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Cancer Immunotherapy: The Role Regulatory T cells Play and What can be Done to Overcome their Inhibitory Effects

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INTRODUCTION

Regulatory T cells

In 1909, Paul Ehrlich first postulated that the immune system works to eliminate malignant cells as they develop. He posited that in the absence of an effective immune system tumors would develop more frequently because transformed cells arise continuously during cell division. This hypothesis was experimentally confirmed in the 1950s by Burnet and Thomas, who postulated the existence of immune surveillance; a system of immune cells that provides a first “line of defense” against malignant cells (1). The immune surveillance hypothesis implied that tumors expressed distinct structures (antigens), that could be recognized by the immune system. Thus, the immune system could recognize malignant cells and destroy them before they developed into detec4 tumors (2). In the 1970s it was suggested that “suppressor” T cells contributed to the development of malignancies by limiting immune effector function (3). However, controversy surrounding the description and characterization of these cells resulted in the concept being effectively dismissed by the mainstream immunology community. In 1995, Sakaguchi and colleagues reawakened the “suppressor” field by identifying CD25 as a marker of T cells with the capacity to suppress autoimmune disease in mice (4). Subsequent studies provided evidence that these suppressor or Regulatory T cells (Treg) play important roles in regulating immunity to self, alloantigens, infectious agents, the fetus and cancer (reviewed in (5)). Given this background, the development of clinically applicable strategies to reduce or interfere with Treg function has become an area of active investigation.

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There are two major types of Treg. Naturally occurring Tregs (nTreg) arise from the thymus as a distinct lineage of CD4+CD25+ T cells with a diverse TCR repertoire (6). These cells acquire their regulatory phenotype in the thymus where they undergo positive selection, a consequence of recognizing antigens presented by the thymic epithelium (7). nTreg appear to recognize self antigens preferentially and are thought to play an important role in preventing autoimmunity (6,8). Since many of the currently recognized tumor-associated antigens are self antigens that are over-expressed by tumors (e.g. Her2/neu in breast cancer), or are selectively expressed by tissues (gp100 in melanoma and pigmented tissue), nTreg may play a critical role in limiting the immune response against these targets. In contrast, the second type of Treg, induced-Treg (iTreg) are not Treg when they leave the thymus, but acquire what has been called a suppressive phenotype in the periphery after stimulation of their TCR with non-self and/or self antigens (9–11). iTreg can be distinguished from nTreg by their requirement for further differentiation, a consequence of exposure to antigen (12). CD4+ iTreg can be subdivided into two major populations. One population constitutively expresses CD25 and FoxP3 while the other up-regulates FoxP3 through TCR stimulation and typically express high levels of the immune suppressive cytokines IL-10 and TGF-β. Both iTreg sub-populations can be generated in vitro following exposure to antigen or mitogen in the presence of the appropriate amount of immunosuppressive cytokines or factors. For example, in vitro culture with IL-10 can lead to the induction of iTreg (Tr1) cells (13), and CD4+CD25+ Foxp3- cells can be induced to become Foxp3+ iTreg T cells when stimulated in the presence of TGF-β (9,14,15). Prostaglandin E2 can also generate iTreg with in vitro suppressor function (16).

The Treg cells found at the tumor site (tumor-associated Treg) may include both natural and induced Treg (Figure 1). Since tumors can express IL-10, TGF-β and/or PGE2, in addition to tumor-associated antigens, the tumor milieu can be an efficient breeding ground for iTreg. In addition to overexpressed self antigens, iTreg may be induced in response to non-self antigens derived from virus infected cells (17) or from mutated self antigens (18,19).

Mechanisms of Suppression

The initial data describing mechanisms of Treg-mediated immune suppression were derived primarily from models of autoimmunity. While these data provide a strong basis on which to build a consensus model, drawing conclusions about Tregs and cancer from autoimmunity models may include a critical bias. As discussed above, nTreg are considered primarily to prevent autoimmunity, while tumor-associated Treg likely also contain iTreg that may function differently than nTreg. Nonetheless, there is general agreement that both nTreg and iTreg can block productive immune responses. However, the point or points where Treg exert their suppressive functions in vivo and the mechanism(s) by which suppression is achieved need further clarification in order to provide effective means to counteract Treg-mediated suppression.

