CXCR3⁺ T regulatory cells selectively accumulate in human ovarian carcinomas to limit type I immunity

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Abstract

Anti-tumor Type I T cell responses involving IFN-γ production are critical to control cancer, but the efficacy of this response is limited by a variety of immunosuppressive mechanisms that promote tumoral immune escape. One critical mechanism involves the accumulation of FOXP3+ T regulatory cells (Treg), a class of suppressive T cells that prevent excessive tissue destruction caused by unchecked immune responses. Recent studies have revealed that FOXP3+ Treg include distinct subsets specifically controlling over the corresponding effector subset. In particular, CXCR3+ Treg have been described as a subset specialized in the control of type I T cell responses in vivo. Here we show that CXCR3+ Treg are highly enriched in human ovarian carcinomas, particularly in solid tumor masses, where they represent the majority of Treg. Tumor-associated CXCR3+ Treg co-express T-bet but do not secrete IFN-γ ex vivo and suppress proliferation and IFN-γ secretion of T effectors. In addition, they co-express Helios, suggesting that they originate from natural Treg. Finally, we show that the proportion of CXCR3+ Treg at tumor sites is directly correlated with that of CXCR3+ T effectors, consistent with expression of CXCR3 ligands. Together, our findings support the concept that natural CXCR3+ T-bet+ Treg selectively accumulate in ovarian tumors to control type I T cell responses, resulting in the collateral limitation of efficient anti-tumor immunity.
Introduction

Anti-tumor type I immune responses to human ovarian cancers have the potential to control disease progression, as the presence of intra-tumoral T cells, as well as the expression of IFN-γ, have been reported to correlate with improved clinical outcome (1, 2). The clinical efficacy of type I immune responses in ovarian cancer, however, is limited by immune regulation mechanisms at the tumor site, including tumor-associated FOXP3+ Treg, that are abundant, particularly in late stage tumors, their frequency correlating with reduced survival (3-5). It has been reported that ovarian cancer-associated Treg express CCR4 and accumulate at tumor sites attracted by the CCR4 ligand CCL22, secreted by tumor cells and environmental macrophages. On the basis of these findings, it has been proposed that blockade of CCL22 may inhibit Treg trafficking into ovarian tumors (3, 6).

Recent studies have revealed a significant phenotypical and functional heterogeneity in FOXP3+ Treg, similar to that found in conventional CD4+ T cells. CD4+ T cell populations, both conventional and Treg, have been identified that co-express more than one lineage-specific transcription factor, exhibit hybrid features, and could represent differentiation intermediates and/or exert specialized functions, underlining the complexity and finesse of the immune response (7, 8). An emerging concept is that subpopulations of FOXP3+ Treg co-expressing other lineage-specific transcription factors specifically control over the corresponding conventional effector subset (9). In this context, a recent study has identified, in mice, a subset of FOXP3+ Treg expressing the TH1-associated chemokine receptor CXCR3 and the transcription factor T-bet (10). The subset is highly expanded during type I inflammation and exhibits homeostatic and migratory properties optimized for the suppression of TH1 responses in vivo. Lately, the existence of CXCR3+ T-bet+
FOXP3+ Treg has been demonstrated in humans, both in healthy individuals and in patients with autoimmune diseases (11-13). However, the presence of CXCR3+ Treg in tumors has not been addressed so far.
Materials and methods

Patients and healthy donors samples

Surgical tumor specimens and peripheral blood samples were obtained at the Roswell Park Cancer Institute (Buffalo, NY) from ovarian cancer patients upon approval by the institutional review board and signed informed consent. Tumor specimens were processed by mechanical dissection and single-cell suspensions were cryopreserved. Cells from ovarian cancer ascites were isolated by centrifugation and cryopreserved. Peripheral blood samples from healthy donors were obtained from the Etablissement Français du Sang Pays de la Loire (Nantes, France) upon signed informed consent and approval by the Institutional Review Board. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient sedimentation using LSM 1077 lymphocyte separation medium (PAA Laboratories GmbH).

Cell purification, *ex vivo* phenotypic assessment and cell sorting

$CD4^+$ T cells were enriched by positive selection from PBMC, single-cell suspensions from ovarian solid tumors or ascites, by magnetic cell sorting (Miltenyi Biotec). $CD4^+$ T cells were assessed phenotypically by staining with fluorochrome-labeled mAb (from BD Biosciences unless indicated otherwise) specific for CD4, CD45RA, CCR7, CD25 (Beckman Coulter), and CD127 together with mAb specific for CXCR3, CCR4, CCR6, FOXP3 (eBioscience), T-bet (eBioscience) and Helios (BioLegend), as indicated, and analyzed by flow cytometry (FACSAnia, BD Biosciences). For *ex vivo* flow cytometry cell sorting, enriched $CD4^+$ T cells were stained with fluorochrome-labeled mAb specific for CD8, CD4, CD45RA, CD25, CD127, CCR7 and CXCR3. After gating on $CD4^+CD8^-$ lymphocytes, cells were
separated into the indicated populations to high purity (>97%) by flow cytometry cell sorting (FACSria, BD Biosciences).

