Differences in Tumor Regulatory T-Cell Localization and Activation Status Impact Patient Outcome

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Abstract

The presence of regulatory T cells (Treg) has been described in a large panel of solid tumors. However, their impact on tumor progression differs according to the tumor type analyzed. We recently obtained evidence in breast carcinoma that Treg localized within lymphoid aggregates, but not in the tumor bed, have a negative impact on patients' survival. Moreover, we showed selective Treg recruitment through CCR4/CCL22 in the lymphoid aggregates upon contact with dendritic cells (DC), where they became strongly and selectively activated (ICOS+), and block conventional T-cell response. Here, we discuss the meaning and potential implication of these novel findings. [Cancer Res 2009;69(20):7895–8]

Background

Cancer immunosubversion is a process by which tumor cells escape destruction by the immune system through a variety of mechanisms, including the production of immunosuppressive cytokines and the alteration of dendritic cell (DC) function (1). Several studies have shown that immune cells are present and functional in solid tumors and may promote both humoral and cellular antitumor responses. As an example, in several cancers high numbers of CD8+ T cells infiltrating the tumor are associated with a better clinical prognosis (2). However, in most of the cases these T cells are unable to counteract tumor progression. In cancer patients, increased levels of CD4+CD25+ regulatory T cells (Treg) expressing FOXP3, a lymphocyte counterpart with immunosuppressive properties (for review see ref. 3), are described in the peripheral blood, the primary tumor microenvironment, and in the draining lymph nodes, supporting a role for Treg in cancer-induced immunosuppression. However their effect on tumor progression varies according to the tumor type in humans. Treg have a negative impact on survival in lung, pancreatic, gastric, liver, and ovarian carcinoma patients (4–8), whereas they may exert a beneficial role in B-cell lymphoma, head and neck, and colon carcinoma (9–11), or have no effect on survival in colon, prostate, renal, and anal squamous cell carcinoma (12–15). However, in most of these studies the authors only analyzed the presence or absence of Treg regardless of their location within the tumor.

The localization of immune cell populations within tumors strongly influences prognosis of patients with colorectal and non–small cell lung carcinoma (2, 16). The coordination of the immune reaction among tumor regions may be required to mount an effective immune response, and the immune compartments in each tumor region may control different tumor events.

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Findings

As in others solid tumors, in breast cancer (BC), the number of circulating Treg is increased and Treg are also present within primary tumors. A negative impact of intratumoral FOXP3+ Treg (Ti-Treg) in BC patient outcome was recently reported (17). To better define the role of Ti-Treg in BC tumor immune suppression, we did immunohistochemistry on two different areas (tumor bed and lymphoid aggregates) by tissue microarray (TMA; n = 191; ref. 18). High numbers of FOXP3+ Ti-Treg were detectable in the lymphoid infiltrates or within the tumor area, and their presence in both locations was significantly correlated with high Scarff Bloom and Richardson (SBR) histoprognostic grade, HER2/neu amplification, and lack of ER/PgR expression, whereas no correlation was found with lymph node or tumor size involvement (18).

Importantly, whereas the presence of FOXP3+ Ti-Treg within the tumor bed did not affect the patients’ clinical evolution, their detection in lymphoid-enriched areas correlated in univariate analysis with a higher risk of relapse, a shorter relapse free survival, and overall survival (OS). Using a multivariate analysis introducing classical histopathologic parameters, FOXp3 expression, in lymphoid infiltrates only, was found to be an independent prognostic factor for OS along with the SBR grading and tumor size.

Analyses of enzymatic disaggregation of fresh primary tumors confirm that whereas all immune subpopulations are detectable, functionally suppressive Ti-Treg represented a high percentage of CD4+ tumor-infiltrating T cells. The high number of Ti-Treg present in the breast tumor environment raises the question of their recruitment. Their decreased expression of CD62L and CCR7 is in agreement with an active recruitment. Moreover, patients’ blood Treg expressed high CCR4 levels and selectively migrated in response to CCR4 ligands produced in the tumor microenvironment. In BC, Ti-Treg expressed low to undetectable CCR4 levels resulting from their internalization consecutive to an active recruitment through CCL22 but not CCL17 (Fig. 1). The importance of CCL22 in recruiting Ti-Treg within lymphoid infiltrates was strengthened by TMA analyses showing that only CCL22 produced in the tumor strongly correlated with the presence of FOXP3+ Ti-Treg in lymphoid infiltrates, but not in the tumor area.