Three points have been identified where Treg cells can suppress the immune response (19). Treg may block anti-tumor immunity byany or all of the following:

1. Direct suppression of effector T cell function
    - Contact dependent
    - Contact independent
2. Suppression of the antigen-specific priming of naïve T cells.
3. Modulation of the function of antigen-presenting cells (APC), which results in inefficient priming and maturation of effector T cells.

Direct Mechanisms of Suppression

One of the earliest mechanisms proposed to explain suppression was that IL-2 receptor+ (CD25+) cells acted as an IL-2 “sink”, soaking-up IL-2 and preventing expansion of antigen-primed T cells and ultimately inducing apoptosis of effector T cells. While this is not necessarily a contact-dependent mechanism, the cells acting as the IL-2 sink probably need to be “close” to mediate their effect. In some tumor models Treg cells use granzymes and perforin, mediators of cytolysis, to eliminate effector T cells (20) directly. These first two mechanisms are well established, but recently, two other mechanisms based on “metabolic disruption” were identified. Treg cells can generate adenosine that can suppress responding T cells (21) or Tregs could deliver cAMP, an inhibitory second messenger, which could suppress effector T cell function (22).

Mouse models indicate that Treg cells dampen the immune response of other T cells largely by inhibiting activation and/or proliferation. TGF-β, IL-10 and IL-35 are three cytokines produced by Treg that can mediate this inhibition. TGF-β which has been commonly implicated in Treg-mediated suppression, is a pleotrophic cytokine expressed in different isoforms by different tissue and cell types. TGF-β 1 is the most common isoform expressed by cells of the immune system (reviewed in (23)). Several model of autoimmunity have shown that nTreg cells suppress in a TGF-β dependent mechanism (24–26). While TGF-β is an important mediator of suppression, there is in vitro data showing that Treg could suppress even when responder T cells were insensitive to TGF-β (27). This is also consistent with our data in a B16BL6 tumor model where iTreg suppress the priming of therapeutic T cells even when naïve T cells are insensitive to TGF-β (Petrausch et al., manuscript submitted).

IL-10 plays a crucial role in the induction of iTreg (Tr1) cells and is an important mediator of suppression for these iTreg. IL-10 can be produced by tumor cells (28,29), as well as by macrophages (30), DCs (31) and activated CD4+CD25+ T cells (32). It is unclear whether IL-10 alone is efficient in activating natural Tregs and/or recruiting new Tregs from CD4+ T cells or whether IL-10 needs to be combined with TGF-β from the tumor cells (33), the surrounding stroma (34) or infiltrating macrophages (30). IL-35 is a recently described cytokine that is also expressed by Treg cells and can inhibit proliferation of T cells. Similarly to TGF-β and IL-10, IL-35 can induce naïve T cells to become functional Treg cells. Together, TGF-β, IL-10 and IL-35 are three soluble mediators by which Treg cells can limit the anti-tumor function of effector T cells (35).

Indirect mechanisms of suppression

Although there are a number of mechanisms by which Treg can directly inhibit effector T cell function, Treg cells can also inhibit anti-tumor immunity indirectly by blocking the priming of tumor antigen-specific effector T cells. For example, depletion of CD25 cells led to enhanced priming of T cells when RNA-pulsed DCs were used to prime T cells (36). The potential for Treg to affect DCs became better appreciated once intra vital microscopy was employed to study in vivo interactions between these two cell types. This technology has shown, as expected, that in vivo effector T cells that interact with Treg cells exhibit reduced effector function (37), other studies reported that Treg interact with DC in vivo (38,39). Together these observations provide insight into the multiple levels available for Tregs to exert their suppressive effect as well as suggest potential opportunities to overcome or disrupt these interactions.

CTLA-4 is constitutively expressed by Treg and provides a ligand for interactions with CD80 and CD86 expressed on DC. It has been reported that Treg suppressive function correlates with intracellular CTLA-4 expression in $CD4^{+}CD25^{+}$ T cells (40,41). Further, CTLA-4–deficient Treg exhibit reduced capacity to suppress DC and CTLA-4 stimulation of

DC can induce production of indoleamine 2,3-dioxygenase (IDO). IDO appears to promote tolerance in two ways. First, IDO stimulates the tryptophan catabolism pathway that has a potent immunosuppressive activity on effector T cells. Second, DC expressing IDO preferentially promote the generation of iTreg instead of effector T cells (42). Together, this combination of suppressing effector T cells and promoting iTreg provides a potentially important mechanism for Treg to influence the generation of anti-tumor immunity.