**In vitro differentiation of NTreg and conventional naïve CD4⁺ T cells**

Ex vivo-sorted conventional naïve CD4⁺ T cells and NTreg (3 × 10⁴/well in 96-well U-bottom plates) were stimulated in vitro with anti-CD2/CD3/CD28–coated microbeads according to the manufacturer’s instructions (Miltenyi Biotec) in the absence or presence of recombinant human (rh)IL-12 (10 ng/mL; R&D Systems) and rhIFN-γ (20 ng/mL; R&D Systems) alone or in combination. Cells were maintained in culture in IMDM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) containing rhIL-2 (100 IU/mL; Chiron). Aliquots of day 12 cultures were either stained with anti-CXCR3 mAb and analyzed by flow cytometry or assessed for cytokine production and transcription factors expression, following stimulation with PMA and ionomycin, as well as for suppressive function, as detailed below.

**Assessment of cytokine production, transcription factors expression and suppressive function**

Cytokine production by CD4⁺ T cell populations was assessed by intracellular staining after stimulation with PMA (100 ng/mL; Sigma Aldrich) and ionomycin (1 μg/mL; Sigma Aldrich) during 4 and 6 h for in vitro-stimulated cultures and ex vivo CD4⁺ T cells, respectively, as previously described (7, 14). Brefeldin A (10 μg/mL; Sigma Aldrich) was added 1 h after the beginning of the incubation. Cells were then fixed, permeabilized, and stained with mAb specific for FOXP3, T-bet, Helios, and IFN-γ and analyzed by flow cytometry. The ability of ex vivo-sorted Treg from PBMC and from OC tumors as well as in vitro-stimulated CD4⁺ T cell populations to
 suppress the growth of responder CD4⁺CD25− T cells was assessed by co-culture of CFSE-labeled responder conventional CD4⁺ T cells with or without test populations in the presence of irradiated monocytes, and PHA, as previously described (15, 16). Growth of responder cells was assessed by flow cytometry analysis of CFSE dilution in day 5 cultures. The growth (% divided cells) of the wells with test population (experimental group) was compared with that of the wells without test population (control). The percentage of suppression was determined as follows: 100 – ((growth of experimental group/growth of control) x 100). In some experiments, in order to assess the ability of Treg to suppress IFN-γ production by responder cells, IFN-γ was measured in day 5 culture supernatants by ELISA (Invitrogen). The percentage of IFN-γ production suppression was determined as follows: 100 – (([IFN-γ; ng/mL] in experimental group cultures/[IFN-γ; ng/mL] in control cultures) x 100).

**Assessment of chemokines mRNA expression by PCR and migration assay**

Human ovarian cancer cell lines SK-OV-3 and SK-OV-6 (kindly provided by Dr. Lloyd J. Old) and the human colon carcinoma cell line HT-29 (ATCC) as well as monocytes (CD14⁺), enriched by positive selection from HD PBMC by magnetic cell sorting (Miltenyi Biotec), were treated for 24 h in the absence or presence of rhIFN-γ (20 ng/mL) and rhTNF-α (50 ng/mL; R&D Systems) alone or in combination, as indicated. RNA was extracted from ovarian tumor specimens, cells isolated from tumor ascites, tumor cell lines and monocytes using the NucleoSpin® RNA II kit (Macherey-Nagel). Reverse transcription of RNA was performed using Promega Reverse Transcription System kit (Promega). Quantitative PCR (qPCR) was performed using a TaqMan assay on an ABI Prism 7000 sequence detection system (Applied Biosystems) using assays-on-Demand Gene Expression probes for
CXCL10 (Hs00171042_m1), CCL22 (Hs01574247_m1) and ACTB (Hs99999903_m1) (Applied Biosystems). Relative mRNA expression was calculated as $2^{(Ct_{ACTB} - Ct_{test\ gene})}$. Conventional PCR was performed using GoTaq® Flexi DNA Polymerase kit (Promega). cDNA integrity was tested by amplification of ACTB (β-actin) mRNA (15) and CXCL10 and CCL22 mRNA expression was assessed using previously validated primers (17, 18).

