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Shifting the equilibrium in cancer immunoediting: from tumor tolerance to eradication

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Summary: The continual interaction of the immune system with a developing tumor is thought to result in the establishment of a dynamic state of equilibrium. This equilibrium depends on the balance between effector and regulatory T-cell compartments. Whereas regulatory T cells can infiltrate and accumulate within tumors, effector T cells fail to efficiently do so. Furthermore, effector T cells that do infiltrate the tumor become tightly controlled by different regulatory cellular subsets and inhibitory molecules. The outcome of this balance is critical to survival, and whereas in some cases the equilibrium can rapidly result in the elimination of the transformed cells by the immune system, in many other cases the tumor manages to escape immune control. In this review, we discuss relevant work focusing on the establishment of the intratumor balance, the dynamic changes in the populations of effector and regulatory T cells within the tumor, and the role of the tumor vasculature and its activation state in the recruitment of different T-cell subsets. Finally, we also discuss work associated to the manipulation of the immune response to tumors and its impact on the infiltration, accumulation, and function of tumor-reactive lymphocytes within the tumor microenvironment.

Keywords: *costimulation, cancer, tumor microenvironment, immunotherapy, vaccines*

The immune system and cancer

The relative contribution of the immune system to the control of cancer growth and its spread has been debated for many years. The 'cancer immunosurveillance' hypothesis, initially postulated by Burnet and Thomas in the late 1950s, proposed that as tumors grew they could elicit efficient immunity which prevented clinical manifestation and that the immune system had evolved, at least in part, to control malignant cell outgrowth (1). Subsequent attempts to prove this hypothesis showed that mice with an impaired immune system were more susceptible to tumors, but controversy persisted as these findings were mostly limited to chemically or virally induced tumors. In the case of virus-associated tumors, it was argued that the results could be attributed to virus-mediated transformation consequent upon impaired immunity against the virus rather than as a direct effect of the impaired immune response

directed towards the cancer cells *per se*. Later work further fueled the debate, as a series of experiments comparing wild-type and nude mice showed no difference in the incidence of non-virally derived tumors (2, 3). Only in the last two decades has the concept of cancer immunosurveillance been more fully accepted following a series of publications demonstrating that mice genetically deficient in an array of key components of the immune response ($RAG^{-/-}$, $RAG^{-/-}STAT^{-/-}$, $PFN^{-/-}$, $IFN\gamma^{-/-}$, and $IFN\gamma R^{-/-}$) had higher susceptibility to spontaneous, transplantable, and chemically induced tumors (4–7). The concept of cancer immunosurveillance has evolved into a larger and more complex 'cancer immunoediting' model, initially introduced by Ikeda, Old, and Schreiber (1, 8), and defined by three key events: elimination, equilibrium and escape. In this model, the 'elimination' phase corresponds to cancer immunosurveillance, where tumors are detected and destroyed by various components of the immune response. During the 'equilibrium' phase, a balance is established between the tumor and the immune system, during which both tumors and immune cells are shaped reciprocally by each other. Finally, the immune system contributes to the selection of tumor variants that will then grow uncontrollably and 'escape' immune control (9).

It is during the equilibrium phase that the interplay between several components of the immune system and the tumor will define the final outcome of the immune response. It is now clear that as tumors develop they can be infiltrated by different subsets of effector, helper, and regulatory T cells (Treg) which, together with myeloid derived suppressor cells (MDSCs), can shape the microenvironment into one less permissive for effector T-cell (Teff) function. Furthermore, transition through the equilibrium phase not only depends on the extrinsic control exerted by Treg cells and MDSCs but also on the intrinsic regulation of T-cell function by co-inhibitory and costimulatory receptor–ligand pairs. Understanding the key factors involved in maintaining the balance during the equilibrium phase and recognizing ways to interfere with them will help us devise new therapeutic strategies capable of tilting this balance towards elimination instead of escape.

Tumor-specific tolerance – general principles

Progression of cancer may depend on multiple changes within the tumor, including changes intrinsic to the tumor cells resulting in loss or attenuation of immunogenicity (as proposed in immunoediting models), and changes that the tumor cells induce in the surrounding microenvironment or more broadly exert on host immunity to induce a state of immunological tolerance. Support for these latter mechanisms comes from studies such as those of Willimsky and Blankenstein (10) using a mouse model in which the viral SV40 large T cancer-promoting gene was controlled to activate rarely in random tissues. Although immune responses to the SV40 large T protein were initially detected in such mice, they subsequently developed immune tolerance, whereas the tumors remained capable of eliciting vigorous immunity when transferred into identical but tumor-free mice. Although tumor-specific immunity is compromised in tumor-bearing mice, there is often not generalized immune deficiency (11), indicating that tumors can specifically suppress the induction of effective antitumor immunity, subjugating host responses to create isolated nodes of immune privilege within otherwise immunologically intact hosts. Thus, in models of concomitant immunity, mice injected with a tumor are capable of rejecting a subsequent challenge with the same tumor at a distant site, despite continued growth at the site of initial challenge (12–14). Such concomitant immunity is eventually subverted during primary tumor progression by the establishment of $CD4^+$ T-cell-mediated immune suppression (15), which has more recently been shown to be mediated largely by $CD4^+CD25^+Foxp3^+$ Treg cells (16).

