CD25 Identifies a Subset of CD4⁺FoxP3⁻ TIL That Are Exhausted Yet Prognostically Favorable in Human Ovarian Cancer

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Abstract

CD25, the alpha subunit of the IL2 receptor, is a canonical marker of regulatory T cells (Treg) and hence has been implicated in immune suppression in cancer. However, CD25 is also required for optimal expansion and activity of effector T cells in peripheral tissues. Thus, we hypothesized that CD25, in addition to demarcating Tregs, might identify effector T cells in cancer. To investigate this possibility, we used multiparameter flow cytometry and IHC to analyze tumor-infiltrating lymphocytes (TIL) in primary high-grade serous carcinomas, the most common and fatal subtype of ovarian cancer. CD25 was expressed primarily by CD4⁺ TIL, with negligible expression by CD8⁺ TIL. In addition to conventional CD25⁺FoxP3⁻ Tregs, we identified a subset of CD25⁺FoxP3⁻ T cells that comprised up to 13% of CD4⁺ TIL. In tumors with CD8⁺ TIL, CD25⁺FoxP3⁻ T cells showed a strong positive association with patient survival (HR, 0.56; P = 0.02), which exceeded the negative effect of Tregs (HR, 1.55; P = 0.09). Among CD4⁺ TIL subsets, CD25⁺FoxP3⁻ cells expressed the highest levels of PD-1. Moreover, after in vitro stimulation, they failed to produce common T-helper cytokines (IFNγ, TNFα, IL2, IL4, IL10, or IL17A), suggesting that they were functionally exhausted. In contrast, the more abundant CD25⁻FoxP3⁻ subset of CD4⁺ TIL expressed low levels of PD-1 and produced T-helper 1 cytokines, yet conferred no prognostic benefit. Thus, CD25 identifies a subset of CD4⁺FoxP3⁻ TIL, that, despite being exhausted at diagnosis, have a strong, positive association with patient survival and warrant consideration as effector T cells for immunotherapy. Cancer Immunol Res. 3(3):245–53. ©2014 AACR.

Introduction

Studies over the past decade have highlighted the strong influence of the immune system on the survival of patients with cancer. In particular, CD8⁺ tumor-infiltrating lymphocytes (CD8⁺ TIL) are associated with improved survival in virtually every human solid cancer studied, including ovarian cancer (1, 2). Importantly, however, CD8⁺ TIL do not act in isolation but rather through cooperative interactions with other immune cells (3). Thus, to enhance the beneficial effects of TIL, it is imperative to understand how different TIL subsets work together to mediate tumor immunity.

Gooden and colleagues (4) performed a meta-analysis of 52 studies investigating the prognostic significance of TIL expressing the T-cell markers CD3, CD8, CD4, and FoxP3. Intriguingly, CD3⁺ TIL showed a stronger prognostic effect than CD8⁺ TIL (pooled HRs of 0.58 and 0.71, respectively), suggesting that other CD3⁺ TIL, in particular CD4⁺ T cells, might contribute to the favorable prognosis. The prognostic influence of CD4⁺ TIL has been difficult to assess directly as they include both effector and regulatory subsets (5). Furthermore, CD4 is also expressed by macrophages in humans, thus complicating the histopathologic scoring (6). Owing to this complexity, CD4 as a standalone marker has shown no association with patient survival in ovarian cancer (7, 8).

CD4⁺ TIL have been divided into several distinct functional subsets based on cytokine secretion patterns and phenotypic markers (5). For example, IFNγ-producing CD4⁺ TIL (Th1 cells) have been identified in ovarian cancer (9), and Th1-like gene expression signatures have been reported as prognostically favorable in many cancers (1). More recently, IL17-producing CD4⁺ T cells (Th17 cells) have been identified in several cancers (1). The prognostic effect of Th17 cells appears to depend on tumor site, ranging from unfavorable in colorectal cancer (10) to favorable in ovarian and gastric cancer (11, 12). CD4⁺ TIL with a regulatory phenotype (Treg) are also prevalent in human cancers. Regulatory T cells (Treg) are commonly defined by coexpression of the IL2 receptor (IL2R) alpha subunit (CD25) and the transcription factor FoxP3. These cells can inhibit tumor immunity by a variety of mechanisms, including the production of immunosuppressive cytokines, depletion of extracellular ATP, and inhibitory cell contacts (13). Accordingly, Tregs have been associated with poor prognosis in ovarian cancer (8, 14–16) and many other cancer types (1, 17). Thus, CD4⁺ TIL represent complex mixtures of T-cell subsets with both positive and negative influences on tumor immunity.