To assess the ability of T cells to migrate toward CXCL10, CXCR3⁺ and CXCR3⁻ MTreg and Mconv populations sorted from CD4⁺ T cells of HD were stimulated with plate-bound anti-CD3 and -CD28 mAb (BD Biosciences) and maintained in culture in the presence IL-2. Aliquots of day 10 cultures were stained with anti-CXCR3 mAb and analyzed by flow cytometry. Cells from day 10 cultures (5 x 10⁴ / well) were added to the top chambers of 96-transwell plates (Corning Costar; 5-μm pore polycarbonate filter) and culture medium containing or not CXCL10 (100 ng/mL; R&D Systems) was added to the lower chambers. After 2 h incubation at 37°C, cells were recovered from lower chambers and counted by flow cytometry. The migration index for each population was calculated as the ratio of the number of cells that migrated in the presence of CXCL10 to that of cells that migrated in the absence of CXCL10.
Results

CXCR3\(^+\) Treg are the prevalent tumor-infiltrating Treg subset in ovarian carcinomas

We initially assessed the proportions of CD4\(^+\) Treg in T lymphocytes infiltrating ovarian tumor solid masses (tumor infiltrating lymphocytes, TIL) or isolated from tumor ascites (tumor associated lymphocytes, TAL), using anti-CD25 mAb in combination with anti-CD127 mAb as described (19). TIL contained high proportions of CD25\(^+\)CD127\(^-\) Treg, whereas the proportion of Treg in TAL was significantly lower and, in average, similar to that found in circulating CD4\(^+\) T cells of patients or healthy donors (Fig. 1). Tumor Treg in both TAL and TIL exhibited a more differentiated phenotype as compared to coexisting CD25\(^-\) conventional CD4\(^+\) T cells (Tconv), and were, for the largest part, CD45RA\(^-\)CCR7\(^-\) effector memory cells (Supplementary Fig. S1). We then evaluated the relative proportions of Treg expressing CXCR3, CCR4 or CCR6, by co-staining with specific mAb. Similar to healthy donors, circulating memory Treg (MTreg) from patients contained in average 25% CXCR3\(^+\) cells (Fig. 2A and B). Strikingly, we found that the majority of MTreg in TIL were CXCR3\(^+\). The proportion of CXCR3\(^+\) MTreg was lower in TAL than in TIL, but was, in average, significantly higher than in circulating CD4\(^+\) T cells. It is noteworthy that CXCR3\(^+\) cells were also highly enriched in CD25\(^-\) conventional memory CD4\(^+\) T cells (Mconv) from TAL and TIL as compared to circulating CD4\(^+\) T cells. In contrast, the proportion of MTreg expressing CCR4 in TIL was lower as compared to circulating CD4\(^+\) T cells and TAL (Supplementary Fig. S2A). The proportion of MTreg expressing CCR6 in TAL and TIL was also lower than in circulating CD4\(^+\) T cells (Supplementary Fig. S2B). Thus, CXCR3\(^+\) Treg, and not CCR4\(^+\) Treg or CCR6\(^+\) Treg, were enriched in T lymphocytes infiltrating ovarian carcinomas.
**CXCR3⁺ Treg in ovarian tumors express T-bet but do not secrete IFN-γ and are highly suppressive ex vivo**

Expression of CXCR3 in T helper 1 (T_H1) is associated to that of the transcription factor T-bet (10). To assess if, and to what extent, FOXP3⁺ Treg expressing CXCR3 also expressed T-bet, we performed a combined analysis of CD25, CD127, FOXP3, T-bet and CXCR3 expression in TIL and TAL samples, by combined surface and intranuclear staining with specific mAb. As expected, we found that the large majority of CD25⁺CD127⁻ cells in TIL and TAL were FOXP3⁺ (Supplementary Fig. S3A) whereas very low proportions of FOXP3⁺ cells were contained in the CD25⁻ population. The proportions of CXCR3⁺FOXP3⁺ cells in TAL and TIL (Supplementary Fig. S3B) were therefore similar to those of CXCR3⁺CD25⁺CD127⁻ cells (Fig. 2). CXCR3⁺ MTreg in ovarian tumors expressed T-bet ex vivo (Fig. 3A). The expression levels of T-bet in tumor-associated CXCR3⁺ MTreg were similar to those found in the co-existing CXCR3⁺ Mconv populations and were higher in TIL than in TAL (Fig. 3B). FOXP3 and T-bet, however, control distinct transcriptional programs that can result in opposite effects. Namely, whereas T-bet directs IFN-γ secretion, FOXP3 instead suppresses IFN-γ production. To address the functional consequences of FOXP3 and T-bet co-expression in tumor Treg, we stimulated CD4⁺ T cells isolated from tumors ex vivo with PMA and ionomycin and assessed the secretion of IFN-γ with respect to the expression of FOXP3 and T-bet. As illustrated in Figure 4A, Mconv in the tumors contained large proportions of IFN-γ-secreting cells ex vivo. However, tumor-associated FOXP3⁺ Treg, despite high expression of T-bet, failed, for the largest part, to secrete IFN-γ. To further assess CXCR3⁺ Treg functionally, we isolated them ex vivo, from PBMC and OC tumors, by flow cytometry cell sorting, and stimulated them.
in the presence of CFSE-labeled responder CD4+ T cells. As shown in Figure 4B and C, CXCR3+ Treg from PBMC and OC tumors suppressed proliferation and IFN-γ production of stimulated responder T cells as efficiently as CXCR3- Treg. In addition, it is noteworthy that CXCR3+ Treg suppressed proliferation of both CXCR3+ and CXCR3- responder T cells (Supplementary Fig. S4). Together, these results indicate that CXCR3+ FOXP3+ CD4+ T cells that accumulate at sites of ovarian carcinomas have full functional characteristics of Treg, as they do not secrete IFN-γ and exert vigorous suppressive functions ex vivo.