Changes occurring during the escape phase of tumor growth that contribute to the development of functional tolerance may be broadly considered as those intrinsic to the tumor cells and those involving the local tumor microenvironment. For example, increased expression of T-cell inhibitory molecules, such as programmed cell death ligand 1 (PD-L1), B7-H3, B7x, HLA-G and HLA-E, by the tumor cells or surrounding parenchyma [stromal or antigen-presenting cells (APCs)] can directly inhibit Teff cell function, and expression levels by the tumor or its microenvironment correlate inversely with outcomes in many epithelial tumors (17–25). Similarly, soluble suppressive factors such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), or gangliosides may be elaborated by either the tumor cells or parenchyma (26–32). Indoleamine 2,3-dioxygenase (IDO) expression by tumor cells or IDO-competent APCs can also contribute to acquired tolerance, both by direct suppression of T cells and by enhancement of local regulatory T-cell-mediated suppression (33, 34). IDO catalyzes the rate-limiting step in tryptophan degradation, and the combination of local reduction in tryptophan levels and the production of immunomodulatory tryptophan metabolites appears to exert tolerogenic activity. Furthermore, IDO-expressing plasmacytoid dendritic cells (pDCs) resident within tumor-draining lymph nodes appear to directly activate mature Treg cells, which can subsequently

cause upregulation of PD-L1 by other DCs which in turn inhibits Teff proliferation (35). The presence of an array of other cell types capable of actively suppressing immune responses such as $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells, IL-10-secreting Treg, CD1d-restricted natural killer T (NKT) cells, immature DCs and pDCs, and MDSCs has been demonstrated to be pivotal for the induction and/or maintenance of local immune privilege in a number of animal models (34, 36, 37). Such cells may be preferentially recruited to these sites or expanded or induced therein.

Extrinsic suppressors: T cells

$CD4^+$ T cells can in many ways be considered as master regulators of immune responses, contributing both to development of effector and suppressor activities. The dominant inhibitory potential of Treg cell populations in murine models of malignancy is well established (38), and a similar potential role in human malignancies has been suggested (39). The mechanisms driving Treg cell expansion and accumulation in patients with cancer are not fully understood, but both proliferation of pre-existing Treg and conversion from naive precursors are likely to be involved (40–42). Suppressor populations fall broadly into one of two categories: a thymically derived population that appear crucially dependent on the expression of the X-linked forkhead/winged helix transcription factor Foxp3 for their development (so-called 'naturally occurring' Treg) (43–49), and a peripherally induced population which arise from naïve $CD4^+$ T cells as a result of 'tolerogenic' encounters. These 'inducible' Treg include IL-10-producing Tr1 cells (50–52), TGF-β-producing Th3 cells, which are mostly associated with oral tolerance (53, 54), and extrathymically or *de novo* generated $CD4^+$ $CD25^+$ $Foxp3^+$ inducible Treg (iTreg) cells (55–60). The acquisition of regulatory phenotype by conventional non-regulatory $CD4^+$ T cells appears important for the maintenance of T-cell homeostasis and control of inflammation. Assuming antigen encounter is required for conversion, it is likely that the regulatory pool expands at the expense of potential Teff, since precursors recognizing tumor antigens may be redirected into a suppressor rather than effector phenotype (40, 61, 62). Factors such as suboptimal antigen stimulation in combination with TGF-β appear to be important in driving peripheral conversion, both of which are likely to be relevant within the tumor microenvironment (57, 63).

Extrinsic suppressors: APCs

Suppressive APC populations have been postulated to play a part in the generation of local immune privilege within tumors. Developing tumors may selectively recruit suppressive APCs or convert stimulatory APCs into suppressors, mirroring the situation with suppressive T-cell populations. The molecular mechanisms underpinning active immune suppression by DC and myeloid populations have not been fully elucidated but include secretion of IL-10 and TGF-β, expression of FAS ligand, PDL1, and elaboration of intracellular IDO (64–68). IDO-competent DCs can induce apoptosis of activated T cells or either T-cell anergy or conversion of effectors into iTregs, as previously outlined (35, 69–71). The local balance of stimulatory versus suppressive APCs is probably critical in determining the eventual outcome of T-cell encounter with antigen in these sites. It has also become clear that the interaction between DCs and Tregs is likely a two-way process (72–74). MDSCs are a heterogeneous group of cellular precursors of macrophages, granulocytes, DCs, and myeloid cells at earlier stages of differentiation (75–77). Specific phenotypic markers that are reflective of suppressor function remain relatively poorly defined (78). MDSC numbers may correlate with clinical outcomes in human cancer (79). Several tumor-derived cytokines have been implicated in the expansion of MDSCs, including VEGF, IL-1β, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (80–82). The mechanism of MDSC-mediated suppression is complex, involving contributions from either inducible nitric oxide synthase or arginase 1 (65, 83–86), which enable MDSCs to inhibit T-cell responses in various ways, including induction of apoptosis, inhibition of proliferation, or induction of a regulatory phenotype. Type 2 macrophages found at tumor sites have also been implicated in suppression of tumor immunity and seem to share some functional properties with immature myeloid cells (87, 88).

Effector-intrinsic tolerance

The existence of co-inhibitory receptors mediating direct downregulation of lymphocyte activation and/or effector function is a well-recognized feature of the immunoglobulin superfamily. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is expressed by activated $CD4^+$ and $CD8^+$ T cells, though its surface expression is tightly regulated with a short half-life. It influences some the earliest events in T-cell activation (89, 90), being rapidly mobilized from intracellular vesicles to the immune synapse after T-cell receptor (TCR) engagement (91). It is constitutively expressed by natural and inducible $Foxp3^+$ Tregs, although the majority of CTLA-4 is again found intracellularly. CTLA-4 shares the B7-1 (CD80) and B7-2 (CD86) ligands with CD28, a critical costimulatory molecule. Ligation of CD28 in concert with TCR stimulation enhances

