whether the tumor pDC numbers would also correlate with clinical outcome in EOC patients. 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
pDCs driven Treg cell stimulation in ovarian cancer

progression-free survival compared to 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% confidence interval, 2.2–70.2). Thus our data identify the presence of ICOS⁺ Treg cells and pDCs in the tumor microenvironment as predictor of poor clinical outcome in EOC patients.
DISCUSSION

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

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

Survival, proliferation and function of ICOS+ Foxp3+ 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 demonstrate that ICOS costimulation may also promote tumor immune-suppression and progression, suggesting that ICOS could represent a new potential target for therapeutic intervention in EOC.
Several findings suggest that, within the tumor microenvironment, effective activation and expansion of ICOS\(^+\) Foxp3\(^+\) 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, co-culture 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 EOC. 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).
pDCs driven Treg cell stimulation in ovarian cancer

Several mechanisms for pDC recruitment into peripheral tissues have been proposed. pDCs selectively express chemokine like receptor 1 (CMKLR1), 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 chemoattractants (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 EOC. One strategy would be to block ICOS-L/ICOS costimulation 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 EOC (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 EOC patients 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 demonstrated in several cancers (13, 38-42).

In conclusion, we indentified a new mechanism how pDCs drive immunosuppression in EOC 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 (43) and breast cancer (44).
ACKNOWLEDGEMENTS, GRANT SUPPORT

This work was funded by a PO1 grant from the NIH (CA128913), the American Cancer Society (RSG-06-173-01-LIB), the MDACC Ovarian Cancer SPORE, and the Gillson-Longenbaugh Foundation to MG.
REFERENCES


FIGURE LEGENDS

Figure 1: Accumulation of plasmacytoid dendritic cells in malignant ascites of ovarian cancer patients. (A) Phenotypical analyses of plasmacytoid (pDC) and conventional dendritic cells (cDC) isolated from malignant ascites of ovarian cancer patients. Plasmacytoid dendritic cells were identified as lineage-; HLA-DR+; CD123++; cells that co-express BDCA2, BDCA4, CD4, CD45RA, but lack expression of CD11c. Conventional dendritic cells were identified as lineage-; HLA-DR+; CD123- cells that lack BDCA2, BDCA4, CD4, CD45RA, but express CD11c. (B) Accumulation of plasmacytoid dendritic cells in malignant ascites of ovarian cancer as compared to peripheral blood of patients (n=11) and healthy volunteers (n=7). (C) Decreased percentage of conventional dendritic cells in malignant ascites as compared to peripheral blood of ovarian cancer patients (n=11) and healthy volunteers (n=7). * p<0.005, ** p<0.01

Figure 2: Paralleled accumulation of plasmacytoid dendritic cells and Foxp3+ T regulatory cells in malignant ascites of ovarian cancer patients. (A) Representative FACS analyses of different immune cells types - including plasmacytoid (pDC) and conventional dendritic cells (cDC), CD4+ T cells, CD8+ T cells, Foxp3+ T regulatory cells, B cells, and NK cells - isolated from malignant ascites (Tumor) and peripheral blood (Blood) of ovarian cancer patients. (B) Tumor to peripheral blood-ratio for analyzed immune cells showed associated accumulation of both Foxp3+ T regulatory cells and plasmacytoid dendritic cells in the malignant ascites. (C and D) Immunohistochemical stainings for plasmacytoid dendritic cells (BDCA2, C) and Foxp3+ T regulatory cells (Foxp3, D) in the tumor microenvironment of peritoneal metastases. TIL = Tumor infiltrating lymphocytes
**Figure 3: Mainly ICOS+ Foxp3+ T regulatory cells accumulate in ovarian cancer. (A)**

Representative FACS analysis of ICOS- and Foxp3-expression on CD4+ T cells isolated from malignant ascites and peripheral blood of the same patient. (B) Double-colour immunohistochemical staining of tumor microenvironment for Foxp3 (red) and ICOS (blue). ICOS- Foxp3+ T regulatory cells appear single positive for Foxp3 (*arrow heads*) and ICOS+ Foxp3+ T regulatory cells appear double positive for ICOS and Foxp3 (*arrows*). (C) Among total CD4+ Foxp3+ T regulatory cells, the ICOS+ subset shows an increased percentage in malignant ascites as compared to peripheral blood. FACS analyses were confirmed by histological quantifications. (D and E) Increased percentage of ICOS+ (D) and ICOS- (E) Foxp3+ T regulatory cells per total CD4+ T cells in malignant ascites as compared to peripheral blood. No difference could be seen between peripheral blood of ovarian cancer patients (n=8) and healthy volunteers (n=11). * p<0.05, ** p<0.0001, *** p<0.005