**CXCR3+ Treg co-express the natural Treg marker Helios ex vivo and are efficiently generated from natural naïve Treg stimulated under Th1 priming conditions**

FOXP3+ Treg include natural Treg, generated as such in the thymus, and adaptive Treg, generated in the periphery from conventional CD4+ T cells after exposure to Ag (20). The two subsets have been long considered as phenotypically identical, but it has been recently inferred that expression of Helios, a transcription factor of the Ikaros family, can distinguish natural from adaptive Treg (21). To get insight into the origin of Treg in ovarian tumors, natural or adaptive, we assessed expression of Helios in tumor Treg by co-staining CD4+ T cells isolated ex vivo from tumors with FOXP3 and Helios specific mAb. This revealed that the large majority of FOXP3+ Treg in TAL and TIL were Helios+ (Fig. 5A). Co-staining with CXCR3, FOXP3 and Helios specific mAb confirmed high expression of Helios in CXCR3+ Treg in TAL and TIL (Fig. 5B) suggesting that the latter are, for the most part, natural Treg. To further address if tumor-associated CXCR3+ Treg may originate from natural Treg, we isolated NTreg, a population of human natural naïve
(CD45RA+CCR7+) FOXP3+ Treg, that we have previously identified (15) from CD4+ T cells of healthy donors, ex vivo by cell sorting (Fig. 6A), and stimulated them, along with naïve conventional CD4+ T cells (Nconv), under neutral (IL-2 only) or Th1 polarizing conditions, in the presence of IFN-γ and IL-12, alone or in combination. As summarized in Figure 6B, expression of CXCR3 was induced in a large fraction of NTreg and in a lower fraction of Nconv by stimulation in the presence of IL-2, was increased in Nconv by IFN-γ and was increased in both NTreg and Nconv by IL-12, alone or with IFN-γ. Expression of T-bet was induced by IL-12 alone or with IFN-γ, but not by IFN-γ alone, in NTreg and Nconv. It is noteworthy that a large fraction of NTreg stimulated in the presence of IL-12 alone or with IFN-γ remained FOXP3+ and Helios+ and acquired T-bet, but remained IFN-γ- (Fig. 6C) and suppressive (Fig. 6D). Thus, stimulation of NTreg under Th1 conditions resulted in the efficient induction of CXCR3+ Treg, similar to those found in ovarian tumors, namely co-expressing high levels of FOXP3, T-bet and Helios, unable to secrete IFN-γ and endowed with suppressive capacity.

The proportion of CXCR3+ Treg in ovarian carcinomas is directly related to that of CXCR3+ T effectors and is consistent with the expression of CXCR3 ligands