Although IL2 can serve as a growth factor for virtually all T-cell subsets, Tregs exhibit the most obvious IL2 dependency in vivo.
This was revealed most strikingly when IL2- and IL2R-deficient mice were first generated. Rather than being immune compromised as many had expected, these animals develop a lethal, systemic autoimmune syndrome that is attributable to the loss of Tregs (18). Thus, CD25 serves not only as a marker of Tregs but also as a component of an essential developmental and homeostatic signaling pathway for these cells (18). Accordingly, several strategies have been developed to deplete Tregs in patients with cancer by targeting the IL2 pathway. For example, IL2 conjugated to diphtheria toxin has been used to deplete Tregs in vivo (19, 20). Tregs can also be depleted with antibodies to CD25 (21). Although these agents have been shown to reduce the number of circulating Tregs in patients with cancer, and improve responses to tumor-specific vaccines, this has generally not resulted in major antitumor effects. Indeed, in the settings of transplantation and autoimmunity, these agents have proven efficacious at inhibiting T-cell responses rather than reversing immune suppression (22).

Consistent with this, a closer examination of IL2- or CD25-deficient mice has revealed an important role for IL2 in the expansion of effector T cells in nonlymphoid tissues such as gut and lung epithelium (23, 24). Moreover, we found in a mouse model of advanced ovarian cancer that IL2 signaling, though dispensable for the initial expansion of CD8+ T cells, was essential for CD8+ T-cell responses in the tumor environment (25). Indeed, systemic IL2 infusion can have potent antitumor effects as a monotherapy and in the setting of adoptive immunotherapy (26). In ovarian cancer, intraepithelial IL2 administration yielded a 25% objective response rate in early-phase clinical trials (27, 28). Thus, the physiologic role of IL2 is highly context-dependent, and a better understanding of its role in the tumor microenvironment is needed if we are to rationally manipulate this signaling axis.

Toward this end, we evaluated the phenotype and prognostic significance of CD25+ TIL subsets in high-grade serous carcinoma (HGSC), the most common and lethal form of ovarian cancer. In accord with prior reports (8, 14–16), CD4+CD25+FoxP3+ Tregs were a prominent component of CD4+ TIL in many patients, and their presence trended toward decreased patient survival. Unexpectedly, we also identified a CD4+CD25+FoxP3+ TIL subset that had a highly exhausted phenotype yet was strongly associated with patient survival. Our results reveal a potentially beneficial role for CD4+CD25+FoxP3+ T cells in tumor immunity and provide new insights into immune modulatory strategies for HGSC and related malignancies.

Materials and Methods

Patient characteristics and biospecimens

All specimens and clinical data were obtained with either written informed consent or a formal waiver of consent under protocols approved by the Research Ethics Board of the British Columbia Cancer Agency (BCCA) and the University of British Columbia (Vancouver, British Columbia, Canada). All patients in this study were diagnosed with advanced stage HGSC. Survival analyses were performed with a previously described retrospective cohort comprised of 187 HGSC cases (Table 1; refs. 7, 29). Briefly, a tissue microarray (TMA) with 0.6-mm cores was constructed from formalin-fixed paraffin-embedded tumor samples obtained at the time of primary surgery from patients seen at the BC Cancer Agency from 1984 to 2000 (OvCaRe Ovarian Tumour Bank). Patients in this cohort were deemed optimally de-bulked, meaning they had no macroscopic residual disease.

Flow cytometry studies were performed with viable tumor and blood specimens collected at the time of primary surgery from previously untreated patients with HGSC admitted to BCCA from 2007 to 2010. Tumor tissue samples were mechanically disaggregated in RPMI media containing 0.5 μg/mL collagenase Type I (Sigma-Aldrich), 0.5 μg/mL collagenase Type IV (Sigma-Aldrich), 0.25 μg/mL hyaluronidase (Sigma-Aldrich), and 0.1 μg/mL DNase I (Sigma-Aldrich) and incubated for 12 hours at 4°C. Single-cell suspensions were prepared by passing digested tissue through a 100-μm filter. Cellular yield and viability were determined by flow cytometry, and aliquots of 1 × 106 cells were frozen in RPMI media containing 50% FBS (Fisher Scientific) and 10% DMSO (Sigma-Aldrich).