T-cell proliferation by inducing production of IL-2 and antiapoptotic factors, decreasing the number of ligated TCRs that are required for a given biological response (92). CTLA-4 has significantly higher affinities for both B7 ligands than does CD28 and may therefore outcompete CD28 for the ligand (93). Furthermore, CD28 recruitment to the immunological synapse can be disrupted by CTLA-4, which forms extended high affinity lattices of alternating CTLA-4 and B7-1 homodimers (94). CTLA-4 ligation by B7 ligands also induces decreased production of cytokines (particularly IL-2 and its receptor) and cell cycle arrest. Finally, in addition to its role in controlling Teff function, CTLA-4 has an important role in Treg-mediated suppression (95), as further evidenced by the recent demonstration that Treg-specific CTLA-4 deficiency in conditional knockout mice is associated with a profound reduction in their suppressive capacity (96). The function of CTLA-4 as a negative regulator of CD28-dependent T-cell responses is most strikingly demonstrated by the phenotype of CTLA-4 knockout mice, which succumb to a rapidly lethal polyclonal CD4-dependent lymphoproliferation within 3–4 weeks of birth (97, 98). The role of CTLA-4 in controlling antitumor responses has been demonstrated in many preclinical models of cancer, thus suggesting that interfering with this pathway in cancer patients may also result in improved survival. This was recently demonstrated in a randomized phase III trial in advanced melanoma. Patients receiving a blocking monoclonal antibody against human CTLA-4 (ipilumimab), either alone or in combination with a gp100 peptide vaccine, demonstrated superior overall survival when compared to patients receiving only the vaccine (99). This is the first randomized trial to ever demonstrate that blockade of an immune inhibitory pathway can be used as an effective cancer therapeutic.

PD-1 is more broadly expressed than CD28 or CTLA-4. It can be detected on activated $CD4^+$ and $CD8^+$ T cells, as well as B cells, monocytes, and at lower levels on NKT cells. It binds

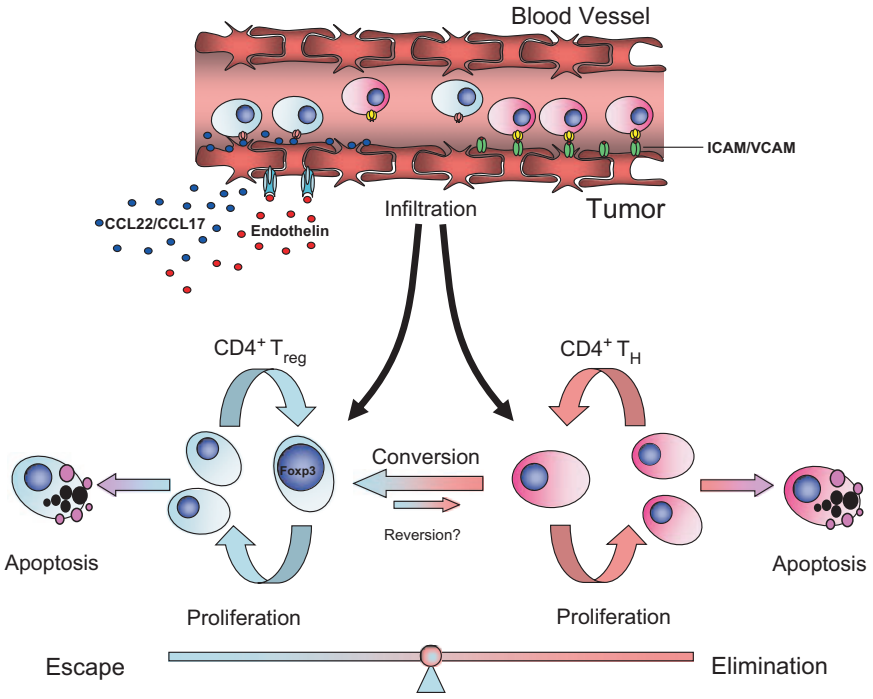

***Fig. 1.* The intratumor balance of effector and regulatory T cells (Treg).** The infiltration of tumors by different subsets of lymphocytes can be initially affected by the activation state of the tumor vasculature as well as by the tumor microenvironment. During tumor progression and under non-inflammatory conditions, a microenvironment rich on CCL22 and or CCL17 will favor recruitment of $CD4^+$ $Foxp3^+$ Treg cells via engagement of chemokine receptor 4 (CCR4) on their cell surface. Furthermore, endothelin-1 produced by tumors will engage endothelin B receptor on the tumor endothelium and reduce infiltration by activated effector T cells (Teff) through downregulation of intercellular adhesion molecule (ICAM) on the tumor vasculature. Conversely, activation of the tumor vasculature by inflammatory mediators or by immunotherapy can increase the expression of adhesion molecules such as ICAM and vascular cell adhesion molecule (VCAM), thus favoring tumor infiltration by Teff. Once within the tumor, the balance is maintained by expansion and contraction of both regulatory and effector T cells. Low levels of costimulation, absence of Th1-type inflammation, and presence of cytokines such as TGF-β may further contribute to conversion of $CD4^+$ $Foxp3^-$ naive or helper T cells into $CD4^+$ $Foxp3^+$ Treg, thus tipping the balance towards tumor tolerance and escape. Although still debated, changes in the stability of Foxp3 may result in Treg reverting into Teff. In addition, an increase on the levels of costimulatory molecules or inflammatory intermediates will also contribute to the accumulation of Teff and reduction of Treg within the tumor, consequently leaning the balance towards tumor elimination.

to two ligands, PD-L1 and PD-L2, which exhibit distinct expression profiles (68). PD-L1 is broadly expressed and can be detected on resting and activated T cells (including $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs), B cells, macrophages, DCs, and mast cells. In addition, its expression on non-hematopoietic cells (including cornea, lung, pancreatic islets, placental synctiotrophoblast, keratinocytes, and vascular endothelium) may have relevance to the function of this receptor–ligand pair. This broad non-hematopoietic expression pattern suggests that inhibition through the PD-L1/PD-1 axis may not be restricted solely to the interaction of T cells and professional APCs but that it may also be relevant during the effector phase of the immune response in peripheral tissues, perhaps helping to prevent immune-mediated tissue damage directly at the tissue interface. By comparison, PD-L2 has a much more limited expression profile. It is not expressed on naive or activated T cells but is instead restricted to activated macrophages, myeloid DCs, and mast cells, suggesting that it fulfills a role that differs from that of PD-L1. The phenotype of PD-$1^{-/-}$ mice provides perhaps the most direct evidence for an inhibitory role of this receptor (100, 101). These mice can develop an array of autoimmune pathologies characterized by high titers of auto-antibodies.