Expression of CXCR3 in tumor MTreg suggested that this population is chemoattracted at tumor sites associated with type I inflammation by CXCR3 ligands, to control type I T effectors. By assessing isolated CXCR3- and CXCR3+ Treg, we confirmed that, similar to Th1 cells, the latter efficiently migrate toward the CXCR3 ligand CXCL10 (Supplementary Fig. S5). It was therefore of interest to assess the presence of CXCR3+ MTreg in ovarian tumors in relation to that of CXCR3+ T effectors and of CXCL10. We found that the proportion of CXCR3+ MTreg in both TIL
and TAL was directly related to that of co-existing CXCR3+ Mconv (Fig. 7A), supporting the concept that these two populations are co-attracted at tumor sites by CXCR3 ligands. We obtained similar results by assessing the relationship between T-bet expression levels in MTreg and Mconv in TIL and TAL (Fig. 7A). We then assessed the expression of CXCL10, that is induced by IFN-γ, by conventional and qPCR, using specific primers. We found no significant expression of CXCL10 in normal ovarian tissue (Fig. 7B and C) but detected high expression in CD14+ monocytes and ovarian cancer cell lines after stimulation with IFN-γ and TNF-α. In addition, we detected significant expression of CXCL10 in the majority of ovarian tumors both in solid tumor masses (11/12) and in ascitis-associated cells (13/15). Thus, expression of CXCR3 ligands in ovarian tumors, induced by type I cytokines, was consistent with the recruitment of CXCR3+ T effectors and Treg at tumor sites. It is noteworthy that we detected expression of the CCR4 ligand CCL22 only in a minority of samples (3/15 ascites and 1/12 tumors, Supplementary Fig. S6A and B).
Discussion

FOXP3+ Treg have emerged as potent immunosuppressive cells in cancer, raising considerable expectations about the possibility to therapeutically target them to boost anti-tumor immunity. Recently, however, it has become increasingly appreciated that Treg are a complex population, composed, similar to conventional CD4+ T cells, of various subpopulations that differentiate and migrate in response to signals from the immune milieu to exert specialized functions (22). For example, Treg expressing CD103 and CCR4 migrate to and are retained in the skin, and deletion of these molecules in Treg results in the development of skin-specific autoimmunity (23, 24). Loss of CCR7 prevents Treg migration to the lymph nodes and impairs their protective function in experimental colitis (25) and expression of CCR6 by Treg is indispensable for their recruitment at sites of Th17 mediated inflammation in experimental autoimmune encephalomyelitis (26). Expression of IRF4, involved in controlling Th2 differentiation and IL-4 production, has been shown to be required for Treg-mediated control of Th2-type inflammation (8). Similarly, it has been shown that Treg expressing CXCR3 and T-bet accumulate at sites of Th1-type inflammation and that T-bet-deficient Treg cells fail to control IFN-γ-producing Th1 cells when transferred into FOXP3-deficient scurfy mice (10). From these studies, a model is emerging in which Treg subsets expressing Th lineage-specific chemokine receptors and transcription factors specifically suppress the corresponding Th subset, likely through complex mechanisms involving differential modulation of migration, homeostasis and function. An important implication of this concept in cancer is that the subset of Treg that suppress Th1 type immune responses, associated with efficient anti-tumor responses, should be specifically targeted to enhance anti-tumor immunity.
A previous study from Curiel et al. has documented the accumulation of Treg in human ovarian cancer along with their association with poor clinical outcome (3). In support of the relevance of Treg in ovarian tumors, we found highly increased proportions of Treg in TIL as compared to the periphery. The frequency of Treg in TAL, however, was lower and, in our sample group, not significantly different from the periphery. A major finding of our study is that CXCR3⁺ Treg are the prevalent subset of tumor-infiltrating FOXP3⁺ Treg in ovarian cancer. Namely, whereas CXCR3⁺ Treg constitute, in average, one of four circulating memory Treg, they are significantly enriched in TAL and represent the large majority of Treg in TIL ex vivo, indicating that this particular Treg subset selectively accumulates in ovarian tumors. The study from Curiel et al. reported that tumor-associated Treg express CCR4 and accumulate in ovarian tumors attracted by the CCR4 ligand CCL22, produced by ovarian cancer cells and tumor-associated macrophages. We found, however, lower proportions of CCR4⁺ than CXCR3⁺ Treg in ovarian tumors, particularly in TIL, and less frequent expression of CCL22 than of CXCL10.

Along with increased proportions of CXCR3⁺ Treg in ovarian tumors, we found high proportions of CXCR3⁺ Mconv, supporting the concept that ovarian tumors are predominantly associated with a T₇₁ type inflammatory milieu. A recent study using a murine model of lethal infection with *Toxoplasma gondii* has shown that the strong T₇₁ environment triggered by the infection impaired Treg through several mechanisms, including their conversion into T₇₁-like effectors, expressing T-bet and secreting IFN-γ (27). We found, however, that tumor-associated CXCR3⁺ Treg express high levels of T-bet but do not secrete IFN-γ (or other inflammatory cytokines, not shown) ex vivo and strongly inhibit proliferation and IFN-γ production of Tconv, demonstrating that they fully maintain their suppressive capacity in the
ovarian cancer environment. It is noteworthy that an important role in the subversion of Treg during *Toxoplasma gondii* infection is played by decreased production of IL-2, that is crucial for Treg homeostasis. We found, however, that tumor-associated Mconv contain significant proportions of cells secreting IL-2 (not shown), which may explain the ability of Treg to survive and maintain their phenotype in the ovarian cancer environment.