Flow cytometry

Tumor-cell suspensions were thawed at 37°C followed by washing and a 2-hour rest at 37°C in RPMI media (Life Technologies) containing 10% FBS (Fisher Scientific), L-glutamine (Life Technologies), sodium pyruvate (Life Technologies), β-mercaptoethanol (Sigma-Aldrich), and HEPES (Life Technologies). Cells were stained with fluorochrome-conjugated monoclonal antibodies to the following cell surface markers: CCR4, CCR6, CD3, CD4, CD8, CD25, CD127, CD357 (GITR), CTLA-4, CXCR3, CXCR5, LG-3, OX40, PD-1, and TIM-3 (Supplementary Table S1). To analyze cytokine production, bulk tumor preparations were stimulated with phosphor myristate acetate (PMA; 50 ng/mL, Sigma-Aldrich) and ionomycin (1 μmol/L, Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences) for 3 hours at 37°C. Cells were then fixed and permeabilized with pre-treatment solution (eBioscience) and stained with fluorochrome-conjugated monoclonal antibodies to IFNγ, TNFα, IL2, IL4, and IL17 (Supplementary Table S1). Analysis of FoxP3 and Helios was performed simultaneously with intracellular cytokine staining. For analysis of T-bet, GATA-3, and RORγt, unstained bulk tumor preparations were stained using the Transcription Factor Buffer Set (BD Biosciences) according to the manufacturer’s protocol. To generate positive controls for T-bet and GATA-3 staining, Th1 and Th2 lines were produced by stimulating healthy donor peripheral blood mononuclear cells (PBMC) for 5 days with anti-CD3 and anti-CD28 in the presence of IL2 (30 U/mL) + IL12 (20 ng/mL) or IL4 (10 μg/mL), respectively. Flow cytometry was performed using eight-channel Influx or FACSCalibur instruments (BD Biosciences), and data were analyzed with FlowJo software v10.0.7 (TreeStar Inc.).

IHC

Multicolor IHC was performed as previously described (30). Briefly, TMA sections were stained with pan-cytokeratin alone or the following antibody combinations: (i) CD25, CD8, and FoxP3 and (ii) CD3, CD8, and FoxP3 (Supplementary Table S1). All slides were deparaffinized, treated with Diva Decloaker in a Decloaking Chamber for antigen retrieval, and then blocked with Peroxidase-1 and Background Sniper. All staining was performed at room temperature for one hour for the primary antibodies and 30 minutes for secondary amplification. For the pan-cytokeratin stain, the primary signal was amplified using the MACH-2 Mouse-AP polymer Kit and visualized with Warp Red (10 minutes). The first antibody combination utilized anti-CD25 (clone 4C9) and anti-CD8α (clone SP16) antibodies, the MACH-2

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polymer kit Double Stain 1, and the chromogens Warp Red and Betazoid DAB, respectively. Slides were denatured at 50°C for 45 minutes to remove the primary and secondary antibodies (31). The anti-CD25 antibody (clone 4C9) was reapplied (to boost the signal) together with the anti-FoxP3 antibody (clone SP97), followed by the secondary polymer Kit MACH-2 Double Stain 2 and Warp Red and Vina Green, respectively. For the second antibody combination, we used the primary antibodies anti-CD3 (clone SP7) and anti-CD8a (clone C8/144B), the secondary polymer kit MACH-2 Double Stain 2, and Warp Red and Betazoid DAB, respectively. After denaturation, the second round of staining used anti-FoxP3 antibody (clone SP97), the secondary polymer MACH-2 Double Stain 2, and Vina Green. Where indicated, slides were counterstained with hematoxylin. Other than antibodies (Supplementary Table S1), all IHC reagents and equipment were obtained from Biocare Medical.

Image analysis and scoring

Images were captured using an Olympus BX53 microscope equipped with a motorized stage (Quorum Technologies Inc.) and the Nuance multispectral imaging system (CRI). Pan-cytokeratin–stained TMA cores were captured as a single image at 200× magnification. For cores stained with the CD8/CD25/ FoxP3 and CD3/CD8/FoxP3 combinations, each chromogen was scanned at its optimal wavelength and 400× magnification. Images were then combined electronically using MetaMorph software (Quorum Technologies Inc.). To aid the identification of intraepithelial TIL (as opposed to stromal lymphocytes), cytokeratin-positive tumor regions were overlaid on serial sections stained for TIL markers (Supplementary Fig. S1). To enumerate TIL, multispectral images were deconvoluted into individual components using Nuance software. Using Metamorph software (Quorum Technologies Inc.), images containing three chromogens were re-created and used to visually count cells with the indicated phenotypes.

