Altering regulatory T cell function in cancer immunotherapy: a novel means to boost the efficacy of cancer vaccines

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1. ABSTRACT

Cancers express tumor associated antigens that should elicit immune attack, but spontaneous immune rejection of established cancer is rare. Recent data demonstrate that specific and active tumor-mediated mechanisms hinder host anti-tumor immunity. $CD4^+CD25^+$ T regulatory cells (Tregs) are important mediators of active immune evasion in cancer. Disrupting tumor-mediated mechanisms hindering host immunity is a novel approach to tumor immunotherapy. Treg depletion improves endogenous anti-tumor immunity and the efficacy of active immunotherapy in animal models for cancer, suggesting that inhibiting Treg function could also improve the limited successes of human cancer immunotherapy. We have identified five strategies to block Treg activity: depletion, interference with trafficking, inhibition of differentiation, blockade of function or raising the effector T cell threshold for suppression. Discovery of additional regulatory cell populations expands the potential targets for these approaches. The fusion toxin denileukin diftitox (Ontak) reduces Treg numbers and function in the blood of some patients with cancer. We discuss specific strategies to block Treg activity and present some of our preliminary data in this area. Combining Treg depletion with active vaccination and other approaches poses additional challenges that are discussed.

2. INTRODUCTION

Malignant tumors express numerous tumor-associated antigens (TAA), yet rarely elicit endogenous immunity that effectively eradicates established tumors. Much data suggest that early stage cancers are eliminated by immune surveillance, whereas established tumors more likely induce immune tolerance (1). Recent work

demonstrating that a multitude of tumor-derived factors contribute to tumor microenvironmental immune tolerance and immunosuppression (2) helps elucidate the relative lack of effective immune surveillance in tumors at later stages.

Earlier enthusiasm that boosting the numbers or function of effector cells alone would be clinically useful is now tempered by the realization that active tumor-mediated programs can inhibit anti-tumor immunity. Thus, effective anti-cancer immunotherapy must overcome tumor microenvironmental immunosuppression for optimal utility.

Strong anti-tumor immunity often requires breaking peripheral tolerance to tumor associated antigens (TAA), which may be mediated by regulatory T cells (also called T regulatory cells or Tregs (3-5) as we refer to them in this review). Although CD4+ Tregs have been extensively characterized (reviewed in (6,7), many questions remain regarding their origins and functions. The most-studied Treg population is CD4+CD25+ and expresses the forkhead/winged helix nuclear transcription factor FOXP3. These Tregs can inhibit tumor specific CD4+ (8) or CD8+ (9) T effector cell function through incompletely understood mechanisms that include soluble factors and cell-to-cell contact (6, 7, 9-11). Functional Tregs are increased in peripheral blood of patients suffering from many types of cancers (12-16) and are also found in the solid tumor mass and draining lymph nodes (13). The accumulation of human CD4+CD25+ Tregs as well as FOXP3 expression in the tumor environment portend reduced survival in ovarian cancer (13,17). Tregs are considered important mediators of tumor microenvironmental immunosuppression in patients with cancer by inhibiting TAA-specific immunity. In support, experimental depletion of Tregs in mouse models of cancer improves endogenous immune-mediated tumor clearance (18) and TAA-specific immunity (19), and boosts the potency of tumor immunotherapy, including vaccination (20) or CTLA-4 blockade (21). Nonetheless, Tregs also control self-reactive T cells present in essentially everyone, including the Tregs mediating homeostatic peripheral tolerance. Thus, the therapeutic efficacy of TAA-reactive Tregs depletion may be tempered by induction of pathologic autoimmunity if homeostatic Tregs maintaining normal peripheral tolerance are also eliminated.

Therapeutic tumor vaccines effect some positive clinical responses, but their usefulness has been modest thus far. Significant and durable clinical benefits have generally not been observed, despite the fact that many vaccines engender TAA-specific immunity. It has recently been recognized that tumor-associated Tregs may contribute to the failure of some vaccines.