The finding that CXCR3+ T-bet+ Treg are the main Treg subset in ovarian tumors prompted us to address their origin, from natural Treg, generated in the thymus, or adaptive Treg, generated in the periphery from Tconv. Previous studies addressing the origin of tumor-associated Treg in mouse models have suggested that both Treg types can contribute to tumor-specific tolerance (28-30). Analysis of the TCR repertoires of tumor-infiltrating Treg and Tconv in murine carcinogen-induced tumors, however, has revealed a minor overlapping between the two TCR repertoires, suggesting that Treg and Tconv arise from different populations with unique TCR repertoires (31). In addition, in support of the concept that most Treg in tumors are thymically-derived, in a mouse model of glioblastoma multiforme, thymectomy prior to tumor implantation highly decreased the number of Treg infiltrating brain tumors (32). The relative contribution of the natural and adaptive subsets to Treg populations infiltrating human cancers has remained unexplored, mainly because of the lack of specific markers that distinguish them. Recently, however, expression of Helios, a transcription factor of the Ikaros family, has been proposed to differentiate thymically-derived from peripherally-induced Treg (21). We found that tumor CXCR3+ Treg express high levels of Helios, suggesting that they are natural Treg. In line with this conclusion, we found that stimulation of NTreg under Th1 conditions results in the efficient induction of CXCR3 and T-bet. In
addition, similar to Treg in ovarian cancers, a large proportion of NTreg differentiated under T_h1-polarizing conditions were FOXP3^+ and Helios^+, did not secrete IFN-γ and were highly suppressive. Together, these data are in line with the previous conclusions from the study of Koch et al. that CXCR3^+ Treg controlling type I inflammatory responses are derived from CXCR3^− T-bet^− FOXP3^+ natural Treg precursors and not peripherally-induced from naïve CD4^+ FOXP3^− T cells (10). Lately, however, it has been argued that expression of Helios may also be induced following activation by Ag, depending on specific conditions (33-35). Thus, although we did not observe significant induction of Helios or FOXP3 expression in Nconv in our in vitro differentiation system, additional studies comparing the TCR repertoire or/and the antigen specificity of Treg and Tconv in ovarian tumors will be of interest, to further support the conclusion that tumor-associated Treg originate from natural Treg.

We found that both CXCR3^+ Treg and CXCR3^+ Tconv were enriched at ovarian tumor sites and, consistently, detected frequent expression of the CXCR3 ligand CXCL10 in ovarian tumors. Together, our data depict a scenario in which CXCR3^+ Treg migrate and accumulate in ovarian tumors in response to type I inflammation, to control it, which collaterally results in the limitation of type I anti-tumor immunity. It is noteworthy that, in line with our findings, a study from Wolf et al., that independently of Curiel et al. (3) confirmed the correlation between FOXP3 expression and survival in ovarian cancer, also found FOXP3 expression, unexpectedly, to positively correlate with expression of T_h1-associated factors, and in particular the IFN-γ-regulated gene IRF-1 (4). Whereas the same group had previously documented a positive correlation between high IFN-γ expression and survival in ovarian cancer (2), the study showed that, in patients with high IFN-γ expression, a subgroup with
concomitant high FOXP3 expression was characterized by a significantly inferior overall survival.

Together, the findings reported in the present study confirm the relevance of Treg in human ovarian cancers. However, they also make it indispensable to modify the current paradigm concerning this population, particularly with respect to approaches aimed at interfering with Treg-mediated suppression of anti-tumor immunity (36). First, our data showing that CCR4+ Treg are less frequent than CXCR3+ Treg, particularly in solid tumor masses, make it unlikely that CCL22 blockade alone, as proposed (3, 6) would suffice to prevent the recruitment of Treg in ovarian tumor tissues. Targeting CXCR3 would simultaneously prevent the recruitment of T effectors in tumors and would therefore be equally inadequate. Second, the finding that the large majority of Treg in ovarian tumors are Helios+, and therefore could mostly correspond to natural and not to induced Treg, has also several implications. Indeed, if tumor Treg are mostly natural Treg, approaches aimed at interfering with peripheral conversion of Tconv into Treg, such as those targeting for example the indoleamine-2,3-dioxygenase (IDO) pathway, would not give the expected results, although the same pathways may also affect natural Treg (36-38). On the other hand, because natural Treg are believed to be self-reactive and should be distinct from tumor-specific T effectors in terms of TCR repertoire and antigen specificity, combined approaches aimed at depleting Treg concomitant with elicitation or reinforcement of tumor antigen-specific T cell responses with highly immunogenic cancer vaccines (39-41), could be particularly promising in ovarian cancer. Finally, further assessment of tumor-associated CXCR3+ Treg may reveal specific “Achilles’ heels” of this population that will, hopefully, help the selection of
new approaches for their therapeutic targeting to improve the clinical outcome of patients with ovarian cancers.
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References