Cases were considered positive for a given TIL subset if at least one cell was present per 0.6-mm core. The density of each intraepithelial TIL subset was calculated by normalizing cell counts by epithelial area as described. The density of intraepithelial TIL was inferred for the cohort by subtracting the mean number of CD8+/CD25−FoxP3− T cells seen with the CD25/CD8/FoxP3 IHC combination from the mean number of CD3+CD8−FoxP3+ T cells seen with the CD3/CD8/FoxP3 combination.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0. Survival analysis was performed using Kaplan–Meier plots and log-rank tests. Pearson correlations were used to assess associations between TIL subsets. The χ² analysis was used to assess the association of TIL subsets with MHC class II. P values of less than 0.05 were considered statistically significant.

Results

CD25 and FoxP3 define four subsets of CD4+ TIL.

To assess which populations of TIL express CD25, we performed multiparameter flow cytometry on disaggregated tumor samples from 12 patients with HGSC. CD25 was expressed by a small but significant proportion of CD3−TIL (mean, 12%; range, 3%–20%) and was not found on any non-T (CD3+) cells. The majority of CD25+ T cells were CD4+CD8− (mean, 84%; range, 78%–91%), and a small proportion were CD4+CD8− (mean, 3%; range, 1%–7%; Fig. 1A and D). The remaining CD25+ cells were CD4+CD8 or CD4+CD8+. Among the CD4+ TIL, CD25 was expressed by an average of 20% of cells (range, 4%–28%; Fig. 1B and D). To investigate whether these were Tregs, we stained for the transcription factor FoxP3. Consistent with prior reports (8, 14–16), cells with a canonical Treg phenotype (CD25+FoxP3+) constituted a significant fraction of CD4+TIL (mean, 13%; range, 3%–25%) and are hereafter referred to as Tregs. Notably, a substantial fraction of CD25+ TIL did not express FoxP3 (mean, 7%; range, 2%–13%; Fig. 1C and D). The remaining CD4+ TIL were CD25+FoxP3− (mean, 7%) or CD25−FoxP3+ (mean, 74%; Fig. 1C and D). Among CD8+ TIL, only a small percentage expressed CD25 (mean, <1%), FoxP3 (mean, 2%), or both (mean, <1%; data not shown). Similar T-cell subsets were found in peripheral blood samples from healthy donors except that, in line with the findings of others (32), CD4+CD25+FoxP3− T cells were rare and CD4+CD25+FoxP3+ T cells were absent (data not shown).

Prognostic significance of TIL subsets

To investigate the prognostic significance of TIL subsets, a 187-case HGSC TMA was stained with antibodies to CD8, CD25, and FoxP3 (Fig. 2A). Because CD4 is also expressed by macrophages (6), it is difficult to score CD4+TIL directly. Instead, we assumed that any CD25+ and/or FoxP3+ cell that did not express CD8 was a CD4+ T cell, an assumption that was justified by the flow cytometry results shown in Fig. 1. With this staining combination, we could directly visualize all CD8+ TIL subsets, as well as the CD25+FoxP3−, CD25+FoxP3+, and CD25−FoxP3− subsets of CD4+ TIL. To infer the number of CD4+CD25+FoxP3+ TIL, we employed a second IHC combination involving antibodies to CD3, CD8, and FoxP3. We determined the number of CD4+FoxP3− cells, which appeared as CD3−CD8+FoxP3− cells. From this, we subtracted the number of CD25+FoxP3− cells determined from the first staining combination. This yielded an estimate of the number of CD4+CD25−FoxP3− cells. Our detection and scoring approach proved valid, as the results obtained by multicolor IHC and flow cytometry were concordant (Fig. 1D and E).

We stained an adjacent section of the TMA for cytokeratin to unequivocally identify tumor epithelium versus stroma; for all subsequent analyses, we focused on intraepithelial TIL, as these have the greatest prognostic significance (2).