A subpopulation of Treg can also express LAG-3, a CD4 homologue that binds MHC class II and is thought to facilitate Treg interactions with class II+ DC. LAG-$3^{-/-}$ mice were used to demonstrate that this MHC class II ligand was important for a subset of regulatory T cells in the mouse (43). Further, this same group showed that antibodies to LAG-3 could inhibit the suppressive function of Treg cells. In contrast to studies in mice, in humans soluble LAG-3 protein can promote a TH1 immune responses, a response that is generally appreciated to be therapeutic in a majority of tumor models (44). Additional investigations will be required to determine the role for LAG-3 in generating DC that are suppressive or promote the development of a therapeutic immune response.

CD25+ Treg: Preclinical studies

Tumor models show that the frequency of CD4+CD25+ T cell is increased in tumor-draining lymph nodes and spleens of tumor-bearing mice (45) and functional data indicate that concomitant immunity to tumors is prevented by the presence of CD4+CD25+ T cells (46). Depletion of CD25+ cells led to regression of established tumor in one model (47) and in vaccinated animals, promoted increased priming of therapeutic T cells for adoptive immunotherapy (48) (Poehlein, et al., manuscript submitted) and increased protection in a tumor challenge model (49). Interestingly, while we have not seen reproducible increases in numbers of CD4+CD25+ Treg in the spleen of mice bearing systemic B16BL6 tumor, the CD25+ cells are functionally suppressive and depletion of CD25+ cells recovers therapeutic anti-tumor immunity (Poehlein, et al., manuscript submitted). While these studies successfully used anti-CD25 to eliminate Treg cells, the reliance on CD25, a marker of both activated T cells and Treg cells is a major drawback.

Identification of FoxP3 as a marker of Treg cells

Expression of the transcription factor forkhead box p3 (Foxp3) has been reported to be necessary and sufficient for the development of Treg in mice (50). Supporting this link between FoxP3 and Treg function is the observation that mice and humans that lack functional FoxP3 develop profound autoimmune disease and fail to thrive (males with immune dysregulation, X-linked syndrome). Furthermore, transfection of CD4+CD25-T cells with FoxP3 induces Treg function (8,51). An impediment to using the intracellular transcription factor, Foxp3, as a marker is that detection of FoxP3 requires permeabilization of the cell membrane, which results in cell death. To identify viable FoxP3+ cells, transgenic (Tg) knock-in mice were engineered so that whenever FoxP3 was expressed, a fluorescent protein was also expressed. This allowed analysis and manipulation of Foxp3 Treg cells while maintaining their viability (52). Using this strategy, Fontenot et al., demonstrated that Foxp3 is a lineage-specific marker of Treg and that IL-2 is critical for their maintenance (52). In another example, Kapp et. al., crossed the Foxp3-GFP Tg mouse with a T cell receptor (TCR) Tg mouse and showed that in the presence of TGF-β, antigen up-regulates CD4+Foxp3+ Treg cells that inhibit proliferation of antigen-specific CD8+ T cells (53).

While still relatively new, these FoxP3-GFP mice have confirmed findings using CD25 to detect Treg cell and have provided significant new insights into the complexities and regulation of Treg cells. One of the next steps will be to employ FoxP3-GFP Tg mice to

develop strategies to limit the development, trafficking and/or persistence of FoxP3+ Treg cells that inhibit the efficacy of cancer immunotherapy.

