Review

Cancer Research

Regulatory T-cell Modulation Using Cyclophosphamide in Vaccine Approaches: A Current Perspective

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Abstract

Regulatory T cells (Treg) have become an important player in regulating anticancer immune responses. In fact, published studies describe a correlation between tumor-infiltrating Tregs and poor prognosis. Once called "suppressor T cells," these T cells evaded isolation because of a lack of known markers that distinguished them from other T cells. However, the biology of these T cells is currently a major focus of immunologic research. Markers have since been discovered that identify these T cells and provide insights into how these T cells are regulated. Despite these advances, much needs to be learned about the subsets of Tregs and their specific roles in regulating immune responses. In addition, specific agents that target Tregs are currently unavailable. Cyclophosphamide has emerged as a clinically feasible agent that can suppress Tregs and allow more effective induction of antitumor immune responses. This review focuses on the use of cyclophosphamide in targeting Tregs to augment cancer vaccine approaches. However, these principles can also be applied to other immunotherapy strategies. *Cancer Res; 72(14); 3439–44. ©2012 AACR.*

Introduction

Cancer vaccines have come of age, with the first vaccine approved for prostate cancer treatment. Yet, the survival benefit as a single agent is modest. Accumulating evidence now supports multiple mechanisms of immune tolerance that inhibit the most potent antitumor immune responses and reinforce the use of a multipronged approach, which combines agents that prime and expand the best tumor-specific T cells with agents that target immune-suppressive factors. Combinatorial vaccination strategies are under development, testing immune adjuvants that recruit and activate antigen-presenting cells (APC) and agents that provide additional activating signals to APCs and T cells. Also under development are antagonist antibodies that target inhibitory signaling pathways, promoting checkpoint blockade of signals that T cells receive from APCs, tumors, and Tregs. However, currently no agents specifically inhibit Tregs. Cyclophosphamide is the agent used most extensively to inhibit Tregs, not only because it is widely available and inexpensive, but increasing evidence suggests that it has multiple immune-modifying properties. Entire reviews have focused on this multifaceted aspect of cyclophosphamide and have enumerated the vast number of proposed mechanisms responsible for its immune properties. This review focuses specifically on the use of cyclophosphamide to inhibit Tregs in the context of cancer vaccines.

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Historical Perspective

Cyclophosphamide has many purported immune-modulatory mechanisms. Sistigu and colleagues recently reviewed many mechanisms that include TH2/TH1 to TH17 shifts in cytokine production, induction of TH17 cells, inhibition of Tregs, enhancement of T-cell proliferation and survival, and resetting of dendritic cell homeostasis (1, 2). However, the earliest mechanism proposed was the inhibition of a population of suppressor T cells. These T cells were difficult to isolate because of the lack of a marker specific for this population until the discovery of FOXP3, a transcriptional regulator of what are now known as Tregs. Multiple subsets of Tregs, constitutive and inducible, $CD4^+$ and $CD8^+$, $FoxP3^+$ and $FoxP3^-$, have since been described in the context of malignancy. Most studies associate the presence of $CD4^+CD25^+FoxP3^+$ Tregs in tumors with poor prognosis. This finding has been shown in a number of cancers, including breast, ovarian, and pancreatic cancers (3–5). In some cancers, such as Hodgkin lymphoma, Tregs in the tumor microenvironment have been associated with improved clinical outcomes (6). More recent studies suggest that it is T-effector (Teff)–Treg ratios that correlate with effective antitumor responses (7). In fact, a natural response to vaccination is the concurrent induction of Teffs and Tregs. Thus, it is likely that the balance of these T-cell subsets influences outcomes.