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**Figure legends**

**Figure 1.** CD25+CD127- Treg accumulate in ovarian tumors. CD4+ T cells isolated from healthy donors’ (HD) and ovarian cancer (OC) patients' PBMC as well as from OC TAL and TIL were stained with anti-CD4, anti-CD25 and anti-CD127 mAb and analyzed by flow cytometry. CD25- conventional CD4+ T cells (Tconv) and CD25+CD127- Treg were analyzed as shown in the representative dot plot corresponding to CD4+ T cells from TAL. Data corresponding to the percentage of Treg in CD4+ T cells from HD PBMC (n=15), OC PBMC (n=15), OC TAL (n=17) and OC TIL (n=12) are shown. Statistical analyses were performed using a two-tailed t-test. ***P < 0.001.

**Figure 2.** MTreg in ovarian tumors are highly enriched in CXCR3+ cells. CD4+ T cells isolated from OC patients’ PBMC as well as from OC TAL and TIL were stained with CD4, CD45RA, CCR7, CD25, CD127 and CXCR3 specific mAb and analyzed by flow cytometry. A, Dot plots gated on memory (CD45RA-CCR7+/- and CD45RA+CCR7-) CD4+ T cells show CXCR3 expression with respect to that of CD25 in representative PBMC, TAL and TIL samples. B, The proportions of CXCR3+ cells in memory CD25+CD127- Treg (MTreg) and memory conventional CD25-CD4+ T cells (Mconv) from OC patients’ PBMC (n=14) and OC TAL (n=16) and TIL (n=12) are summarized in box and whiskers plots. Statistical analyses were performed using a two-tailed t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 3.** OC CXCR3+ FOXP3+ MTreg express the Th1 lineage-specific transcription factor T-bet ex vivo. CD4+ T cells isolated from TAL and TIL were stained with
CD45RA, CCR7, CD25, CD127, FOXP3, CXCR3 and T-bet specific mAb and analyzed by flow cytometry. A, Left, dot plot for one representative TIL sample showing FOXP3 and CXCR3 expression gated on total memory CD4+ T cells. Right, expression of T-bet in the indicated populations, defined according to the expression of CXCR3 and FOXP3. B, Mean fluorescence intensity (MFI) of T-bet staining in CXCR3+ and CXCR3− MTreg (FOXP3+ CD25+ CD127+) and Mconv (FOXP3− CD25−) populations is summarized for all PBMC (n=4), TAL (n=4) and TIL (n=4) assessed. Statistical analyses were performed using a two-tailed t-test. *P < 0.05, **P < 0.01.

Figure 4. OC FOXP3+ T-bet+ MTreg do not produce IFN-γ and are highly suppressive ex vivo. A, CD4+ T cells from OC TAL and TIL were stimulated for 6 hours with PMA and ionomycin, stained with CD4, FOXP3, CD127, IFN-γ and T-bet specific mAb and analyzed by flow cytometry. IFN-γ production is shown for a representative TIL with respect to FOXP3 expression gated on total CD4+ cells in the left dot plot and with respect to T-bet expression gated on FOXP3+ and FOXP3− cells, as indicated, in the middle and right dot plots. The MFI of T-bet staining and the proportions of cells producing IFN-γ in MTreg and Mconv from PBMC (n=6), TAL (n=15) and TIL (n=10) are summarized in the box and whiskers plots. Statistical analyses were performed using a two-tailed t-test. ***P < 0.001. B, CD4+ T cells from PBMC (n=3), TAL (n=3) and TIL (n=1) were stained with CD4, CD45RA, CCR7, CD25, CD127 and CXCR3 specific mAb, CXCR3+ and CXCR3− MTreg and total Mconv populations were sorted by flow cytometry and co-cultured with CFSE-labeled conventional CD4+ T cells, at the indicated suppressor-to-responder cell ratios, in the presence of irradiated monocytes and PHA. CFSE-dilution was assessed in day 5 cultures by flow cytometry. Histograms show CFSE-dilution in the absence or
presence of the indicated test populations at 1:1 ratio (numbers in histograms correspond to the percentage of divided cells) and percent suppression, calculated as detailed in the Methods section, is shown for all test populations and ratios assessed. Statistical analyses were performed using a two-tailed t-test to compare, at each suppressor-to-responder cell ratio, CXCR3⁺ and CXCR3⁻ MTreg. C, IFN-γ was measured by ELISA in day 5 supernatants of cultures assessed as in B. Percent suppression of responder cells’ IFN-γ production by the indicated MTreg populations was calculated as detailed in the Methods section.