**Figure 4: Plasmacytoid dendritic cells provide optimal stimulus for and correlate with ICOS+ Foxp3+ T regulatory cells in the tumor microenvironment. (A)** FACS analyzes of plasmacytoid dendritic cells isolated from both peripheral blood and tumor ascites express high levels of ICOS-L and show no expression of co-stimulatory CD80 and low levels of CD86. (B) Different immune cells and ovarian cancer cells isolated from malignant ascites were analyzed for ICOS-L and CD86 expression by FACS. (C) Representative FACS plots of ICOS-L expression on plasmacytoid dendritic cells (CD123), conventional dendritic cells (CD11c) and monocytes (CD14) isolated from malignant ascites of ovarian cancer patients. (D) Ovarian cancer tissue was stained with antibodies against BDCA2 (green), ICOS (blue) and Foxp3 (red). Using triple color confocal microscopy, pDCs were found to be in close vicinity of ICOS+ Foxp3+ Tregs within the
tumor microenvironment. (E) Percentages of plasmacytoid dendritic cells correlate with percentages of ICOS\(^+\) Foxp3\(^+\) T regulatory cells within the tumor microenvironment of ovarian cancer. *B; data depicted show one representative of repeated independent experiments. pDC = plasmacytoid dendritic cells, cDC = conventional dendritic cells, Mono = monocytes*

**Figure 5: Survival and function of ICOS\(^+\) Foxp3\(^+\) T regulatory cells are dependent on ICOS-L provided by plasmacytoid dendritic cells.** (A) ICOS\(^+\) and ICOS\(^-\) Foxp3\(^+\) T regulatory cells isolated from peripheral blood were co-cultured with or without plasmacytoid dendritic cells (pDC) in the presence or absence of neutralizing anti-ICOS-L antibodies or anti-CD80/CD86 antibodies. Intracellular production of IL-10 and IFN-\(\gamma\) assayed by FACS 6h after re-stimulation of primed T cells with PMA and ionomycin. The percentages of each population are indicated in the plots. (B) The immunosuppressive function of ICOS\(^+\) Foxp3\(^+\) T regulatory cells was tested using a mixed lymphocytic reaction after co-culture with conventional dendritic cells (cDC) or plasmacytoid dendritic cells in the presence or absence of anti-ICOS-L antibodies. Proliferation was assessed by \([3H]\)thymidine incorporation and representative experiment is shown. (C) ICOS\(^+\) and ICOS\(^-\) Foxp3\(^+\) T regulatory cells isolated from tumor ascites were co-cultured with or without autologous plasmacytoid dendritic cells in the presence or absence of neutralizing anti-ICOS-L antibodies as described. Data depicted represent mean numbers of viable cells after four days of culture. Similar results were obtained in three independent experiments. (D) Numbers of viable ICOS\(^+\) Foxp3\(^+\) T regulatory cells isolated from malignant ascites after co-culture with or without autologous plasmacytoid dendritic cells from either peripheral blood or malignant ascites in the presence or absence of neutralizing anti-ICOS-L antibodies. * \(p<0.01\), ** \(p<0.05\), *** \(p<0.02\)
Figure 6: Accumulation of ICOS⁺ Foxp3⁺ T regulatory cells and plasmacytoid dendritic cells predict poor clinical outcome in ovarian cancer patients. (A) Significances for numbers of tumor infiltrating total Foxp3⁺ T regulatory cells, ICOS⁺ and ICOS⁻ Foxp3⁺ T regulatory cell subsets, and plasmacytoid dendritic cells as predictors of disease progression using Cox hazard model. (B and C) Kaplan-Meier curve for disease free survival by number of tumor-infiltrating ICOS⁺ Foxp3⁺ T regulatory cells (B) and plasmacytoid dendritic cells (C) in 31 individuals with ovarian cancer. Patients were divided into two groups according to cells numbers within the tumor microenvironment as indicated.
Figure 1

A

Tumor ascites
ovarian cancer

CD3/14/15/16/20

HLA-DR

gated on Lin- DR+ cells

CD123

BDCA-2
BDCA-4
CD4
CD45RA
CD11c

pDC

C

* n.s.

n.s.