As early as 1974, Polak and Turk postulated that cyclophosphamide reversed immune tolerance in a guinea pig–sensitization model through inhibition of a yet-to-be-identified suppressor T-cell population (8). Throughout the 1980s, experiments done by North and colleagues suggested that cyclophosphamide augmented adoptive immunotherapy by inhibiting suppressor T cells. In 1988, Awwad and North (9) published an article on the effects of cyclophosphamide (150 mg/kg i.v.) in combination with passively

transferred tumor-sensitized T cells in a cyclophosphamide-resistant tumor model. Adoptively transferred T cells were administered 1 hour after cyclophosphamide. Because the tumor was resistant to cyclophosphamide, this model suggested that cyclophosphamide's effects were through the inhibition of suppressor T cells, rather than a direct effect on tumor burden. That same year, Berd and Mastrangelo used a regimen of low-dose cyclophosphamide (300 mg/m^2 i.v.), given 3 days prior to vaccination with autologous melanoma cells admixed with Bacillus Calmette-Guerin (BCG), to treat patients with melanoma (10). Cyclophosphamide plus vaccine resulted in a decrease in the proportion of CD4$^+$ T cells expressing 2H4 (CD45), which they stated was an identifier of "inducers of suppression." The reduction did not become apparent until day 28 and became statistically significant on day 49. Interestingly, they did not see an effect of cyclophosphamide on the suppressor population expressing the interleukin-2 receptor (CD25).

Machiels and colleagues evaluated the potential of several chemotherapies, including cyclophosphamide, doxorubicin, and paclitaxel, to potentiate the effects of granulocyte macrophage colony-stimulating factor (GM-CSF)–secreting whole-cell vaccines in the *HER-2/neu* mouse model of mammary cancers (11). They determined that both the dose and sequence of drug administration in relation to vaccine delivery was important in mediating enhancement of vaccine effects. Again, the mechanism for cyclophosphamide's effect was not due to direct cytolytic effect on cancer cells, but through cyclophosphamide's influence on immunity. When cyclophosphamide was given at a dose range between 50 and 150 mg/kg 1 day prior to vaccine, the combination controlled tumors more effectively than either agent alone. The same treatment 7 days later was ineffective. Higher doses of cyclophosphamide did not enhance and often abrogated vaccine efficacy. The improved efficacy of lower doses of cyclophosphamide supported that the antitumor effects were not mediated through cyclophosphamide's cytolytic capacity. In these studies, cyclophosphamide was shown to amplify the T-helper 1 neu-specific T-cell response.

In 2004, Ghiringhelli and colleagues (12) showed that a single administration of cyclophosphamide at 25 to 30 mg/kg in rats depleted CD4$^+$CD25$^+$ T cells and delayed the growth of colon carcinomas. In addition, cyclophosphamide given prior to tumor cells mixed with BCG resulted in complete regression of tumors. Furthermore, cyclophosphamide induced a decrease in the CD4$^+$CD25$^+$/CD4$^+$ splenic T-cell ratio in the spleen resected 7 days after a single dose. This decrease was also seen with methotrexate and anti-CD25 monoclonal antibody. In this model, the CD4$^+$CD25$^+$/CD4$^+$ ratio reached its nadir at 7 days.

After Hori and colleagues (13) identified the transcription factor FoxP3 as a key regulator in Treg development, reports followed showing cyclophosphamide-mediated reductions in FoxP3$^+$ Tregs. Subsequent studies showed that, in addition to cyclophosphamide's effect on decreasing Treg number, low-dose cyclophosphamide decreased the functionality of Tregs (14). Lutsiak and colleagues (14) isolated Tregs from untreated and cyclophosphamide-treated (2 mg intraperitoneally) mice 2 and 10 days after cyclophosphamide treatment and evaluated the Tregs in suppression assays. Cyclophosphamide-treated Tregs had significant impairment in their suppressive capacity, which returned by day 10 after treatment. Cyclophosphamide also interfered with homeostatic proliferation of Tregs, increased their susceptibility to apoptosis, and decreased their expression of suppression markers, including glucocorticoid-induced TNF receptor–related protein (GITR) and FoxP3.