Measurement of Regulatory T cells

Murine Treg are generally characterized as CD4+CD25+Foxp3+ T cells (52), but CD8+CD25+FoxP3+ T cells with suppressive activity have also been described (54). Foxp3+ is also expressed on both CD4+ and CD8+ T cells in humans,. However, in contrast to the mouse, it appears that FoxP3 expression on human T cells can be associated with activated, $CD4^+$ and $CD8^+$ T cells that lack suppressive activity (55–57). Therefore, the definition of human Treg as CD4+CD25+FoxP3+ T cells has to be viewed with some caution. Unfortunately, the poor reproducibility of "functional" Treg assays coupled with the requirement for substantial amounts of blood to perform the studies has limited the number of clinical reports with a description of functional Treg cells. Nonetheless, we believe an assessment of Treg "suppressive" function should be required as a gold standard when phenotypic evidence suggests that Treg are increased.

A number of reports have indicated that some cancer patients exhibit increased frequencies of Treg (Table 1) and increased non-Treg:Treg cell ratios are associated with a good prognosis in patients with ovarian cancer (58,59). Further, several groups have identified the suppressive function of tumor-associated Treg in humans. This includes Treg isolated from NSCLC (41) and head and neck cancer patients (60,61). Unfortunately, due to limited accessibility to adequate tumor samples, most clinical studies are limited to enumeration of Treg cells in the peripheral blood. The correlation between the number or function of Treg in the peripheral blood and at the tumor remains to be explored. We consider it likely that the primary inhibitory effect of Treg occurs in the tumor microenvironment, where, in addition to regulating the immune response, tumor-induced Treg are likely also being induced by the regulatory-cytokine rich environment. Based on preclinical studies, we consider tumor-induced Treg cells to be the biggest hurdle to current state-of-the-art cancer immunotherapy. However, the absence of a surface marker that can distinguish natural and induced Treg has hindered this area of investigation. While no unique marker of iTreg has been identified, some Treg express other non-exclusive markers including; GITR (62), CD39/CD73 (21,63), CD223 (43), CD134 (64–66), Folate Receptor FR4 (67) and CD127 (68). The heterogeneity of expression of these markers on Treg suggests that there may be subsets with different functional properties. Further characterization of these subpopulations may identify novel biomarkers of iTreg cells that may be useful in the treatment of patients with cancer.

Tumor-induced Treg cells

Using a preclinical B16BL6 melanoma model, Turk et al., reported that concomitant tumor rejection was impaired by the presence of tumor for only a few days (46). This rapid onset of suppression was suggested to be the result of pre-existing natural Treg cells. In a model employing a B cell lymphoma that expressed a viral hemagglutinin antigen (HA), a role for both natural and adaptive Treg in tumor-induced immune suppression was identified (69,70). Given the differences in cytokines and factors produced by different tumors and what is known about the role of TGF-β, IL-10 and PGE-2 in developing iTreg, it is reasonable to assume that some tumors will generate more iTreg than others. While there may be generalizations across a specific tumor histology, we hypothesize that there may also be striking differences between different metastatic sites of tumor in one patient. This may also explain the variations in reports of Treg numbers from different investigators evaluating tumors of the same histology. We have also considered that differences or polymorphisms in immune relevant alleles could alter T cells, B cells or DC and provide an environment that favors the generation of iTreg in response to tumor. For example, breast cancer patients who

possess a mutated Tlr4 allele had a significantly shorter metastases–free survival than women with the normal allele (71). While strong evidence was presented that DC containing a mutant Tlr4 allele were less effective stimulators of effector T cells, it is possible they might be more efficient at triggering Treg cells. In this senario, increased priming of iTreg would lead to reduced priming of tumor-specific effector T cells and decreased metastases-free survival.

Vaccine-induced Treg cells

In retrospect, it should not be surprising that immunization with any antigen may induce Treg cells that limit the magnitude of the resulting immune response. Pre-clincal data from Zhou and co-workers and our laboratory show that repetitive vaccinations can induce Treg cells that limit the efficacy of immunotherapy (69) (MGL, manuscript submitted). We have observed a consistent increase in the frequency and absolute number of CD4+CD25+FoxP3+ Treg cells in the peripheral blood of prostate cancer patients two weeks following administration of their initial vaccine (Thompson et al., manuscript in preparation, Curti et al, manuscript in preparation). While we are unaware of any clinical evidence that vaccines have induced Treg that are suppressive in vitro or in vivo, the recent report of negative results from several large Phase III clinical trials of cancer vaccines (Eggermont et al., abstract reported at ASCO 2008) underscore the potential significance of these findings. Based on these preclinical and clinical findings we recommend that the next trials of therapeutic cancer vaccines carefully consider combining vaccines with an agent that can reduce Treg cell number or function. There are a few agents that can be combined with vaccines or co-stimulatory antibodies (eg., anti-CTLA-4) in patients (vida infra). While some of these are limited by intellectual property issues and business development concerns, several agents are already approved, tested in preclinical models and could be combined with vaccines in pilot phase I studies.