Thus, interfering with tumor microenvironmental Treg function could improve the efficacy of tumor immunotherapy. We define five general strategies to reduce Treg function. The first strategy is to eliminate Tregs, which can be achieved through monoclonal antibodies, targeted toxins, certain chemotherapeutic agents or other

molecules. Additional strategies include blocking Treg function and trafficking, inhibiting the interactions between dendritic cells and Tregs, and raising the effector T cell threshold for suppression. Some of these concepts have already been tested clinically, whereas others are in preclinical stages. We now discuss each strategy with reference to CD4$^+$CD25$^+$ Tregs, although they could also be applied to other types of regulatory cells. Additional considerations pertaining to the combination of Treg reduction and active immunizations will be addressed at the end of the review.

3. DEPLETION OF REGULATORY T CELLS

3.1. Anti-CD25 antibodies

CD25 is the α-chain of the IL-2 receptor, and is constitutively expressed on many tumor-associated Tregs. The anti-CD25 monoclonal antibody PC61 has been shown to deplete CD4$^+$CD25$^+$Treg function rapidly and efficiently (19) and augment tumor rejection in mice (18). However, recent data suggest that PC61 mediates functional Treg inactivation, not depletion (22).

Daclizumab (Zenapex) and basiliximab (Simulect) are anti-human CD25 antibodies approved for use in autoimmune diseases, transplantation and cancers including HTLV-1 induced adult T-cell lymphoma/leukemia (reviewed in (23)). To our knowledge there are no reports published on whether these anti-CD25 antibodies deplete Tregs in humans although anecdotal reports suggest limited, if any, such activity. A definitive study testing whether these antibodies affect tumor-related Tregs would be very helpful.

3.2. Denileukin diftitox

Denileukin diftitox (Ontak, DAB$_{389}$IL-2) is a recombinant protein fusing the active domain of diphtheria toxin to human interleukin (IL)-2. The United States Food and Drug Administration has approved it to treat cutaneous T cell leukemia/ lymphoma (24). It is targeted to the IL-2 receptor (25,26) and is proposed to be internalized through CD25 (the IL-2 receptor alpha chain) by endocytosis. It inhibits protein synthesis and induces apoptosis. Denileukin diftitox also targets cells through the beta and gamma subunits of the IL-2 receptor (CD122 and CD132, respectively) in addition to CD25 (26,27) (and Curiel, *et al.*, unpublished results).

Because denileukin diftitox is approved for treatment of CD4$^+$CD25$^+$ cutaneous T cell leukemia/lymphoma , we hypothesized that it would deplete phenotypically similar CD4$^+$CD25$^+$ Tregs and undertook a clinical trial demonstrating that denileukin diftitox depletes functional Tregs in blood of human patients with cancer, correlating with improved immunity (28) and clinical benefit (29). Another clinical trial (30) demonstrated that denileukin diftitox pretreatment improved immunogenicity of a vaccine comprised of RNA-transfected dendritic cells, augmenting proliferation of tumor-specific T cells compared to vaccination alone in patients with renal cell cancer. This study also showed that denileukin diftitox reduces functional blood Tregs and the

authors speculated this reduction as its mechanism of action. Denileukin diftitox reduces tumor-associated Tregs and augments immune-mediated tumor rejection of mice xenografted with syngeneic breast cancer cells (31). Nonetheless, a definitive relationship between Treg reduction and immune or clinical benefits in humans remains to be determined.

Another recent clinical study showed that denileukin diftitox treatment did not effect objective clinical responses in human melanoma or renal cell carcinoma, or inhibit $CD4^+CD25^+$ cell-mediated suppressive action *in vitr* (32). Reasons for a lack of denileukin diftitox effects on Treg function in this study are unknown, but could be due to differences in dosing or scheduling, differences in cancer immunobiology, prior IL-2 treatments, or other factors. Prior IL-2 treatment may be significant as IL-2 augments blood CD4+CD25+FOXP3+ Treg numbers in renal cell carcinoma and melanoma (33), and pediatric sarcoma (34) patients and signaling through the IL-2 receptor on Tregs increases Treg survival and suppressor function (35). In addition, a report just published showed that denileukin diftitox reduces Treg numbers and function in melanoma with improved melanoma-specific immunity (36).