Low- Versus High-Dose Cyclophosphamide

Depending on the dose administered, cyclophosphamide's antitumor effects are either through immune potentiation or direct cytolytic activity. In the *HER2/neu* mouse model, cyclophosphamide was most effective in enhancing vaccine effects given at a dose range between 50 and 150 mg/kg (11). Higher doses hampered vaccine-induced immunity by causing bone marrow suppression. This finding was further supported by Motoyoshi and colleagues, who showed that low- (20 mg/kg), but not high-dose (200 mg/kg), cyclophosphamide selectively suppressed CD4$^+$CD25$^+$ T-cell numbers, sparing conventional CD4$^+$ and CD8$^+$ T cells and preventing murine hepatoma growth (15). In the low-dose group, the decline in CD4$^+$CD25$^+$ T cells was more profound and recovered more slowly than CD4$^+$ T cells, resulting in lower ratios of CD4$^+$CD25$^+$/CD4$^+$ T cells for longer periods of time. In contrast, in the high-dose group, all T-cell subsets and the ratio were severely decreased. Low and high doses of cyclophosphamide were also compared in immuncompetent and nude mice. Although low doses were effective in treating tumors only in immunocompetent mice, the high doses worked in immunocompetent and nude mice. This finding suggested that low-dose cyclophosphamide contributes to antitumor immunity, whereas high-dose cyclophosphamide works solely through its cytotoxic effects. Low-dose cyclophosphamide also resulted in higher intratumoral lymphocyte infiltration. Repletion of CD4$^+$CD25$^+$ T cells abolished the antitumor effect of low-dose cyclophosphamide.

Emens and colleagues (16) conducted a trial in patients with breast cancer to address the question of dosing. In this study, an allogeneic, HER2-positive GM-CSF–secreting breast tumor vaccine was given alone or in sequence with low-dose cyclophosphamide and doxorubicin. The study used a factorial design to identify the cyclophosphamide and doxorubicin dose combination that maximized vaccine-induced immune responses. The range of cyclophosphamide doses tested was 200 mg/m^2 to 450 mg/m^2. On the basis of the *HER2/neu* mouse model, cyclophosphamide was given 1 day prior to vaccine at the time of T-cell priming, and doxorubicin was given on day 7 at the time of T-cell expansion (11). Immune readouts included assessment of delayed-type hypersensitivity (DTH) responses to HER2 human leukocyte antigen–class II–restricted peptides and measurement of HER2 antibodies. The addition of 200 mg/m^2 cyclophosphamide had no impact on the rate of DTH development, but cyclophosphamide doses higher than 200 mg/m^2 suppressed vaccine-induced DTH responses, compared with vaccine alone. Furthermore, induction of HER2-specific humoral immunity was optimally enhanced at the 200-mg/m^2 dose and decreased with higher cyclophosphamide doses. Although this study assessed the combination of

cyclophosphamide and doxorubicin and not cyclophosphamide alone, the results suggest that cyclophosphamide doses above 200 mg/m^2 may abrogate immune responses induced by vaccination and that the optimal cyclophosphamide dose for enhancing vaccine-induced immunity in humans is 200 mg/m^2 or lower. Lower doses were not tested in this study. However, Greten and colleagues (17) evaluated single-agent cyclophosphamide doses of 150, 250, and 350 mg/m^2 in patients with hepatocellular carcinoma and reported that the 2 lower doses induced a decrease in the absolute and relative frequency of Tregs in the blood of patients with hepatocellular carcinoma, and the 250 mg/m^2 dose impaired suppressor function and showed decreased Treg frequency out to day 71. Alpha-fetoprotein–specific T-cell responses were also induced in the lower treatment arms (17). On the contrary, a previous report testing an allogeneic melanoma cell vaccine in patients identified 300 mg/m^2 given 3 days prior to vaccine as the optimal dose (18). The other doses tested were 150 mg/m^2 and 75 mg/m^2. However, the immune readout was the reduction in peripheral CD8$^+$CD11B$^+$ suppressor cells. A second melanoma study evaluating the addition of melanoma-associated helper peptides and cyclophosphamide, 300 mg/m^2 i.v. 3 days prior to a melanoma vaccine, concluded that cyclophosphamide did not augment T-cell responses to that vaccine (19).

Given the findings in murine studies and the clinical trials assessing actual effector responses, future studies should focus on evaluation of the lower range of cyclophosphamide doses typically used to inhibit Tregs (range, 150–1,000 mg/m^2 i.v.). Additional studies are needed to better understand the effects of cyclophosphamide at lower doses.