PD-L1 is expressed by a variety of human and murine tumors, and PD-1 expressed by tumor-infiltrating lymphocytes, suggesting that they may be important in restricting intratumor Teff responses. In humans, myeloid DCs isolated from tumor or lymph nodes from ovarian carcinoma patients express high levels of PD-L1 and are capable of enhancing T-cell activity only following PD-L1 blockade (102). Likewise, pDCs in tumor-draining lymph nodes produce high levels of IDO, which results in Treg cell activation, upregulation of PD-L1 on the DCs, and negative regulation of T-cell responses (35). PD-L1 is expressed on several human carcinomas (mammary, cervical, lung, ovarian, colonic, renal), as well as melanoma, glioblastoma and some hematopoietic malignancies (18, 103–107). Its expression has been directly correlated with poor prognosis in bladder, breast, kidney, gastric, and pancreatic cancer (19, 105, 108). Forced expression of PD-L1 on murine tumor lines diminished T-cell activation and tumor killing *in vitro* and markedly enhanced tumor growth *in vivo*, while anti-PD-L1 antibodies blocked these effects (109, 110).

PD-L1 was recently demonstrated to bind B7-1 with an affinity intermediate between those of CTLA-4 and CD28 for B7-1 (111). This interaction is specific and bidirectional, allowing suppression of T-cell proliferation and cytokine production either through B7-1 or PD-L1. T-cell activation signals delivered through the TCR and CD28 will thus be integrated with cell-intrinsic co-inhibitory signals delivered through CTLA-4 and PD-L1 (via B7-1 on the APC and potentially also via B7-1 and PD-1 on other T cells), and PD-1 and B7-1 (via PD-L1 on the APC and potentially via CTLA-4, PD-1, and PD-L1 on other T cells). Finally, inhibitory signaling through PD-1 and B7-1 (via PD-L1 on non-hematopoietic tissues) may influence the final outcome of antigen encounter in the periphery. It is likely that there is some redundancy within such complex and apparently overlapping systems. The physiological relevance of some of these findings remains uncertain, but members of the PD-1:PD-L1/PD-L2 grouping clearly make attractive therapeutic targets for attempts to enhance antitumor immunity. Recent data highlight the relevance of this pathway to chronic T-cell responses to pathogens (112–115). During chronic viral infection, antigen-specific $CD8^+$ T cells are impaired. These 'exhausted' T cells demonstrate a selective upregulation of PD-1, and *in vivo* administration of anti-PD-L1 antibodies restores their activity, as indicated by increased proliferation and cytokine production, and by a significant reduction in viral load (112). Similarly, upregulation of PD-1 on HIV-specific $CD8^+$ T cells has been associated with T-cell exhaustion and disease progression in humans (113, 116). Together these data suggest that blockade of PD-1 and/or PD-L1 can restore functionality of the T-cell compartment and could be applied not only to reinvigorate responses to chronic infections but also to enhance T-cell activity towards other chronic pathologies such as cancer.

The intratumor balance of Teff and Treg cells

The Teff/Treg ratio

As mentioned earlier, several layers of regulation can restrict or prevent immunity against tumors. Tregs play a pivotal role in the control of autoimmune diseases and infections, and several studies have also demonstrated their role controlling antitumor immunity (39, 61, 117–121). Together with controlling the initiation of the immune response in peripheral lymphoid organs, Tregs also accumulate at tumor sites in mice and in humans (118, 121–125) where they can regulate helper and Teff responses (126, 127). The attention of immunotherapists has therefore been focusing on the events taking place within the tumor microenvironment. Whereas the proportion of Tregs in peripheral lymphoid organs averages 5–10% of the total $CD4^+$ T-cell compartment, this proportion is significantly increased at tumor sites, amounting to 20–30% dependent on the type of tumor (121). This is an important observation, since all *in vitro* and *in vivo* data suggest that Tregs suppress in a dose-dependent manner. Nevertheless, tumor

infiltration is not restricted to Tregs, as other T-cell subsets such as $CD4^+$ $Foxp3^-$ as well as $CD8^+$ T cells can also be found within tumors. Prior to the description of Foxp3 as a key marker for Tregs, many studies had demonstrated that the presence of tumor-infiltrating lymphocytes (TILs) correlated with a favorable overall survival (128–130). Later work from Ohtani's group (131, 132) studying both murine and human cancers went further in the analysis of the TILs and concluded that whereas tumors lacking T-cell infiltrates were most likely to progress, it was the presence of a $CD8^+$ T-cell infiltrate and proliferation of such cells that best correlated with favorable prognosis. Further critical insights were provided by Sato *et al.* (133), who incorporated an additional variable to the analysis of TILs. By analyzing the levels of $Foxp3^+$ T-cell infiltrates, they demonstrated that broad characterization of $CD3^+$ T-cell infiltrates was not sufficient to determine correlation with survival but instead a high ratio of $CD8^+$ T cells to $Foxp3^+$ Treg cells was clearly associated with favorable prognosis in epithelial ovarian cancers (133). This major breakthrough in the characterization of TILs was not limited to humans. It was also observed in murine models of cancer. Using the transplantable B16 melanoma (121) and TRAMP-C2 prostate cancer cell lines (authors' unpublished data), we observed that untreated tumors were predominantly infiltrated by $CD4^+$ T cells, of which the majority were $CD4^+$ $Foxp3^+$ Tregs. The relative abundance of $CD8^+$ T cells was severely reduced within tumors, where $CD8^+$ T cells co-existed with Tregs in similar numbers. Together these data underscored the relevance of the intratumor balance between effector and Treg (*Fig. 1*) and posed the question of whether tipping this balance towards the Teff compartment would prevent tumor escape while favoring elimination.