A large proportion of cases (74%) scored positive for CD8+ TIL (Fig. 2B), and consistent with the flow cytometry data, only a small number of CD8+ TIL expressed CD25 and/or FoxP3 (data not shown). Over 90% of cases with CD8+ TIL also had CD4+ TIL.
CD3 + CD8 -/CD3 + CD8 + cells); conversely, almost all cases with CD4 + TIL also had CD8 + TIL (Fig. 2B). Thus, CD8 + and CD4 + TIL were strongly associated with one another. Tregs (defined as CD8 -/CD3 + CD25 + FoxP3 + cells) were the most prevalent subset of CD4 + TIL, being present in 67% of cases (Fig. 2B). The CD25 + FoxP3 - and CD25 + FoxP3 + subsets were present in 55% and 43% of cases, respectively (Fig. 2B). Thus, the different subsets of CD4 + TIL were usually found together, although there were exceptions. All subsets of CD4 + TIL showed a strong association with MHC class II expression by tumor cells (determined in a previous study of this cohort; ref. 7; P < 0.01 for all subsets).

As expected, CD8 + TIL were strongly associated with disease-specific survival (HR, 0.33; P < 0.0001; not shown) and progression-free survival (HR, 0.38; P < 0.0001; Fig. 3A). The strong prognostic effect of CD8 + TIL can confound the analysis of closely associated TIL subsets, such as Tregs (30). Therefore, to determine the prognostic significance of CD4 + TIL subsets, we restricted our

**Figure 1.**

CD25 and FoxP3 define four subsets of CD4 + TIL. Multiparameter flow cytometry was performed on bulk disaggregated tumor samples from 12 patients with HGSC, and data for one representative case are shown. A, CD25 + TIL predominantly consisted of CD3 + CD4 + T cells. B, approximately 20% of CD3 + CD4 + TIL expressed CD25. C, within the population of CD3 + CD4 + TIL, expression of CD25 and FoxP3 defined four subsets, indicated by the quadrants. D, average proportions of the four CD4 + TIL subsets (± SEM) for the 12 cases analyzed by flow cytometry. E, average proportions of the four CD4 + TIL subsets (± SEM) for 181 evaluable cores of the TMA analyzed by multicolor IHC. For flow cytometry experiments in plots A to D, events were gated on live cells with forward and side scatter characteristics of lymphocytes. The numbers shown indicate the percentage of events falling within an indicated gate.

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**Figure 2.**

Analysis of TIL subsets by multicolor IHC and spectral image analysis. A 187-case TMA was triple stained with antibodies to CD8, CD25, and FoxP3 and counterstained with hematoxylin. A, brightfield (upper left plot) and pseudo-colored images from a representative tumor core showing examples of four TIL subsets: (a) CD8 -/CD3 + CD25 + FoxP3 +, (b) CD8 +/CD3 + CD25 - FoxP3 -, (c) CD8 +/CD3 + CD25 + FoxP3 -, and (d) CD8 -/CD3 + CD25 + FoxP3 -. Magnification, ×400. B, Venn diagram showing the distribution of the five indicated TIL subsets, with numbers indicating the percentage of cases scoring positive for each TIL subset.
analysis to cases that were positive for CD8$^+$ TIL. As expected, cases with a higher than median ratio of Tregs to CD8$^+$ TIL trended toward decreased survival (HR = 1.55; \(P = 0.09\); Fig. 3B). In contrast, a higher than median ratio of CD25$^+$FoxP3$^-$ to CD8$^+$ TIL was strongly associated with survival (HR = 0.85; \(P = 0.02\); Fig. 3C). The prognostic effect of CD25$^+$FoxP3$^-$ TIL was even more pronounced in cases with lower levels of Tregs (HR = 0.29; \(P = 0.003\); Fig. 3D). No other CD4$^+$ TIL subsets showed an association with survival (Fig. 3E and F). Thus, the CD25$^+$FoxP3$^-$ subset was unique among CD4$^+$ TIL in showing a positive association with patient survival.