Moreover, unlike CD4+ conventional T cells (Tconv) that displayed a resting phenotype, most of the Ti-Treg expressed activation markers (HLA-DR, GITR, ICOS). This finding was strengthened in situ on primary BC tumor sections where all Ti-Treg and only Ti-Treg in the lymphoid aggregates coexpressed ICOS, whereas Treg detected within the tumor area did not. This result shows that only Ti-Treg infiltrating lymphoid aggregates at the periphery of the tumor are activated (Fig. 1).

Moreover, in contrast to peripheral blood Treg, a substantial part of Ti-Treg proliferated in situ as shown by flow cytometry. Furthermore, as shown by immunohistochemistry, part of ICOS+ Ti-Treg expressed KI67 within lymphoid aggregates, whereas Treg within the tumor bed were all negative.
Implications

Treg recruitment: a role for CCL22 but not CCL17. The down-regulation of CD62L, CCR4, and CCR7 on Ti-Treg compared with peripheral blood suggests, as in lymphoid organs, their cooperation in the recruitment of Treg from the blood to the lymphoid infiltrates surrounding the tumor. In line with this hypothesis, experiments using Treg from CCR4\(^{-/-}\) mice or conditional CCR4 knockout mice in the FOXP3+ Treg compartment have recently identified the critical role of CCR4 in Treg trafficking in secondary lymphoid organs or tissues (19).

Although several studies suggested a role for CCR4 in Treg migration within tumors, our results identify CCL22 as the critical CCR4 ligand in mediating Ti-Treg recruitment, although CCL22 and CCL17 are both intensely produced by tumor cells. Although they share the same receptor, CCL22 but not CCL17 induced CCR4 internalization, probably resulting from differences in affinity for CCR4 (20). The preferential role of CCL22 in the migration of Treg was also suggested in ovarian tumors using ascitis (4). CCL17 produced in the tumors may play an alternative role within the tumors by attracting other T-cell subsets such as Th2 CD4\(^+\) T cells. Whereas CCL22 is mainly produced by the tumor cells, its secretion is only correlated to the presence of Treg in the lymphoid aggregates, suggesting cooperation, probably through CCR7, with chemokines (i.e., CCL19/CCL21) produced within lymphoid infiltrates (21), which could retain Ti-Treg that no longer expressed CCR4 at the cell surface in the infiltrates.

As we have shown, CCR4 is specifically expressed and required for intratumoral Treg migration. Therefore, targeting CCR4 or its ligand CCL22 via monoclonal antibodies could be a way to block their recruitment in the tumor area and help to restore antitumor immunity.

Localization and proliferation status of Treg. We showed the presence in lymphoid aggregates, but not in the tumor area, of ICOS\(^{high}\) Ti-Treg that are in proliferation. FoxP3\(^+\) Treg colocalized with both mature DC (DC-Lamp\(^+\)) and plasmacytoid DC (Ti-pDC) in lymphoid aggregates but not in the tumor bed (Bendriss-Vermare N., Unpublished data) (Fig. 1). Both DC subsets have been reported to be involved in Treg proliferation and survival (for review see refs. 22, 23), suggesting that Treg proliferation could result from presentation by DC of tumor-associated antigens (TAA), resulting in TAA-specific Treg expansion in agreement with the observed skewed TCR repertoire of Ti-Treg. Until now very few studies have analyzed the Treg specificity in human tumors. Although in invaded melanoma lymph nodes, Treg with polyclonal TCR V\(\beta\) chain repertoire were reported (24), Ti-Treg clones specific...
for LAGE-1 or ARTC1 were described in primary melanoma (25), and human papilloma virus (HPV)-specific Treg were reported in cervical cancer biopsies and their associated lymph nodes (26). Finally, in metastatic ovarian tumors, the recruitment of Treg capable of blocking Her2/neu-specific T proliferation was highlighted (4).

DC vaccination offloaded with tumor antigens has been used to overcome tumor progression, but with few positive results (27). As these DC can stimulate both effector T cells and Treg, the Ti-Treg–antigen specificity could lead to a better understanding of the conditions required for their in situ proliferation, and possibly neutralize it.