**Figure 5.** OC CXCR3⁺ Treg express the transcription factor Helios ex vivo. A, CD4⁺ T cells from OC TAL and TIL were stained with CD4, FOXP3 and Helios specific mAb and analyzed by flow cytometry. Expression of FOXP3 and Helios in one TIL is shown in the dot plot gated on CD4⁺ T cells and the proportions of Helios⁺ cells in FOXP3⁺ and FOXP3⁻ CD4⁺ T cells from all TAL (n=15) and TIL (n=10) assessed are summarized in the box and whiskers plot. B, CD4⁺ T cells from TAL and TIL were stained with CD4, CD45RA, CCR7, CXCR3, CD127, FOXP3 and Helios specific mAb and analyzed by flow cytometry. Examples of dot plots showing expression of Helios and CXCR3 gated on FOXP3⁺ and FOXP3⁻ populations, as indicated, are shown for one TIL. The proportions of Helios⁺ cells in CXCR3⁺ MTreg from TAL (n=4) and TIL (n=5) are shown. Statistical analyses were performed using a two-tailed t-test. ***P < 0.001.

**Figure 6.** CXCR3⁺ MTreg can be generated from NTreg in vitro by stimulation under Th1-polarizing conditions. A, CD4⁺ T cells from HD PBMC were stained with CD4, CD45RA, CCR7, CD25 and CD127 specific mAb and naïve (CD45RA⁺CCR7⁺) CD4⁺
T cells were sorted into NTreg and Nconv populations according to CD25 and CD127 expression as shown in the dot plot. An aliquot of CD4+ T cells was additionally stained with anti-FOXP3 mAb. Expression of FOXP3 gated on NTreg and Nconv is shown in the overlaid histogram plots. B, Sorted populations were stimulated in vitro with anti-CD2/3/28-coated beads in the absence or presence of IL-12 and IFN-γ, alone or in combination, and cultured in the presence of IL-2. Aliquots of day 12 cultures were either stained with anti-CXCR3 mAb or stimulated with PMA and ionomycin for 4 h and stained with anti-T-bet mAb and analyzed by flow cytometry. CXCR3 and T-bet expression was also assessed ex vivo in the sorted populations. The proportions of cells expressing CXCR3 and the MFI of T-bet staining are shown for all populations, ex vivo (↓) and in the indicated culture conditions (n=4; mean ± SEM). na = non applicable. C, Cultures obtained as in B were stimulated in the presence of PMA and ionomycin, stained with FOXP3, Helios, T-bet and IFN-γ specific mAb and analyzed by flow cytometry. The proportions of FOXP3+ and Helios+ cells are shown for all populations, ex vivo (↓) and in the indicated culture conditions (n=4; mean ± SEM). The proportions of IFN-γ-producing cells and the MFI of T-bet staining of Helios+ or Helios- FOXP3+ cells in NTreg cultures are summarized (n=4; mean ± SEM). D, The suppressive capacity of cells in the cultures obtained as in B was assessed, as in Figure 4B, by co-culture with CFSE-labeled responder CD4+ T cells. Percent suppression is summarized for all donors (n=3; mean ± SEM) at 1:1 suppressor-to-responder cell ratio for all culture conditions (middle) and at the indicated suppressor-to-responder cell ratios for cells stimulated in the absence or presence of IL-12 (right). Statistical analyses were performed using a two-tailed t-test.
**Figure 7.** The proportion of CXCR3⁺ Treg correlates with that of CXCR3⁺ T effectors in OC tumors and is consistent with CXCL10 expression. A, Scatter plots and linear regression of the correlation between the proportion of CXCR3⁺ cells in MTreg and Mconv as well as the MFI of T-bet staining in MTreg and Mconv from both TIL and TAL. B and C, CXCL10 mRNA expression was assessed by conventional PCR (B) and qPCR (C) in normal ovarian tissue, in OC tumors and ascitis-associated cells, as well as in CD14⁺ cells from HD PBMC and OC tumor cell lines (SK-OV-3 and SK-OV-6), treated or not with IFN-γ and TNF-α, as indicated.
Figure 1
Figure 2
Figure 5

A

B

FOXP3

Helios

FOXP3

Helios

CXCR3

% Helios+

CXCR3

% Helios+

ns

ns

NS

NS
Figure 7

A

B

C

CXCL10 (298 bp)

ACTB (285 bp)

Controls

Tumor

Ascites