Modifying the intratumor balance through costimulation

Both we and others have demonstrated that therapeutic interventions that significantly increase the intratumor Teff/Treg ratio are most likely to result in effective tumor rejection. Combination of a GM-CSF-secreting tumor cell-based vaccine (Gvax) with a blocking anti-CTLA-4 antibody induced substantial tumor infiltration by $CD8^+$ Teff cells, which increased the intratumor ratio of $CD8^+$ Teff/Treg cells and directly correlated with tumor rejection. In contrast, mice treated with either Gvax or anti-CTLA-4 monotherapy showed only a partial increase in the intratumor $CD8^+$ Teff/Treg cell ratio and failed to reject tumors (121). In keeping with this observation, CTLA-4-blockade in combination with a FLT3L-secreting tumor-cell-based vaccine also resulted in significant increases in the $CD8^+$ Teff/Treg ratios and potent tumor rejection (134). Similar results have been observed in cancer patients, where CTLA-4 blockade resulted in increased ratios of effector to Treg (135, 136). The capacity to change the intratumor balance is not restricted to CTLA-4-blockade, as blocking inhibitory signals via PD-1/PD-L1 interactions also resulted in increased Teff/Treg ratios and tumor rejection (137). Combinatorial blockade of both CTLA-4 and PD-1 pathways resulted in an additive effect with significantly higher Teff/Treg ratios and potent tumor rejection. This is encouraging, as a recent study in melanoma patients demonstrated that the majority to tumor infiltrating $CD8^+$ T cells expressed high levels of PD-1, thus suggesting this as a relevant pathway in the regulation of intratumor responses in cancer patients (138).

Enhancing stimulation of T-cell function via the tumor necrosis factor receptor family also modifies the intratumor balance of T cells, as treatment of established tumors with agonistic anti-GITR (139) or with a combination of cyclophosphamide and an OX40 agonistic antibody (140) resulted in significant $CD8^+$ T-cell infiltration with a concomitant reduction in $Foxp3^+$ Treg cells within the tumors.

Although numerous studies have demonstrated a correlation between the changes in the intratumor balance of Teff/Treg cells and tumor rejection, we still lack a clear understanding of the mechanisms driving such changes. In most cases, overt accumulation of Teff cells and reduction of Treg cells at the tumor site are most likely explanations for the increase in the Teff/Treg ratio, but the cellular and molecular mechanisms underpinning these changes remain less clear.

Dynamic changes in the frequency of tumor infiltrating Treg

Treg accumulation

The description of an intratumor balance favored by the natural accumulation of $CD4^+$ $Foxp3^+$ Treg cells and the impact of modification of such tumor balance through immunotherapy gives rise to two major questions: (i) what drives the accumulation of Tregs within tumors during tumor development and (ii) what are the mechanisms driving the increase in the intratumor Teff/Treg cell ratio following immunotherapy.

The most likely explanations for Treg accumulation during tumorigenesis include an increase in Treg infiltration, enhanced proliferation, reduced apoptosis, or *de novo* induction (or conversion) of $CD4^+$ $Foxp3^-$ cells into $CD4^+$ $Foxp3^+$ Treg cells. Clearly these mechanisms are not mutually exclusive. Tumor infiltration driven by the expression of the chemokine receptor 4 (CCR4) on Tregs is considered a major contributor

in some settings. Seminal studies by Curiel *et al.* (39) demonstrated that in human ovarian carcinoma, a high frequency of tumor-infiltrating Treg cells correlated with poor survival. They were able to demonstrate *in vitro* and *in vivo* that Treg infiltration (but not Teff infiltration) depended on CCL22/CCR4 interactions with CCL22 being produced both by tumor cells and by tumor-infiltrating macrophages (39). Subsequent studies in melanoma (141), breast cancer (142), Hodgkin's lymphoma (143, 144), and most recently in human glioblastoma where the presence and frequency of Tregs also correlated with the WHO tumor grade (145), further support a role for TARC/CCL17 and MDC/CCL22 (specific ligands for CCR4) in tumor infiltration by $CD4^+$ $Foxp3^+$ $CCR4^+$ Treg cells. Several strategies including monoclonal antibodies or receptor antagonists are being developed to target $CCR4^+$ Treg cells and prevent tumor infiltration, although their efficacy at preventing or, more importantly, reverting Treg accumulation remains to be fully demonstrated (144, 146, 147).

Less is known about changes of Treg proliferation within tumors. In a model of murine autoimmune diabetes, low levels of IL-2 are required for maintenance of intra-islet Treg homeostasis and survival (148). An equivalent scenario could occur in tumors where low levels of IL-2 would help sustain Treg proliferation and homeostasis. Although this hypothesis has not been formally tested, we have previously demonstrated that untreated B16 melanoma is infiltrated by $CD4^+$ $Foxp3^+$ Treg cells as well as by $CD4^+$ Teff cells (120, 121). Teff cells could be providing the low levels of IL-2 required for Treg survival and proliferation within the tumor microenvironment. In keeping with this, analysis of untreated tumors demonstrates high levels of KI-67 expression by tumor infiltrating Treg cells (120). Finally, IDO produced by either tumor cells or parenchyma also favors the activation and expansion of Treg cells (35, 71, 149).