Recently activated T cells also express high-levels of CD25. Thus, denileukin diftitox could also deplete $CD25^+$ effector cells. In fact, we have noted effector cell reduction in some patients after three or more weekly denileukin diftitox infusions at 12 µg/kg (Curiel, *et al.*, unpublished results). Recent *in vitro* work (30) supports the concept that denileukin diftitox may deplete recently activated T cells. An optimal balance between Treg depletion and effector function may be attained with different denileukin diftitox schedules or doses. We are now testing monthly infusion in ovarian cancer in this regard.

3.3 Cytotoxic chemotherapy
3.1. Cyclophosphamide

Cyclophosphamide is an alkylating agent widely employed in a number of chemotherapeutic regimens. Low-dose cyclophosphamide can decrease Treg numbers and thus may be useful as an immune modulator, even with cyclophosphamide-resistant tumors (37,38), although the mechanism is incompletely understood (39). Treg depletion by cyclophosphamide in a tumor model in rats augments the potency of active tumor-specific immunotherapy (40 and Treg reduction with low-dose, metronomic cyclophosphamide has recently been convincingly demonstrated in humans (41).

3.2. Fludarabine

The purine analogue Fludarabine is used to treat chronic lymphocytic leukemia (42). It decreases or eliminates CD4+CD25+ T cell suppression in patients with chronic lymphocytic leukemia (43). The effect of fludarabine on Tregs in chronic lymphocytic leukemia as well as other malignancies, merits further investigation.

4. BLOCKADE OF REGULATORY T CELL FUNCTION

4.1. Anti-GITR antibody

GITR (glucocorticoid-induced tumor necrosis factor receptor-related gene) is a cosignaling molecule found on murine (44) and human (45) T lymphocytes. It is expressed constitutively at high levels on Tregs and its expression is upregulated further upon T cell activation (46). GITR stimulation using anti-GITR monoclonal antibody reduces the suppressor function of murine Tregs (46,47), but not human Tregs (48). Agonistic anti-GITR monoclonal antibody treatment of tumor-bearing mice elicits potent tumor-specific immunity and eliminates established tumors in the absence of overt pathologic autoimmunity (48). Underlying molecular mechanisms have not been described in detail but likely involve inhibition of Treg–mediated suppression during antigenic stimulation (49). To our knowledge, this strategy has not yet been tested in a human clinical trial.

4.2. TLR signaling

Approximately 15 Toll-like receptors (TLR) are expressed ubiquitously on a variety of mammalian cells, including human Tregs (50). They recognize certain bacterial and viral pathogen-associated molecular patterns, and affect significant elements of specific immunity including dendritic cell maturation (50). Treg-mediated suppression can be inhibited by TLR-derived signals *in vitro* (51). TLR signaling for tumor immunotherapy is important as demonstrated by *in vitro* experiments showing that only vaccines providing appropriate TLR signals can reverse Treg−mediated tolerance. Further, dendritic cell-based vaccines (which lack TLR signals) break tolerance of CD8+ cells only after removal of Tregs or with addition of another TLR agonist (52). These experiments suggest that Treg inhibition using TLR ligation (such as TLR9 ligation by CpG oligonucleotides) may be a novel way to enhance the potency of certain cancer vaccines. Dendritic cell-based vaccines combined with Treg depletion is another alternative strategy, as discussed.

4.3. Anti-CTLA-4 antibodies

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is expressed at high levels on most Tregs (6,7,53,54), but also on certain other CD4+ and CD8+ T lymphocytes (55). As it downregulates T cell responsiveness (56,57), CTLA-4-mediated inhibition can hinder T cell activation in the initiation and progression of anti-tumor immunity (55). CTLA-4 blockade engenders strong anti-tumor immunity in murine melanoma (21). A human anti-CTLA-4 monoclonal antibody has been tested in phase I cancer clinical trials (58) with some encouraging results. Significant pathologic autoimmunity has been observed with this treatment (59), but may possibly be reduced by using different anti-CTLA-4 antibody clones.

A recent study of CTLA-4 blockade suggests that its anti-tumor effects are due to direct effects on $CD4^+$ and $CD8^+$ effector T cells, not through inhibition or depletion of Tregs (59). Thus, although CTLA-4 blockade is a promising immunotherapy candidate, its major mode of

action may not be through direct suppression of Treg function.