Metronomic Oral Cyclophosphamide

Metronomic oral cyclophosphamide is administered in an iterative low-dose fashion. Historically, low doses of chemotherapeutic agents have been given in this manner to inhibit angiogenesis. The potential benefit of an alternative way to administer cyclophosphamide is that a lower, more continuous dosing schedule may allow for more effective and prolonged inhibition of Tregs, as most studies suggest that Treg levels recover 7 to 10 days after i.v. administration. Ghiringhelli and colleagues first evaluated the effects of metronomic cyclophosphamide in patients with advanced solid tumors (20). Patients received cyclophosphamide, 50 mg orally given twice a day, 1 week on and 1 week off for 1 month or more. The number of circulating CD4$^+$CD25^{high} Tregs in the 9 patients studied were higher at baseline compared with numbers in healthy volunteers. After 1 month of cyclophosphamide treatment, CD4$^+$CD25^{high} T cells were decreased both in percentage (7.9%–3.1%) and absolute numbers (28.7–6.4 cells/mm^3). The decrease occurred in all patients. Of the 4 patients with adequate samples for evaluation, the number of FoxP3$^+$ cells also decreased. This decrease was selective and did not occur in other T- or natural killer (NK)–cell subsets.

Cyclophosphamide's effect on T-cell and NK-cell function was also evaluated. In addition to Teff inhibition, Tregs inhibit innate immunity by downregulating NK-cell proliferation and function. NK-cell lytic activity was tested after 1 month of metronomic cyclophosphamide by determining the capacity of patients' NK cells to kill NKG2D ligand-expressing K562 cells. NK activity in patients receiving cyclophosphamide was enhanced and restored to healthy volunteer levels. T-cell proliferation was also tested using carboxyfluorescein diacetate, succinimidyl ester–labeled peripheral blood mononuclear cells, either untreated or depleted of CD25$^+$ T cells, and then cultured with anti-CD3 and anti-CD28 for stimulation. Cyclophosphamide treatment also restored T-cell proliferation.

Metronomic cyclophosphamide was also evaluated in patients with breast cancer, in whom it has historically been used for its antiangiogenic properties. Patients with breast cancer treated with continuous low-dose cyclophosphamide had a transient reduction in Tregs lasting 4 to 6 weeks (21). Patients received cyclophosphamide, 50 mg orally daily for 3 months. Tregs were reduced within 14 days (3.0% vs. 5.1%), remained decreased until day 42, and returned to pretreatment levels by day 84. Interestingly, endogenous breast tumor–reactive T cells were detected in 27% of patients before cyclophosphamide treatment and increased to 73% on day 14, 80% on day 42, and 88% on day 84. A total of 58% of patients had stable disease. An increase in breast tumor–reactive T cells was associated with both stable disease and overall survival. Although Ghiringhelli and colleagues observed diminished functionality of Tregs at 30 days, suppressive function changes were only tested at 84 days and were not seen in this study (20). Despite a transient and minimal effect on Treg numbers and function, metronomic cyclophosphamide stably increased breast tumor–reactive T-cell responses.

The use of metronomic cyclophosphamide combined with active immunotherapy has recently been reported (22). Patients with advanced solid tumors were treated with 3 different regimens of low-dose cyclophosphamide in combination with an oncolytic adenovirus. Cyclophosphamide was given either as oral metronomic (50 mg/day), a single i.v. injection (1,000 mg), or both. Metronomic cyclophosphamide was given starting 1 week before the adenovirus, and i.v. cyclophosphamide was given 1 hour prior to the adenovirus. The adenovirus was injected intratumorally. Metronomic cyclophosphamide (oral and oral + i.v.) decreased Tregs and induced antitumor or antiviral responses. All cyclophosphamide regimens resulted in higher rates of disease control when compared with the rates for the adenovirus vaccine only. The metronomic groups were most effective in decreasing Treg numbers. However, prior studies with i.v. cyclophosphamide would have predicted recovery of Treg numbers at the 30-day time point evaluated. In addition, the dose of 1,000 mg (approximately 600 mg/m^2) is higher than the one used in many studies. The i.v. cyclophosphamide was administered only 1 hour prior to adenovirus administration. This timing may be appropriate, as the kinetics of the immune response induced by virus-induced tumor lysis differ from that of peripherally administered vaccines. Although it may be difficult to directly extrapolate these data to other vaccine strategies, it is important to note that all cyclophosphamide groups did better than the patients getting adenovirus vaccine alone. Although numerically, the best progression-free survival and overall survival were seen in the oral + i.v. group, the study was