**Ti-Treg activation in lymphoid aggregates: mechanisms of suppression.** The presence in primary BC of ICOS$^{hi}$ Ti-Treg in lymphoid aggregates, but not in the tumor bed in the vicinity of pDC known to express ICOSL (Fig. 1), suggests a role of ICOS/ICOSL interaction in the activation of Ti-Treg. ICOS costimulation was required for the expansion of Treg ICOS$^{hi}$ in the periphery (23) to favor Treg homeostasis as illustrated in ICOS$^{−/−}$ or ICOSL$^{−/−}$ mice (28). The reduced capacity of ICOS$^{−/−}$ Treg to inhibit CD4$^{+}$ proliferation also suggests a role in Treg suppressive function (29). Those results have been reinforced in humans as naturally occurring ICOS$^{+}$ Treg suppress DC and T-cell functions through interleukin (IL)-10 and transforming growth factor $\beta$ (TGF-$\beta$), respectively (23).

Importantly, Ti-Treg were found in close contact with resting CD8$^{+}$ T cells, suggesting a role in the abolishment of T-cell response by blocking their reactivation within the lymphoid aggregates. Ex vivo reactivated Ti-Tconv infiltrating BC produced high levels of IL-10 and $\gamma$ interferon (IFN$\gamma$), the latter being blocked in vitro in the presence of Ti-Treg (18). In melanoma, ICOS$^{hi}$ Treg are also detected within the lymphocytic infiltrates, in the vicinity of Tconv expressing IL-10 (30).

Altogether this suggests that Ti-Treg, in addition to blocking Tconv reactivation, in particular in the presence of pDC, could also impact their cytokine secretion pattern to favor a tolerogenic environment.

Further studies are in progress to understand the impact of ICOS expression on Ti-Treg. Unlike the use of blocking antibodies currently used in clinical trials such as anti-CTLA-4 that can induce strong secondary effects (reviewed see ref. 31), blocking ICOS could allow a reduction of Ti-Treg proliferation and therefore immunosuppressive cytokine secretion.

**Variability of the impact of Ti-Treg on patient survival: differences in terms of tumor organ origin or in Treg localization and activation status within the tumor?** As presented in the introduction, on the basis of the tumor analyzed, the impact of Ti-Treg on the patient’s evolution differs between patients. Are these differences associated with differences in terms of the organ from which the tumor derives or a different role depending on the location of Treg within the tumor? Some tumors analyzed originated from organs physiologically infiltrated by T cells (lung, liver, skin, digestive tract, lymphoid organs, head, and neck), whereas other did not (ovary, pancreas, prostate, kidney, and breast). In the first category, Treg are detectable in physiological conditions as they play an important role in controlling environmental immune response to avoid autoimmunity. In the normal gut that responds in a regulated manner to antigens derived from food and microbes, Treg will favor a tolerogenic environment as their depletion promote the development of inflammatory bowel disease in mice. In the second category, infiltration by T cells can only be detected in the tumor counterpart. Treg are detectable in all of these tumors and could represent a strategy used by tumor cells to block the immune response and favor their own progression such as in BC and ovarian and pancreatic carcinoma in which presence of Ti-Treg is associated with poor prognosis. In this category, Ti-Treg were also observed in prostate and renal carcinoma but no correlation with prognosis was observed (13, 32).

In the first category, the interpretation of the results is complicated by the normal tissue-associated Treg. In the digestive tract (colon, refs. 12, 13; anal squamous cell carcinoma, ref. 14; lymphoma, refs. 11, 33; and head and neck associated tumors, ref. 9), infiltration by Treg has been reported as either of no impact or of good prognosis. These findings might reflect that these tumor types could benefit from infiltration of immune effectors acting as a source of growth factors (i.e., IL-10 for lymphoma) or promoting inflammation-mediated angiogenesis. Ti-Treg infiltration, by blocking the function of these immune cells, could become a good prognosis. However, the Treg from the normal peri-tumoral tissue might interfere with the interpretation. For example, a recent publication (10) showed that, in colon carcinoma, Treg localization has a different impact on the prognosis. Their presence in the normal adjacent tissue is associated with a worse prognosis by suppressing antitumor immunity, whereas the presence of Treg within the tumor correlated with a better prognosis.

In contrast, in gastric carcinoma (7), Treg present in the tumor parenchyma were associated with a worse prognosis, whereas those surrounding the tumor cell nests were not.

In conclusion differences in the impact of Ti-Treg might reflect (i) the different contribution of immune environment and inflammation in tumor progression; (ii) different localization of the Treg within the tumor bed, the lymphoid aggregates, or the normal peritumoral tissue; and (iii) variable levels of Treg activation. These elements are linked to the organ origin of the tumor. Thus, in future studies aimed at analyzing the impact of Treg on tumor progression, it is critical to take into account the localization of these FOXP3$^{+}$ Treg as well as their activation status.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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