B

Percentage pDC

0 2 4 6

Tumor Ascites Blood Blood

Ovarian cancer Healthy donor

C

Percentage cDC

0.0 0.5 1.0 1.5

Tumor Ascites Blood Blood

Ovarian cancer Healthy donor
Figure 2

A

Tumor

- pDC
  - CD11c
  - CD123
  - HLA-DR
  - 6%
- cDC
  - CD11c
  - CD123
  - HLA-DR
  - 0.5%
- CD4/CD8 TIL
  - CD4
  - CD3
  - 38%
- Foxp3 Treg
  - CD4
  - 15%
- NK / B cells
  - CD56
  - 16%
  - CD20
  - 1%

Blood

- CD11c
  - CD123
  - HLA-DR
  - 1.2%
- CD11c
  - CD123
  - HLA-DR
  - 2.1%
- CD4
  - CD3
  - 45%
- Foxp3
  - CD4
  - 3%
- CD56
  - CD20
  - 7%
  - 1%

B

Tumor/blood ratio

- pDC
- cDC
- CD4 TIL
- CD8 TIL
- Foxp3 Treg
- NK cells
- B cells

C

BDCA2

D

Foxp3
**Figure 3**

A. Gated on CD4+ T cells

**Tumor**

<table>
<thead>
<tr>
<th>ICOS</th>
<th>Foxp3</th>
</tr>
</thead>
<tbody>
<tr>
<td>44%</td>
<td></td>
</tr>
</tbody>
</table>

**Blood**

<table>
<thead>
<tr>
<th>ICOS</th>
<th>Foxp3</th>
</tr>
</thead>
<tbody>
<tr>
<td>13%</td>
<td></td>
</tr>
</tbody>
</table>

**B.** ICOS, Foxp3

**C.**

<table>
<thead>
<tr>
<th>Percentage ICOS+ per CD4+ Tregs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Ascites</td>
</tr>
</tbody>
</table>

**FACS**

**Histology**

**D.**

<table>
<thead>
<tr>
<th>Percentage ICOS+ Tregs per CD4+ T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Ascites</td>
</tr>
</tbody>
</table>

**Ovarian cancer**

**Healthy donor**

**E.**

<table>
<thead>
<tr>
<th>Percentage ICOS+ Tregs per CD4+ T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Ascites</td>
</tr>
</tbody>
</table>

**Ovarian cancer**

**Healthy donor**

---

Copyright © 2012 American Association for Cancer Research. Published OnlineFirst on July 31, 2012; DOI: 10.1158/0008-5472.CAN-12-2271
Figure 4

A. Gated on lineage cells

Tumor ascites

Blood

CD123

HLA-DR

ICOS-L

CD80

CD86

B. Percentage of cells expressing ICOS-L

Tumor cells

NK cells

T cells

Monocytes

B0%

20%

40%

60%

80%

100%

Percentage of cells expressing ICOS-L

Percentage of cells expressing CD86

C. ICOS-L

pDC

95%

CD123

cDC

52%

CD11c

Mono

48%

CD14

D. Micrograph

E. Percentage ICOS+ Treg per CD4 T cells

Percentage pDC

p<0.05
Figure 5

A

<table>
<thead>
<tr>
<th>IL-10 (cpm)</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>1:32</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDC</td>
<td>1.4</td>
<td>0.5</td>
<td>6.2</td>
<td>12.3</td>
</tr>
<tr>
<td>pDC + anti-ICOS-L</td>
<td>7.1</td>
<td>4.0</td>
<td>2.4</td>
<td>4.1</td>
</tr>
<tr>
<td>pDC + anti-CD80/CD86</td>
<td>3.8</td>
<td>7.5</td>
<td>14</td>
<td>11</td>
</tr>
</tbody>
</table>

B

**Figure B**

C

<table>
<thead>
<tr>
<th>Tumor Treg cell number</th>
<th>Blood pDC</th>
<th>anti-ICOS-L</th>
<th>pDC</th>
<th>anti-ICOS-L</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICOS+ Treg</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ICOS- Treg</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th>Tumor ICOS+ Treg cell number</th>
<th>Blood pDCs</th>
<th>Ascites pDCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* **p < 0.05**
**p < 0.01**
***p < 0.001**
Figure 6

A

<table>
<thead>
<tr>
<th>Proportional hazard</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDCs</td>
<td>0.013</td>
</tr>
<tr>
<td>Total foxp3+ Tregs</td>
<td>n.s. (0.29)</td>
</tr>
<tr>
<td>ICOS+ foxp3+ Tregs</td>
<td>0.029</td>
</tr>
<tr>
<td>ICOS- foxp3+ Tregs</td>
<td>n.s. (0.481)</td>
</tr>
</tbody>
</table>

B

- low/medium (<13 ICOS+Treg)
- high (≥13 ICOS+Treg)

Progression free survival

Months

C

- low/medium (<10 pDC)
- high (≥10 pDC)

Progression free survival

Months
Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS co-stimulation of Foxp3+ T regulatory cells

Curdin Conrad, Josh Gregorio, Yi-Hong Wang, et al.

Cancer Res  Published OnlineFirst July 31, 2012.

Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-2271
Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2012/07/31/0008-5472.CAN-12-2271.DC1
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Sign up to receive free email-alerts related to this article or journal.
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.