Reduction of Tregs in Clinical Trials

The flood of publications on Treg cells in cancer patients has also reawakened interest in the concept that chemotherapy may work in part by augmenting anti-tumor immunity by reducing or eliminating Treg cells. There is substantial pre-clinical evidence, dating back more than 30 years, that cylophosphamide can eliminate cells with suppressive function and augment the immune response (72–74). Based on these data, a number of clinical trials were initiated to examine the safety and efficacy of chemoimmunotherapy. The earliest trials combined standard cyclophosphamide containing chemotherapy regimens with the current state-of-the-art immunotherapy: BCG (75,76). Subsequent studies more faithfully recapitulated the preclinical models, pretreating with cyclophosphamide alone prior to administration of a cancer vaccine, with some encouraging results (77).

Berd and colleagues showed in vivo treatment with low-dose cyclophosphamide could eliminate some in vitro “suppressor” T cells (Concanavalin A responders) while maintaining other (PHA responder) T cell populations (77). Twenty years later there is additional clinical evidence that low-dose cyclophoshamide reduces the number of CD4+CD25+ cells and also reduces their capacity to suppress in vitro (78,79).

Cyclophosphamide may not be the only chemotherapeutic agent that can eliminate Treg cells. Additionally, since chemotherapy also induces lymphopenia, creating space for homeostasis-driven expansion of T cells, which can also augment the T cell response to vaccination, it is difficult to separate these two immune response promoting activities of chemotherapy. In one of the best studies we know, Machiels and colleagues tested cyclophosphamide, paclitaxel, or doxorubicin in combination with a GM-CSF-secreting tumor vaccine. While low doses of chemotherapy augmented therapeutic immunity, higher

doses eliminated the effect. While this study does not separate the impact of chemotherapy on Treg and/or the creation of space, we find it to be particularly important, as the tumor model is poorly immunogenic and therefore more relevant to issues of vaccines overcoming immunological tolerance (80).

Additional clinical trials, based on substantial preclinical data, have combined cancer vaccines with chemotherapy (81–86). While preclinical models suggest that low immunomodulatory doses of chemotherapy can augment the efficacy of vaccinations, higher doses of chemotherapy have a greater effect if the host is "reconstituted" by the adoptive transfer of T cells prior to vaccination (87,88). Based on these studies, we and others have added the adoptive transfer of peripheral blood T cells to patients lymphodepleted by higher doses of chemotherapy and then vaccinated (89,90) Curti et al, manuscript in preparation. While preliminary, these studies provide a platform for a detailed analysis of the Treg populations and enumeration of tumor-specific T cells. Many chemotherapy agents have a relatively short-term effect on T cells. However, the purine nucleoside analogue fludarabine causes a profound and long-lasting CD4 depletion in humans (91,92). Dudley et. al., provide indirect evidence that the combination of cyclophosphamide and fludarabine further augments the depletion of Treg (93) and promotes the therapeutic efficacy of adoptive T cell immunotherapy (94). Currently, some investigators are combining myeloablative chemotherapy/radiotherapy regimens, stem cell transplant, and adoptive T cell immunotherapy to determine if this provides an additional therapeutic advantage. However, if the underlying hypothesis is that cancer immunotherapy fails because of regulatory T cells, and the dose intensification of chemotherapy alone or combined with whole body irradiation is primarily undertaken to eliminate Treg at the site of tumor, maybe there are alternative approaches that would be as effective but less toxic to the patient. The stratefy would be to deplete Treg cells selectively. We hypothesize that it will not be necessary to eliminate all Treg cells, but it will be important to shift the balance in favor of tumor-specific effector T cells (Figure 1).

Nonchemotherapy approaches to reduce Treg cell activity

Below we describe four general strategies that can modulate Treg function: Depletion, modulation of function, inhibiting Treg induction and interfering with Treg suppressive mechanisms.