5. BLOCKADE OF REGULATORY T CELL TRAFFICKING

5.1. Chemokine signaling

Chemokines are small molecules that modulate trafficking of immune cells between normal and pathologically altered tissues, among other function (60). While normal tissues produce differing chemokines, cancers likewise secrete them (61). We recently demonstrated that the chemokine CCL22 mediates Treg migration into the human ovarian cancer microenvironment (13). We further showed that the CCL22 receptor, CCR4, is expressed on the majority of Tregs in ovarian cancer. Blockade of CCL22 significantly decreases Treg migration into ovarian tumors in an immunodeficient murine xenograft model, accompanied by immune rejection in the presence of anti-tumor effector T cells (13). Thus, blocking Treg trafficking may be useful to treating human cancers. However, CCL22 may also facilitate trafficking of effector T cells. Therefore, any potential benefit of CCL22 blockade, or of interrupting other trafficking signals requires further study as to potential therapeutic utility. While selective small-molecule chemokine receptor antagonists have gone into phase I clinical trials (62), therapeutic utility may be limited by the binding promiscuity of chemokine receptors, the redundancy of chemokine/ligand pairs, and the role of chemokines in normal tissue homeostasis or anti-tumor immunity. These additional effects of chemokine/receptor antagonism must be taken into account in strategies to block chemokines and their receptors.

6. BLOCKADE OF REGULATORY T CELL DIFFERENTIATION

6.1. Dendritic cell-regulatory T cell interactions

Dendritic cells are comprised of diverse populations of antigen presenting cells with diverse anatomic localizations, cell-surface phenotypes, and immunological functions (63). Human dendritic cells are typically categorized into myeloid (MDC) and plasmacytoid (PDC) subpopulations (63). In addition, a novel subpopulation of antigen presenting cells, vascular leukocytes, has been identified recently. These cells resemble dendritic cells as greater than 97% express CD11c and class II major histocompatibility antigens, but are distinct from MDCs or PDCs as they also express endothelial-specific markers (64). Whereas it is well-established that dendritic cells can initiate or upregulate immune responses, it is now becoming clearer that they are also capable of inducing immune tolerance (2).

We have identified distinct mechanisms by which dendritic cells can induce Tregs that could impede tumor immunity (2,65-67). In this regard, it may be possible to reduce Treg suppression in the tumor indirectly by blocking dendritic cell trafficking or function in the tumor environment and thereby reducing Treg differentiation or trafficking

6.1.1. PDC-T Cell interactions

By contrast with normal blood PDCs, PDCs in the tumor microenvironment enhance tumor vascularization (68), and promote differentiation of IL-10-expressing T cells (66). Tumor PDC-activated T cells include CD8+CCR7+CD62L+IL-10+ cells that suppress T effector function through IL-10 (68) and are thus functional CD8^{+} Tregs. Therefore, PDC-CD8^{+} T cell interactions could foster differentiation of CD8^{+} Tregs in tumor and this differentiation could possibly be inhibited by interfering with these tumor PDC-T cell interactions.

The chemokine CXCL12 (stromal derived factor 1, SDF-1), is secreted in enormous quantities by ovarian cancer cells and attracts dysfunctional PDC (67) which can then foster CD8+ regulatory T cell differentiation (66). We demonstrated that CXCR4 blockade with a specific antibody increases tumor PDC apoptosis, while reducing their chemotaxis and adhesion/transmigration *in vitro* (67). Various bicyclam compounds that specifically antagonize CXCR4 signals (AMD3100 (69) or AMD3465 (70)) are useful in certain clinical settings such as mobilizing stem cells or blocking human immunodeficiency virus infection (71). In the treatment of cancer, they may also be useful in the tumor microenvironment by reducing PDC entry, thus reducing PDC-T cell interactions (in addition to reducing CXCR4-mediated tumor metastasis or other detrimental events). In addition, recent studies suggest that IL-2 enhances Treg cell migration into tumors by increasing Treg CXCR4 expression, a receptor for CXCL12 (72). Thus, CXCR4 blockade may decrease Treg numbers or function as well as PDC migration in ovarian cancer.