Numerous studies now support the idea that *de novo* induction (conversion) of $CD4^+$ $Foxp3^+$ Treg cells from $CD4^+$ $Foxp3^-$ precursors contributes significantly to Treg accumulation within tumors. However, distinction between conversion and expansion can be technically difficult due to a requirement for highly purified populations of $CD4^+$ $Foxp3^-$ precursors in many conversion models. Initial studies were based on $CD4^+$ $CD25^-$ purification strategies where there was still a chance for contaminating $Foxp3^+$ cells within the $CD25^-$ population. The use of Foxp3 green fluorescence protein (GFP) knockin mice as more reliable sources of $Foxp3^-$ precursors has only been possible more recently. Studies by the Levitsky group (40) were among the first to demonstrate conversion of $CD4^+$ $CD25^-GITR^-$ HA-reactive $CD4^+$ T cells into $Foxp3^+$ Treg cells in response to HA-expressing B-cell lymphomas (40). Nevertheless, since conversion was induced in response to tumors expressing MHC class II which could be directly presenting antigen to $CD4^+$ T cells, it remains unclear whether this observation is fully translatable to non-hematopoietic class II-negative tumors. Recent studies using Foxp3GFP cells demonstrated that *lamina propria* DCs could promote *de novo* generation of $Foxp3^+$ Tregs upon oral exposure to antigen in a retinoic acid-dependent manner (150). In this model, *in vivo* conversion depended on TGF-β as well as on a lymphopenic environment. Two recent studies suggest conversion as a main mechanism for Treg generation in response to tumors. The first, using a murine pancreatic tumor cell line (Pan02) showed an increase in *de novo* induction of Treg *in vivo* in a TGF-β dependent manner (151). The second demonstrated that conversion of OVA-reactive $CD4^+$ T cells in response to OVA-expressing B16 melanoma was dependent on PD-1/PD-L1 interaction (152). Interestingly in both studies, conversion seemed to depend on host lymphodepletion. TGF-β emerges from these studies as a common requirement for conversion. It is provided by many tumor types, supporting the idea that the intratumor microenvironment can drive conversion. The apparent requirement for lymphopenia in driving conversion in many of these models remains intriguing. Is lymphopenia really required, or are there conditions within the tumor microenvironment that resemble those of a lymphopenic environment? Interestingly, under many circumstances, cancer patients can be partially lymphodepleted either by the effect of chemo-therapeutic or radio-therapeutic interventions or in response to the tumor itself. Perhaps it is in these conditions where *de novo* induction of Treg cells becomes more relevant in the expansion of the regulatory compartment.

Treg reduction

How can this tolerogenic state or balance in the tumors be broken? Increased tumor infiltration by activated Teff cells clearly contributes (121, 139, 140). But besides Teff infiltration, a decrease in the absolute number of Tregs within the tumor can also account for at least part of the increase in the Teff/Treg cell ratio (140, 153, 154). It is not clear what mediates this reduction in Treg numbers. Reduced conversion, increased Treg cell death, impaired infiltration, or even a reduction in the stability of Foxp3 in the regulatory T-cell compartment are all considered potential mechanisms.

Although some studies suggest that manipulation of costimulatory pathways can lead to a reduction in conversion (155–157), it is not clear in the tumor setting if interfering with

those pathways changes the Teff/Treg cell ratio by reducing conversion or by increasing Teff infiltration. An alternative to reduced conversion is the loss of Foxp3 expression, perhaps representing reversion. Treatment of tumor-bearing Foxp3GFP mice with GITR agonistic antibodies resulted in loss of intratumoral Tregs, apparently due to loss of Foxp3. This was verified in histological analysis of tumors, where cells expressing GFP but not Foxp3 were detected, thus supporting the idea of Foxp3 instability (154) In keeping with this finding, a recent study demonstrated that a substantial proportion of $CD4^+$ $Foxp3^+$ cells have unstable expression of Foxp3. Remarkably, loss of Foxp3 expression renders these cells autoreactive as demonstrated by their capacity to mediate autoimmune diabetes (158). The issue remains controversial, however, as a newer study demonstrated great stability of Foxp3 *in vivo* during steady state and after different inflammatory stimuli including autoimmune diabetes (159). Here, the authors point out that one potential explanation for the discrepant outcomes of these studies may reside in different regulation of Foxp3 expression in the bacterial artificial chromosome transgenic mouse (158) versus the endogenous regulation found in the knock in mouse model (159). Finally there is evidence that Foxp3 stability may be dependent on the cell in which it is expressed. A recent study demonstrated that whereas the majority of $CD4^+$ $Foxp3^+$ cells are stable (most of them $CD25^+$), a much smaller subset within the $CD25^-$ population can actually lose and re-acquire Foxp3 expression depending on the environmental cues (160).

Finally, increased Treg death has also been postulated as a potential mechanism accounting for reduced Treg numbers. Although finding apoptotic Tregs has been challenging, a recent paper elegantly demonstrated that a combination of OX40 agonistic antibodies and cyclophosphamide resulted in Treg apoptosis in a murine model of melanoma (140). FAS /FASL interactions have been implicated in increasing Treg apoptosis. In animal models of colitis, inflammatory stimuli were capable of inducing a local FAS-dependent depletion of Tregs without significantly affecting $CD4^+$ Teff cells (161). In a recent publication using a murine model of breast cancer, immunizations with both effector and helper epitopes resulted in significant antitumor responses characterized by a striking change in the intratumor balance of Teff and Treg due to a reduction in tumor-infiltrating Tregs. This reduction was due to apoptosis induced by $CD4^+$ $FASL^+$ T-helper cells induced by the vaccination strategy, and further corroborated after intratumor administration of anti-FASL blocking antibodies prevented Treg apoptosis enabling tumor progression (162). The FAS/FASL hypothesis offers some additional insights into the establishment and resolution of the equilibrium phase. As a tumor develops and Teff cells recognize antigen in absence of costimulation or inflammation, they will succumb to activation-induced cell death, whereas in this same microenvironment, Tregs will tend to infiltrate, expand, and convert, thus tilting the balance towards tolerance and escape. Conversely during immunotherapy, Teff cells will infiltrate and contribute to the elimination of Tregs through FAS-mediated apoptosis. Importantly, several publications suggest that Teff cells primed *in vivo* become resistant to FAS-mediated killing, thus allowing them to survive within the inflammatory milieu generated in the rejecting tumor (163, 164).