CD25$^+$Foxp3$^-$ T cells are phenotypically distinct from other TIL subsets

To better understand the prognostic effect of CD25$^+$Foxp3$^-$ TIL, we used multicolor flow cytometry to assess their activation status and cytokine production profile in comparison with other CD4$^+$ TIL subsets (\(n = 6\) cases; Fig. 4A). CD25$^+$Foxp3$^-$ TIL had an activated phenotype as evidenced by high CD69 and low CCR7 expression (Supplementary Fig. S2B). Similar to Tregs, CD25$^+$Foxp3$^-$ TIL expressed low levels of CD127 and moderate levels of GITR (Supplementary Fig. S2C). However, they expressed lower levels of CITA-4 and OX40 than did Tregs and were negative for Helios (Fig. 4B). These data suggested that CD25$^+$Foxp3$^-$ cells might be Th1 cells, which are widely reported among TIL (1). However, CD25$^+$Foxp3$^-$ T cells expressed only low levels of the Th1 transcription factor T-bet (Supplementary Fig. S3B) and failed to produce any of the hallmark Th1 cytokines IFN\(\gamma\), TNF\(\alpha\), or IL2 after \textit{in vitro} stimulation with PMA and ionomycin (Fig. 4C).

Indeed, Th1 cytokines were only produced by the CD25$^+$Foxp3$^-$ subset (Fig. 4C). None of the CD4$^+$ TIL subsets produced IL4 or IL17A (Supplementary Fig. S2D), and the expression of transcription factors GATA-3 (Th2) and ROR\(\gamma\) (Th17) was restricted to minor subsets of CD4$^+$CD25$^+$Foxp3$^-$ cells (Supplementary Fig. S3D and S3F, and data not shown). All four CD4$^+$ TIL subsets expressed CXCR3 (Supplementary Fig. S3C), which is typical of T cells in inflammatory environments.

Given that CD25$^+$Foxp3$^-$ T cells did not express canonical Th cytokines, we investigated the possibility that they might represent other less common CD4$^+$ T-cell phenotypes. CD4$^+$ cytolytic T cells have been described in cancer (33); however, similar to other non-Tregs, very few CD25$^+$Foxp3$^-$ T cells expressed the cytolytic markers TIA-1, granzyme B, or perforin (Supplementary Fig. S2E). Furthermore, CD25$^+$Foxp3$^-$ TIL did not express CXCR5, a marker of T follicular helper cells (Supplementary Fig. S2F).

On the basis of their lack of discernible functional attributes, we hypothesized that CD25$^+$Foxp3$^-$ TIL might be in a suppressed or exhausted state. In accord with this, we found that CD25$^+$Foxp3$^-$ T cells expressed very high levels of the exhaustion marker PD-1 (Fig. 4D). Indeed, the level of PD-1 expressed by CD25$^+$Foxp3$^-$ TIL was on average 3.1-fold higher than that by Tregs, and 6.6-fold higher than that by CD25$^+$Foxp3$^-$ cells. In addition, CD25$^+$Foxp3$^-$ TIL expressed the exhaustion markers LAG-3 (Supplementary Fig. S2G) and TIM-3, the latter being found on cells with the highest PD-1 levels (Fig. 4D). Thus, despite being prognostically favorable, CD25$^+$Foxp3$^-$ TIL exhibited an exhausted phenotype based on the expression of these markers and deficient cytokine production.
Discussion

Using multiparameter flow cytometry and IHC, we have shown that CD25 and FoxP3 delineate four subsets of CD4+ TIL with distinct functional and prognostic attributes. Consistent with prior reports (8, 14–16), CD25+FoxP3+ Tregs constituted a substantial proportion of CD4+ TIL, expressed canonical Treg markers (Helios, CTLA-4, and OX40), and showed a trend toward decreased patient survival. CD25+FoxP3− T cells constituted the largest subset of CD4+ TIL and were the sole source of Th1 cytokines; however, despite their abundance and functional competence, these cells conferred no apparent prognostic benefit. Instead, the CD25+FoxP3− subset showed a strong association with patient survival, despite expressing very high levels of PD-1 and failing to produce cytokines. This paradoxical relationship between functional status and prognosis might be explained by the fact that PD-1 is a marker of tumor-reactive TIL (34, 35). Thus, the exhausted state of CD25+FoxP3− TIL observed here might reflect ongoing recognition of tumor antigens. Although speculative, this state of exhaustion might subsequently be relieved by the cytoreductive and immune stimulatory effects of surgery and chemotherapy (36), ultimately leading to increased patient survival. Thus, CD25+FoxP3− TIL warrant further investigation for their contribution to spontaneous tumor immunity as well as their potential to serve as effector cells for immunotherapy.