PDCs could alternatively be depleted selectively. Relatively specific antigens expressed on human PDCs (BDCA-2 or BDCA-4) (73) and murine PDCs (mPDCA-1) (74) have been described. While a depleting antibody for mPDCA-1 is available, an effective depleting antibody for human PDC has not been reported to our knowledge. Thus, while manipulating PDC-T cell interactions represents an attractive strategy for tumor immunotherapy, much more research must be done to establish its clinical efficacy and a suitable means to effect it.

6.1.2. MDC-T cell interactions

B7-H1 is member of the B7 family of cosignaling molecules and is upregulated on MDCs in ovarian cancer (65). Signaling through B7-H1 enhances tumor growth (75) by inducing apoptosis of effector T cells (76). Furthermore, B7-H1 signals in the tumor environment induce IL-10 production by T cells, causing immune suppression (65). Blockade of B7-H1 enhances MDC-mediated T cell activation accompanied by downregulation of T-cell IL-10 production and upregulation of T cell IL-2 and interferon-γ (65). B7-H1 signals on endothelial cells can induce CD4+CD25+FOXP3+ Tregs (77). Thus, blockade of B7-H1 signals on MDCs (and perhaps other cells) in the tumor environment could inhibit development or function of tumor-associated Tregs.

6.1.3. Vascular leukocyte-T cell interactions

Vascular leukocytes exhibit properties of both DCs and endothelial-like cells. Vascular leukocytes have been shown to accumulate in the environment of human and murine ovarian cancer and other tumors (64,78). These cells use their vasculogenic potential to develop blood vessels and promote tumor growth *in vivo* (64,78).

The immune function of vascular leukocytes in tumors is not fully characterized. They are tolerogenic, probably through inducing IL-10 secreting T cells and also promote tumor growth. In this aspect, vascular leukocytes resemble tolerogenic dendritic cells that drive Treg development. New data now suggests that vascular leukocytes can contribute to Treg expansion in cancer (G. Coukos, *et al.*, unpublished data). Thus, preventing T cell-vascular leukocyte interactions might decrease Treg numbers or function.

6.1.4. Other strategies

Additional pathways leading to Treg differentiation may be useful to disrupt to attempt to reduce Treg numbers or function. These pathways include signals from IL-2, IL-10, interferon-α, VEGF, TGF-β and prostaglandins among others (2,4,5,7,79,80). Raising the threshold of effector T cell to Treg-mediated inhibition (such as through CTLA-4 signaling blockade) is an interesting concept, however, it could be limited by serious consequences of T-cell overactivation (81).

7 COMBINING DEPLETION OF REGULATORY T CELLS WITH TUMOR VACCINES

As we begin to combine Treg depletion with active cancer vaccination, the following additional considerations must be addressed for optimal effectiveness.

7.1. Antigen specificity of Tregs

Recent evidence demonstrates that Tregs have antigen specificity, and TAA-specific CD4+CD25+ Tregs have been identified (82). A therapeutic modality that selectively depletes TAA-specific Tregs would be highly desirable as this might perturb homeostatic immunity the least. However, such a strategy is dependent on identifying TAA-specific Tregs. Whereas techniques exist to track antigen specific CD4+ and CD8+ T cells *in vivo*, antigen specific Tregs cannot yet be routinely identified *in vivo* . Further, it will be difficult or impossible to relate the effects of Treg depletion to observed immune effects without information regarding the removal of suppressive barriers to activation of TAA-specific effector T cells. Related to this issue is the lack of a clear understanding of what role additional Treg populations might play in suppressing TAA-specific immunity even if these Tregs are not TAA-specific. These additional Tregs could mediate immune suppression through generic suppression of any local T cell, or might inhibit innate immune cells (such as natural killer cells or macrophages) that might otherwise contribute to anti-tumor immunity.

7.2. Expansion of Tregs by cancer vaccines

Recent data suggest that cancer vaccines may contribute to immunosuppression by expanding tumor microenvironmental Tregs (83). These data are consistent with prior observations that specific subsets of dendritic cells can also expand various Treg subsets (65,66). Taken together, these data imply that the capacity of cancer vaccines to induce effective anti-tumor immunity may be impaired by the simultaneous expansion of vaccine-induced Tregs. Therefore, future cancer vaccine trials should consider measuring expansion of TAA-specific Tregs in addition to studying expansion of TAA-specific immunity.