Dissociation of systemic and local responses

Tumor vasculature and microenvironment as barriers to T-cell infiltration

Despite attempts to elicit potent antitumor reactivity through targeting cell-intrinsic and cell-extrinsic regulatory circuits, the responses we are able to generate and quantify in the periphery (i.e. blood and lymphoid structures) have not been mirrored by such promising outcomes in the clinic. In some cases, this could still reflect less than optimal T-cell activation and a lack of durable immunity, but as our understanding of tumor biology grows, we have realized that barriers other than immune-regulatory checkpoints exist. One such barrier is the restriction of efficient redistribution and accumulation of Teff within tumor lesions. Studies in both mice and humans have demonstrated that tumors can continue to grow regardless of detectable levels of tumor-reactive $CD4^+$ and $CD8^+$ T cells in peripheral blood (165–169). One potential explanation is inability of the tumor or tumor antigen to prime a proper T-cell response, either due to lack of costimulatory signals or due to antigen sequestration. This is supported by work in the RIP-Tag5 pancreatic cancer model, the Lewis lung carcinoma model and the B16/BL6 melanoma model showing that presence of tumor did not result in activation of TCR transgenic tumor-reactive $CD8^+$ T cells (168–170). Furthermore, another study by Mark Davis' group (167) identified a group of melanoma patients in whom tumor-specific $CD8^+$ T cells identified by MART1 tetramers displayed a naive phenotype, suggesting lack of T-cell activation even in presence of active disease. Nevertheless, there are studies where good levels of T-cell activation can be detected in absence of tumor regression (171, 172). For instance, in the RIP1-Tag5 model, tumor reactive T cells upregulate the activation marker CD44, dilute CFSE, and acquire cytotoxic activity, suggesting that tumors are capable of eliciting T-cell

activation even in the absence of immunization (172). Despite the presence of activated cytotoxic T cells, tumors continued to grow, suggesting that acquisition of potent effector function by the T-cell compartment is insufficient to drive tumor rejection. On the clinical front, immunizations with the MHC I-restricted peptide from the melanoma differentiation antigen gp100 elicit robust $CD8^+$ T-cell responses against gp100 in peripheral blood samples (173). Strikingly, however, none of the tumor-reactive lymphocytes isolated from the tumors recognized the gp100 peptide used in the vaccine. This data underscores the schism that can exist between responses measured in the blood and those taking place in the tumors.

As novel therapeutics become available, immunotherapists are attempting to better model treatment of disease by treating fully established vascularized tumors. During our attempts to further increase the potency of CTLA-4-blockade to enable the rejection of larger more established tumors, we combined Gvax/anti-CTLA-4 with Treg depletion. Depletion of Tregs is being investigated in pre-clinical models and in clinical trials as a cancer immunotherapy (174, 175). Despite enhancing T-cell activity against a tumor antigen (175), Treg depletion does not increase survival in melanoma patients (174). In agreement, we found that Treg depletion following tumor establishment significantly enhanced peripheral antitumor activity, although increased activity did not protect against tumor outgrowth owing to a lack of intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) expression by the tumor vasculature and poor infiltration of Teff into the tumor (120). Similar observations were made by the Ganss group (176) in the RIP1-Tag5 pancreatic cancer model where they found that treatment with the Toll-like receptor ligand CpG and vaccination against the tumor antigen Tag prevents outgrowth of tumors in 5-week-old mice, whereas therapy fails in 23-week old-mice bearing established tumors despite similar levels of systemic cytotoxicity towards Tag targets. The failure in tumor protection correlated with reduced infiltration of vaccine-generated T cells into the tumor.

Approaches to overcome barriers in T-cell infiltration into tumors

Since irradiation has been demonstrated to enhance infiltration of T cells into tumors and T-cell effector function (177, 178), we adoptively transferred polyclonal $CD4^+$ and $CD8^+$ T cells harvested from mice previously treated with Gvax, anti-CTLA-4, and depleted of Tregs into irradiated mice bearing large tumors (120). This treatment protected mice against tumor outgrowth and was associated with expression of ICAM and VCAM on the tumor vasculature and infiltration of Teff into the tumor. Once again, these findings were in agreement with those from the RIP1-Tag5 model, where irradiation of tumor-bearing mice prior to transfer of tumor-reactive $CD4^+$ T cells resulted in increased infiltration of T cells into the tumor, pro-inflammatory chemokine expression, and ICAM and VCAM expression on the tumor vasculature, resulting in slower tumor progression (179). Although our observations regarding ICAM and VCAM and tumor rejection currently represent correlation rather than causation, it is likely they play a key role in tumor rejection by mediating diapedesis of Teff into the tumor parenchyma (180). The apparent dependence of tumor rejection on ICAM and VCAM expression observed in our model was in keeping with a previous studies demonstrating for the first time an inverse correlation between the expression of the endothelin B receptor (ET_BR) on tumor vasculature and survival of ovarian carcinoma patients (181, 182). Furthermore, they demonstrated that ligation of ET_BR by its ligand ET-1 downregulates ICAM expression and that neutralization of ET_BR using a small molecule inhibitor restores ICAM expression and adhesion of T cells to vessels. When tested in mouse models of cancer, the combination of immunotherapy and blockade of ET_BR synergizes to greatly increase infiltration of T cells into the tumor and reduce tumor outgrowth. Together our data suggest that ICAM expression is an important and perhaps limiting step in tumor elimination, and therapies aiming at increasing its expression on the vasculature may produce better antitumor responses. As a further example, a recent study reported that IL-2 and agonistic anti-CD40 antibodies targeted selectively to the tumor vasculature with a peptide results in enhanced accumulation of T cells in tumors and protection against tumor outgrowth (183). The mechanism of protection involves the anti-CD40 antibody acting directly on $CD40^+$ vessels to upregulate ICAM and VCAM, thus making the tumor receptive to T-cell infiltration. A similar approach involving targeting CpG-loaded liposomes to the tumor vasculature with a peptide also elevated ICAM expression by tumor-associated vessels and made tumors receptive to treatment with immunotherapy (184).