not powered to compare the clinical outcomes between the different groups. Numerous studies are in progress, combining metronomic cyclophosphamide with active vaccination strategies (ClinicalTrials.gov) for a variety of cancers. These studies are incorporating a range of immune analyses. Results from these studies will influence future trial designs.

Downstream Effects of Treg Inhibition

In parallel to studying the optimal cyclophosphamide dose, schedule, and route of administration required to optimally modify Tregs, studies are exploring the mechanisms by which cyclophosphamide modulates antitumor immunity. Enhancement of NK- and T-cell lytic activity and proliferation were described above. To further elaborate on the downstream effects on the antitumor T-cell response, Ercolini and colleagues reported on another mechanism of cyclophosphamide-mediated vaccine enhancement (23). Using *HER2/neu* mice, which are tolerized to neu-expressing tumors, they found that cyclophosphamide inhibited Tregs by selectively depleting the cycling population of $CD4^+CD25^+$ T cells. Tetramer-binding studies showed that cyclophosphamide pretreatment allowed activation of high-avidity HER-2/neu-specific $CD8^+$ T cells, comparable to those generated in the parental strain in which HER-2/neu is immunogenic. The discovery that latent pools of high-avidity tumor-specific T cells can exist in a tolerized host gives further credence to the potential for active immunization in cancer.

The concept that Treg inhibition may recruit higher-avidity effector T cells was further evaluated in a clinical trial in patients with advanced pancreatic cancer, testing an allogeneic, GM-CSF–pancreatic cancer vaccine given alone or in sequence with low-dose cyclophosphamide (24). The GM-CSF vaccine given 1 day after cyclophosphamide resulted in higher rates of mesothelin-specific T-cell responses that were also of higher avidity than those observed in patients treated with vaccine alone. In addition, higher avidity T-cell responses were associated with prolonged progression-free survival and overall survival in a heavily treated patient population (4.3 vs. 2.3 months overall survival).

In subsequent studies in *HER2/neu* mice, cyclophosphamide was shown to exert its effects specifically by depleting a $CD25^{low}$ Treg effector/memory subpopulation, which resides in the tumor microenvironment and preferentially suppresses high-avidity HER-2/neu-specific T cells (25). Effector/memory-like $CD4^+FoxP3^+$ Tregs preferentially hone to nonlymphoid and inflamed tissues and are the predominant cells that traffic into tumors. $CD25^{low}$ Tregs express an activated phenotype with higher levels of ICOS, CD44, CTLA-4, GITR, β1 integrin, LFA1, and CXCR3 and lower levels of CD62L. In contrast, $CD25^{high}$ Tregs are predominantly a lymph-node–residing population. As a result of cyclophosphamide's effects on the $CD25^{low}$ Treg subset, adoptively transferred high-avidity HER-/2neu-specific T cells from vaccine plus cyclophosphamide-treated *HER2/neu* mice expressed higher tumor-trafficking integrins and CXCR3 levels than did the T cells from the no-cyclophosphamide group. This effect was not seen on low-avidity T cells. Specific targeting of the most relevant Treg subsets that are both present in the tumor microenvironment and capable of suppressing high-avidity tumor-specific T cells is extremely relevant in cancer-bearing hosts. Ongoing research dissecting the roles of Treg subpopulations will allow further refinement in approaches targeting these suppressor subsets (Fig. 1).