7.3. Timing of Treg depletion in relation to active vaccination

In a recent report of a mouse model for colon cancer, Treg depletion with PC61 antibody was most effective in augmenting immunity when given just at, versus before or after vaccination (84). These data do not confirm that Treg depletion when combined with active vaccination is always optimal at vaccination. Timing likely will vary depending on the specific vaccine and adjuvant used, the relative potencies of competing TAA-specific effector cells and Tregs in an individual, the method used to deplete Tregs, the specific cancer involved and its stage, in addition to other factors. This study (84) also demonstrated that optimal clinical efficacy depended on multi-modal treatment. A recent clinical trial (30) showed that pretreatment with denileukin diftitox improved the immunogenicity of vaccination with RNA-transfected dendritic cells, significantly improving stimulation of tumor-specific T cells in patients with renal cell cancer compared to vaccination alone. The authors of this study suggest that Tregs should be depleted just before vaccination, as this will most likely avoid collateral effects of the immunotoxin on vaccination-induced effector T cells. This valid concern is supported by *in vitro* data from the same study suggesting that activated T cells, which express CD25, are killed by denileukin diftitox. In addition, we have shown that weekly denileukin diftitox in patients with advanced cancers will also deplete effector cell populations (Curiel *et al.*, unpublished observations). Thus, future strategies for Treg depletion should focus on Treg-specific targeting, which will become more readily available with the identification of Treg-specific surface antigens. Clinical studies investigating Treg depletion at differing times relative to vaccination regimens are also needed to study this effect.

7.4. Pathologic consequences of Treg depletion

It is now well accepted that peripheral tolerance to self antigens in mediated at least in part by CD4+CD25+ Tregs (7). It is therefore conceivable that Treg depletion as a therapeutic strategy could induce pathologic autoimmunity. While autoimmunity associated with Treg depleting interventions in cancer patients has not been reported to date, the potential remains and must be monitored.

7.5. The regulatory cell population targeted for depletion

Our work and that of most investigators studying mice and humans has focused on CD4+CD25+ Tregs.

Nonetheless, other regulatory cell populations proposed to be immunopathogenic in cancer have also been identified, including CD8+ Tregs (66), immature myeloid cells (85), B7-H4+ myeloid cells (86) and NKT cells (87,88). These cells may ultimately be shown to play immunopathologic roles in cancer and undermine the efficacy of tumor immunotherapies. Further studies of their activities, and of effects of their depletion (including in ongoing trials aimed at depleting CD4$^+$CD25$^+$ Tregs) are thus worthwhile.

8 SUMMARY AND CONCLUSIONS

Recent data demonstrate that CD4$^+$CD25$^+$ T regulatory cells (Tregs) can inhibit tumor-specific immunity and that their increased numbers correlate with worsened outcomes in some cancers. Studies in mouse models demonstrate that reducing Treg activity boosts endogenous anti-tumor immunity, and increases the efficacy of active immune interventions. Consequently, inhibiting Treg function is a strategy worth considering in human cancer immunotherapy. Preliminary studies from small human clinical trials have identified denileukin diftitox (Ontak) as an agent useful in this regard in ovarian and renal cell cancer, but not in melanoma. Denileukin diftitox can potentially kill any T cell expressing functional IL-2 receptors (including effector T cells), which may limit clinical utility in some settings. Agents specifically targeting Tregs would be advantageous and are in development in some laboratories, including ours. Identification of Treg-specific markers would also be helpful in this regard. Cyclophosphamide, fludarabine and other agents are also under study to deplete Tregs.

Aside from directly killing Tregs, their function can be reduced by interfering with their suppressor function, trafficking patterns or differentiation. Antigen specificity of Tregs and potential for inducing pathologic autoimmunity are concerns and areas for further study. When designing trials incorporating Treg depletion with active immunization, additional considerations include the timing of Treg depletion relative to vaccination, and the potential for vaccines to induce TAA-specific Tregs (in addition to generation of TAA-specific effector cells).

Finally, we must remain cognizant of the potential immunopathologic roles played by additional suppressor cell populations aside from CD4$^+$CD25$^+$ Tregs, including CD8$^+$ regulatory T cells, several myeloid cell populations and NKT cells, and cognizant of additional tumor-mediated mechanisms of defeating host immunity.

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