Depletion of Treg cells

Since Treg cells characteristically express CD25, it is possible that elimination and or reduction of CD25+ cells will reduce Treg cell numbers. However, activated CD4+ and CD8+ effector T cells can also express CD25. Thus, depletion of CD25+ cells by administering agents that target CD25+ cells risks depleting both effector and regulatory T cells. Because CD25 is preferentially expressed in the early phases of activation, investigators have tried to preferentially effect T-reg by optimizing the timing of depletion. Vieweg and colleagues gave a single dose of denileukin diftitox (Ontak), a recombinant DNA-derived cytotoxic protein composed of the amino acid sequences for diphtheria toxin fragments A and B followed by the sequences for interleukin-2, to reduce Treg in patients with renal cell cancer before vaccination with tumor-RNA-transfected DCs. DC vaccination combined with denileukin diftitox administration led to improved stimulation of tumor-specific T cells when compared with vaccination alone (95). In another study, there was a reduction in Treg numbers following denileukin diftitox in four patients with breast, ovarian or NSCLC; tumor regression was observed in one patient (96). This agent was also administered to patients with melanoma and 5 of 16 patients experienced regression of metastases (97). A similar study did not report cases of tumor regression (98). Another recombinant immunotoxin that may eliminate CD25+ cells in vivo is LMB-2, a single-chain

Fv fragment of the anti-CD25 monoclonal antibody fused to a truncated form of Pseudomonas exotoxin A (99). LMB-2 reduced Treg cells in vitro and transiently reduced the number of Treg in the peripheral blood of patients with melanoma, but did not augment the response to peptide vaccination (100). Since depletion of CD5+ cells in vivo will also deplete recently activated tumor-specific T cells we believe this strategy will work best as a preperative strategy in which Treg cells are depleted prior to vaccine administration. Anti-CD25 antibodies can also be used to eliminate or reduce the function of Treg cells. Although this approach has augmented tumor-specific responses in preclinical models, like the CD25-targeted toxins noted above, anti-CD25 antibody can also delete ag-specific effector T cells (101).

An alternative approach to reduce Treg in a cancer patient and maintain T cell repertoire is to delete $CD25^{+}$ cells from an apheresis product prior to infusing those cells into a lymphopenic patient whose T regs have been effectively depleted. Vaccinations can then be provided to prime efficiently a therapeutic anti-cancer immune response. Preclinical studies in tumor-bearing mice showed that this strategy was highly effective at augmenting a therapeutic anti-tumor immune response (Poehlein, et. al., manuscript submitted). The direct clinical translation of this strategy is underway at our Institute and the Ludwig Maximilians University, Munich, Germany. Two variations on this approach have already been reported. In one study lymphopenic patients were infused with autologous CD25-depleted PBMC and then administered high-dose IL-2. These patients exhibited a rapid reconstitution of Tregs, probably because some CD25+ Treg persisted in the lymphodepleted host and in the apheresis product responded to IL-2 administration (Powell JIT 30 ; 438). In the second study, patients lymphopenic after chemotherapy received a CD25-depleted apheresis product and the recovery of CD25+ cells was monitored (102). One patient exhibited an increased proliferative response to a tumor-associated antigen that coincided with a reduction in Treg numbers. Although this supports the basic concept, both approaches were limited by the transient nature of the depletion of Treg cells.

The absolute number of Tregs may be reduced by attacking a different cell surface target. While there is no Treg specific markers the cells do express CD4. The total number of CD4 T cells can be reduced with an anti-CD4 MAb or immunotoxin. This may reduce CD4 help, an important component of therapeutic immunotherapy in preclinical models (103). Despite this potential drawback, preclinical studies document that this approach can augment the efficacy of immunotherapy (LaCelle, et al submitted) (104).