Accessibility to tumors is not only regulated by ICAM. Regulator of G protein signaling (RGS5) appears to be an additional key player controlling vasculature sprouting and growth (185). In the RIP1-Tag5 system, genetic deletion of RGS5 normalizes tumor vasculature resulting in improved $CD8^+$ T-cell infiltration into tumors after immunotherapy (185, 186). These data suggest that therapeutic manipulation of the RGS5 pathway in combination with immunotherapy may enhance infiltration of vaccine-generated lymphocytes.

Although a great part of many current immunotherapeutic strategies focuses on the generation of more robust T-cell responses, these considerations suggest that combination of such therapies with strategies capable of sensitizing the tumor vasculature and microenvironment will significantly synergize to produce maximal T-cell infiltration and tumor destruction, thus overcoming the observed discordance between local intratumor responses and systemic T-cell activity.

Adoptive cell therapy (ACT) to overcome tolerance to tumors

ACT generally consists of the transfer of large number of activated Teff into lymphopenic tumor-bearing recipients (187, 188). Although ACT may be considered a 'brute force' approach that simply depends on saturating the patient with Teff, study of the mechanisms underpinning the efficacy of ACT have generated significant insights into some of the basic components required for effective rejection of established tumors. A key component of ACT strategies is the state of lymphodepletion induced in the host prior to T-cell transfer. Lymphodepletion eliminates cytokine sinks, myeloid suppressor cells (189), and Treg cells at the same time as providing an environment favorable to homeostatic proliferation (190). Host irradiation, used in many cases to induce lymphodepletion, also contributes by sensitizing the tumor stroma (191) and by inducing the upregulation of adhesion molecules on tumor vasculature, thus rendering the tumor susceptible to T-cell infiltration (177, 179). Furthermore, LPS is released from commensal gut flora following radiation therapy. This allows efficient maturation of DCs carrying tumor antigens which can also be generated as a consequence of irradiation (192). Hence, lymphodepletion acts like a 'reset' button capable of breaking the tolerogenic state originally induced and maintained by the growing tumor. Based on these mechanistic insights, we can postulate that approaches inducing short-lasting lymphodepletion (i.e. radio- or chemo-therapy) will efficiently synergize with active immunization strategies aiming at enhancing T-cell function. As a successful example, combination of an agonistic anti-OX-40 antibody with cyclophosphamide resulted in effective eradication of established melanoma (140).

In addition to lymphodepletion, transfer of large numbers of Teff in the correct stage of activation is crucial for the efficacy of classical ACT (193). For years, ACT and the field of tumor immunology in general have focused on the function of tumor-reactive $CD8^+$ cytotoxic T cells (CTLs) (194) reflecting the fact that $CD8^+$ T cells are considered the ultimate effectors of the immune system, capable of directly engaging and killing their targets. Although it is well known that $CD4^+$ T cells contribute to $CD8^+$ T-cell function (190, 195), more recent studies attribute a potentially more direct role for the CD4 compartment to antitumor immunity (196–198). Thus, *in vitro* expanded and differentiated tumor-reactive Th17 cells are capable of rejecting established melanoma tumors in mice (199). Furthermore, the transfer of high numbers of tumor-reactive $CD4^+$ T cells into a patient with melanoma resulted in a complete response (200). In addition, two recent studies demonstrated that transfer of a small number of naive tumor-specific $CD4^+$ T cells into lymphopenic mice results in rejection of large vascularized melanoma lesions (153, 201). Surprisingly, the antitumor activity was not based in classical T help but dependent in the acquisition of granzyme B-dependent cytotoxic activity by tumor reactive $CD4^+$ T cells (153). The acquisition of cytotoxic activity by transferred tumor-reactive $CD4^+$ T cells distinguished our findings from previous work showing that $CD4^+$ T cells can help rejection of less well-established tumors through indirect effects of IFN-γ (198) on NK cells (197) and tumor-infiltrating macrophages (196, 202–204). Remarkably, $CD4^+$ $Trp1^+$ cells developed all the hallmarks of $CD4^+$ T-helper cells in addition to cytolytic activity. Although $CD4^+$ CTLs targeting viral (205–207) and allo-antigens (208, 209) have been described previously, the demonstration of similar activity in a more physiological model for self/tumor antigen emphasizes the promise of these cells in cancer immunotherapy.

Together these new advances in the understanding of tumor-reactive $CD4^+$ T cells demonstrate their capacity to modify T-cell function as well as the tumor microenvironment, thus becoming a powerful tool in the fight against cancer. Perhaps a better understanding and manipulation of the function of this T-cell subset will provide all the necessary components for the adequate resolution of the equilibrium phase established during cancer immune-editing.

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