Key Findings

Key findings in the use of cyclophosphamide in targeting Tregs include the following: (i) Low-dose cyclophosphamide (i.v.) results in transiently decreased Treg frequencies;

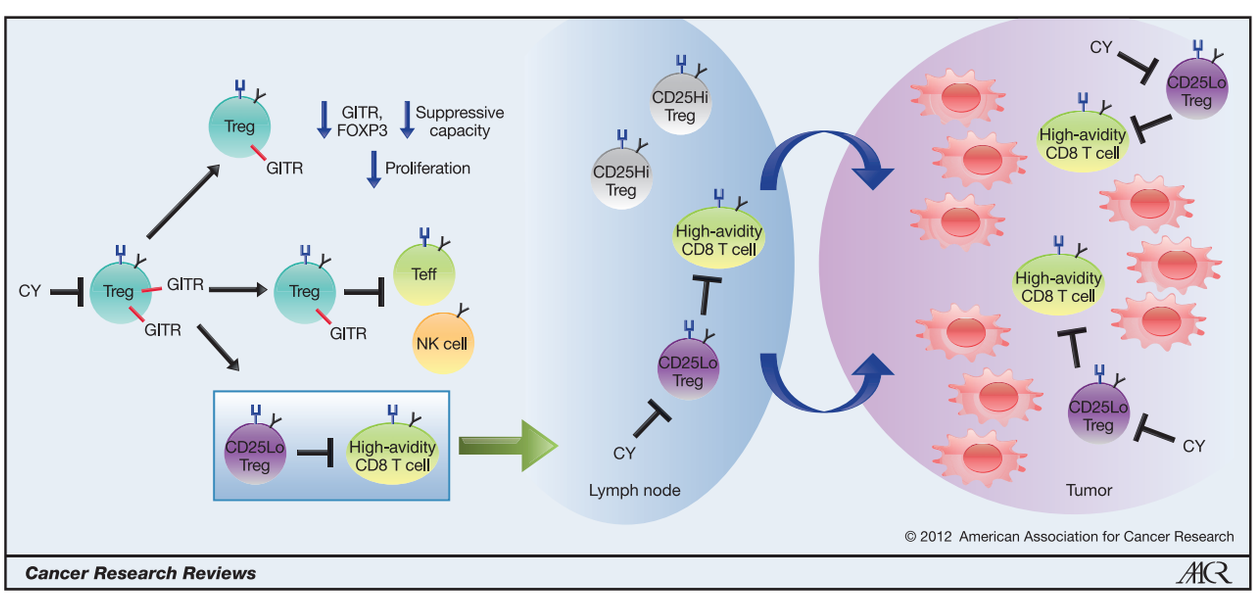

Figure 1. Treg modulation using cyclophosphamide. Cyclophosphamide (CY) decreases Treg numbers and function. The use of cyclophosphamide to preferentially inhibit Treg subsets that suppress high-avidity tumor-specific T cells has implications for cancer immunotherapy.

(ii) metronomic cyclophosphamide results in prolonged Treg suppression, which returns to baseline with continued administration within 4 to 6 weeks; (iii) tumor-specific immune responses are enhanced despite only transient reductions in Treg numbers; (iv) cyclophosphamide-mediated alterations in Treg function may contribute to cyclophosphamide's efficacy; (v) differences in dose, schedule, and routes of cyclophosphamide administration contribute to variable outcomes between studies; and (vi) cyclophosphamide depletion of Tregs can uncover high-avidity tumor-specific T cells.

Future Directions

The results from reported studies are already informing the design of future studies. In addition, preclinical studies are showing efficacy of low-dose cyclophosphamide in combination with other immunotherapeutic agents, such as OX40 receptor ligands and PD-1 antagonists. As these agents make it to the clinics, combinations with cyclophosphamide are likely to follow. Future studies should evaluate cyclophosphamide's effects on Treg-to-T-cell ratios in tumors and on downstream immune responses. These results may be more accurate indicators of clinical outcomes than observing changes in systemic Treg numbers. Finally, the identification of specific Treg subsets responsible for effector T-cell suppression should lead to the development of more specific drugs that alter these populations, leaving in place populations that suppress autoimmunity.

Disclosure of Potential Conflicts of Interest

E.M. Jaffee received research support from Pfizer and has the potential to receive royalties for a patent from BioSante. No potential conflicts of interest were disclosed by the other author.

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