CD25+FoxP3− T cells have been largely overlooked in prior studies of CD4+ TIL, likely because of their low abundance and failure to make cytokines typical of Th1, Th2, Th17, or Tregs. Adding to their inconspicuous nature, these cells are very rare in PBMCs and hence would not be obvious in blood-based immune analyses. Nonetheless, cells with this phenotype are evident in published data of CD4+ TIL in ovarian (14, 37, 38) and other tumors (39). Moreover, their prognostic significance has been directly assessed in one prior study of HGSC. Preston and colleagues (8) used multicolor IHC to enumerate CD4+ CD25+FoxP3− TIL in patients with HGSC who had demonstrated long (>18 months) versus short survival. Although these cells were equally abundant in the two groups, the ratio of CD8+ to CD4+CD25+FoxP3− TIL was modestly higher in the long survival group (0.65 vs. 0.46), leading the authors to propose that CD25+FoxP3− TIL play an inhibitory role in tumor immunity. However, this interpretation could potentially reflect a confounding effect of CD8+ TIL. Specifically, cases exhibiting a low ratio of CD8+ to CD25+FoxP3− TIL were found only in cases with CD8+ TIL (Fig. 3F). Although an analysis to cases that were positive for CD8+ TIL, thereby allowing us to assess any additional prognostic effects contributed by the different CD4+ TIL subsets. The validity of this approach was supported by our finding, with few exceptions, that CD25+FoxP3− TIL were found only in cases with CD8+ TIL. With this correction, a strong positive prognostic effect of CD25+FoxP3− TIL was revealed.

To explain this positive effect, we propose that CD25+FoxP3− TIL might be tumor-reactive T-helper cells that have become functionally impaired or exhausted due to chronic antigen exposure. Indeed, their appearance on flow cytometric plots initially suggested to us that they might represent a CD25hi “shoulder” of the Th1-like CD25+FoxP3− subset. To address this, we assessed the prognostic significance of CD3+CD8+FoxP3− TIL, a grouping that included both the CD25+FoxP3− and CD25+FoxP3− subsets. CD3+CD8+FoxP3− TIL showed no prognostic significance, indicating that the positive effect of CD25+FoxP3− TIL was lost when combined with CD25+FoxP3− TIL (Fig. 3F). Although an
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indirect assessment, this indicates that CD25⁺ FoxP₃⁻ TIL have negligible (or possibly even negative) prognostic significance and that CD25⁺ FoxP₃⁻ TIL make a unique positive contribution to tumor immunity.

At first glance, the above findings may appear inconsistent with prior studies reporting that TIL with Th1, Th17, or other cytokine patterns are prognostically favorable in ovarian (40) and other cancers (1). However, many of these prior studies were based on the analysis of bulk tumor samples for expression of Th1-associated genes, such as IFNγ, TNFα, T-bet, IRF-1, and STAT4 (10, 40). Notably, these genes are also expressed by CD8⁺ T cells and natural killer cells; therefore, gene expression analysis of bulk tumor tissue does not reveal the prognostic influence of CD4⁺ Th1 cells per se. Using a flow cytometric approach, Kryczek and colleagues showed that production of IFNγ, IL2, TNFα, and IL17 by CD4⁺ TIL was restricted to CD25⁺ FoxP₃⁻, and PD-1⁺ cells and that IL17 levels in ascites fluid were associated with survival (37). In accord with their results, we found that cytokine production was restricted to the CD25⁺ FoxP₃⁻/PD-1⁺ subset; however, we failed to detect IL17 production by any CD4⁺ TIL subset. This might reflect a low abundance of Th17 cells in ovarian cancer (as others have also reported; refs. 38, 41), or inadequate sensitivity of our flow cytometric assay for IL17 production. Finally, two studies have used MHC class II tetramers specific for the tumor antigen NY-ESO-1 to isolate CD4⁺ T cells from peripheral blood (42) or tumors (9) of patients with ovarian cancer. NY-ESO-1–specific T cells were found to produce Th1-associated cytokines after in vitro stimulation. However, the Th1 competence of these T cells may reflect the fact that they were derived from peripheral blood, which has negligible CD25⁺ FoxP₃⁻ T cells, or expanded in vitro by phytohemagglutinin stimulation, which can reverse T-cell exhaustion. Our observation that CD25⁺ FoxP₃⁻ TIL express CXCR3 and low levels of T-bet suggests they might represent exhausted Th1 cells. Indeed, others have recently reported that T-bet expression is diminished in dysfunctional effector cells (43). Alternatively, CD25⁺ FoxP₃⁻ TIL could represent early activated Th1 cells that have not yet acquired robust T-bet expression or effector cytokine production, although one would not expect such cells to express multiple exhaustion markers. In summary, our data appear consistent with previous reports of Th1-like TIL in cancer but highlight the exhausted state of these cells at diagnosis.