Modulation of Treg function

Since selective depletion of Treg has proven to be impossible thus far, a second approach has been to employ antibodies or other agents that may reduce the suppressive function of Treg cells (64). Signalling via CD134 (OX40) (105) or CD137 (4-1BB) (106), members of the TNFR super family that are expressed on Treg cells can reduce Treg-mediated suppression. This may have important implications for clinical trials, as CD134 was found to be up-regulated in T cell-infiltrated tumor lesions and in tumor-draining lymph nodes of melanoma and head and neck cancer patients (107). Treatment with a stimulatory mAb to CD134 has increased anti-tumor therapeutic efficacy in some tumor models (108), augmented the development of CD4+ T cell memory (109), supported the differentiation of stimulated CD4+ T cells into Th1 or Th2 helper cells (110) and turned anergic T cells into effector T cells (111). CD137 has similar effects on effector T cell function. It is interesting to speculate that these molecules, originally identified by their costimulatory properties for effector T cells, may deliver a negative signal to regulatory T cells. This possibility is supported by data that suggests that using anti-OX40 to stimulate a CD134+CD4+CD25+ sub-population of Treg in vitro led to their loss of suppressive function (105). Therapeutic enhancement has also been shown in a transgenic tumor model in vivo; OX40 stimulation

had a stimulating effect on CD4+ Th1 helper cells and at the same time appeared to down-regulate the suppressive capacity of CD4+CD25+ Tregs (112). If these preliminary findings can be validated, agonists working through these receptors, with positive co-stimulatory activity on effector T cells and inhibitory effects on Treg cells, may represent the perfect adjuncts to clinical immunotherapy strategies.

Inhibiting induction of Treg

It may be possible to prevent the induction of Treg cells in vivo. For example, the administration of Cox_2 inhibitors may prevent Tregs by reducing the levels of prostaglandin E2 (PGE2), which is secreted by a variety of tumors, and can directly stimulate tumor cell growth (113) and increase Foxp3 and CD25 expression on CD4+ T cells (16,114). Studies are underway to determine whether administration of Cox2 inhibitors prior to surgery reduces the frequency of Treg cells at the tumor site.

Another possibility would be to reduce expression of FoxP3 with a small molecule or siRNA. Generation of an immune response against Foxp3, may reduce the number of cells expressing this marker. Nair and colleagues demonstrated that vaccination against Foxp3 enhanced the immune response against the tumor-associated antigen TRP-2 (104). Although this approach may be possible, the toxicity associated with absence of FoxP3+ cells in mouse and humans, suggests that it will not be clinically applicable. However, if a marker of tumor-induced Treg whose expression is restricted to the induced regulatory cells can be identified, a vaccination strategy targeting that molecule may improve the clinical results of immunotherapy.

Interfering with Treg suppressive mechanisms

Treg function may be inhibited by disrupting the production, secretion or function of its effector cytokines. For example, blocking TGF-β or TGF-β receptor signaling may eliminate a major mechanism Treg use to suppress tumor-specific effector T cells. An additional advantage of this approach is that it may also prevent/reduce the generation of new iTreg Cells. One strategy we have investigated is using an orally bioavailable small molecule, SM16, to interfere with the TGF-β signaling pathway at the time of vaccination. Increased T cell infiltration of the primary tumor, increased IFN-γ and cytolytic activity, and tumor regression resulted from the combination (Rausch et al, manuscript submitted). Antibodies against IL-10, IL-35 and or their receptors may also reduce the induction and function of Treg. These strategies, some of which have already been shown to augment efficacy in preclinical animal models are viable options that may be effective in the clinic.

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Figure 1.

Table

Tumor	Marker at cell level	Method	Reference
Cervical Intraepithelial Neoplasia	CD4+CD25+(bright)	PBMC	(115)
Hepatocellular Cancer	CD4+CD25+FoxP3		(116)
Breast Cancer	CD4+CD25+(bright)		(117)
MDS	CD4+CD25(bright) Foxp3+		(118)
Colorectal Cancer	CD4+CD25(bright) Foxp3+		(119)
Nasopharyngeal Cancer	CD4+CD25(bright) Foxp3+		(120)
NSCLC stage 1 disease	FoxP3	IHC	(121)
Pancreas Cancer	CD4+CD25+FoxP3	IHC	(122)
Pancreas Cancer	CD4+CD25(bright) Foxp3+	PBMC	(123)
Ovarian Cancer	CD4+CD25+	TIL	(59)
Malignant Glioma	CD4+CD25+Foxp3+	TIL	(124)