Although CD25 is a well-established marker of Tregs, to our knowledge, this is the first report of CD25 expression by T cells with an exhausted phenotype. This observation may lead to further insights into the signaling state of CD25⁺ FoxP₃⁻ TIL, as the mechanisms of transcriptional regulation of the CD25 gene are relatively well characterized. The CD25 promoter has both positive and negative regulatory elements, which bind multiple factors, including NFAT, NF-kB, STAT5, and SMAD3/4 (44).

Transient CD25 expression is induced on virtually all T cells by activation of the T-cell receptor (TCR) and the downstream NFAT and NF-kB pathways (44). CD25 can also be induced by a variety of cytokines, including IL1, IL2, IL7, IL12, IL15, TNFα, and TGFβ (44). In particular, IL2 induces CD25 expression via STAT5 (44) and in combination with extensive antigen stimulation induces expression of Blimp-1, which also upregulates CD25 expression (18). Finally, CD25 is expressed at high, constitutive levels by Tregs through a combination of TCR signaling, IL2-induced STAT5 activation, and constitutive expression of FoxP3 (18). In the case of CD25⁺ FoxP₃⁻ TIL, this FoxP3-dependent mechanism would not apply. Rather, CD25 might be induced by antigen stimulation in combination with IL2, a cytokine for which they would be competitive consumers based on their CD25⁺ phenotype. Another candidate is TGFβ, which can induce CD25 (44) and is present in most epithelial tumors. In this regard, it is noteworthy that CD25⁺ FoxP₃⁻ cells expressed high levels of PD-1, which is also induced by TCR stimulation in combination with TGFβ (45). One paradoxical aspect of the phenotype of CD25⁺ FoxP₃⁻ cells is that they somehow maintain CD25 expression despite high levels of PD-1, which normally blunts TCR signaling through SHP-2–mediated dephosphorylation of ZAP70 (46). This suggests that CD25 might be relatively insensitive to PD-1–mediated inhibition, as described for other gene products (47).

The findings reported here have implications for the development of effective immunotherapies for HGSC and related cancers. First, our data indicate that strategies to deplete Tregs by targeting CD25 might have the detrimental effect of also eliminating CD25⁺ FoxP₃⁻ T cells. Indeed, in renal transplantation and autoimmunity, the net effect of anti-CD25 antibody therapy is immune suppression not activation (22). Second, our findings might help to explain the positive clinical effects of IL2 in ovarian cancer, where a 25% response rate was achieved with intraperitoneal administration of IL2 as a monotherapy (26). Third, our data have implications for checkpoint blockade strategies. Because many CD25⁺ FoxP₃⁻ TIL express high levels of CTLA-4 (Fig. 4B), treatment with antibodies to CTLA-4 might inadvertently deplete this TIL subset (48). On the other hand, our data support further investigation of PD-1 blockade in HGSC, given that CD25⁺ FoxP₃⁻ TIL expressed the highest levels of PD-1 compared with all other CD4⁺ TIL subsets (Fig. 4D). That said, our data suggest that PD-1 might not be the only inhibitory mechanism affecting CD25⁺ FoxP₃⁻ TIL, because the stimulatory agents we used (PMA and ionomycin) are expected to bypass PD-1/SHP-2–mediated inhibition, yet failed to induce cytokine production by these cells (Fig. 4C). Finally, our data reveal OX40 as a promising target for immune modulation, because this molecule was highly expressed by Tregs but not the other three CD4⁺ TIL subsets (Fig. 4B). Antibodies to OX40 can directly stimulate effector T cells (39, 49), and they can also enhance antitumor immunity by depleting OX40⁺ Tregs (30). In summary, our study highlights the IL2, PD-1, and OX40 pathways as potential immunotherapeutic targets to differentially enhance the positive effects of CD25⁺ FoxP₃⁻ TIL over the inhibitory effects of Tregs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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