

Old-School Chemotherapy in Immunotherapeutic Combination in Cancer, A Low-cost Drug Repurposed

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Abstract

Cancer immunotherapy has proven to be a potent treatment modality. Although often successful in generating antitumor immune responses, cancer immunotherapy is frequently hindered by tumor immune-escape mechanisms. Among immunosuppressive strategies within the tumor microenvironment, suppressive immune regulatory cells play a key role in promoting tumor progression through inhibiting the effector arm of the immune response. Targeting these suppressive cells can greatly enhance antitumor immune therapies, hence augmenting a highly effective therapeutic antitumor response. Several approaches are being tested to enhance the effector arm of the immune system while simultaneously inhibiting the suppressor arm. Some of these approaches are none other than traditional drugs repurposed

as immune modulators. Cyclophosphamide, an old-school chemotherapeutic agent used across a wide range of malignancies, was found to be a potent immune modulator that targets suppressive regulatory immune cells within the tumor microenvironment while enhancing effector cells. Preclinical and clinical findings have confirmed the ability of low doses of cyclophosphamide to selectively deplete regulatory T cells while enhancing effector and memory cytotoxic T cells within the tumor microenvironment. These immune effects translate to suppressed tumor growth and enhanced survival, evidence of antitumor therapeutic efficacy. This article discusses the reincarnation of cyclophosphamide as an immune modulator that augments novel immunotherapeutic approaches. *Cancer Immunol Res*; 4(5); 377–82. ©2016 AACR.

Introduction

Immunotherapy is emerging as a potent fourth modality for cancer treatment. Successful immunotherapy requires the generation of effector cells that overcome the immunosuppressive strategies within the tumor microenvironment. Fundamental components of the immune suppressive network are the CD4⁺Foxp3⁺ regulatory T cells (Tregs) and the Gr1⁺CD11b⁺ myeloid-derived suppressor cells (MDSCs). In addition to their effects on CD8 T-cell proliferation, these cells secrete cytokines with immunosuppressive functions, such as interleukin 10 (IL10) and transforming growth factor β (TGF β). Expression within the tumor microenvironment of immune inhibitory checkpoint molecules, such as programmed death receptor 1 (PD-1) and cytotoxic T lymphocyte associated protein 4 (CTLA-4), is another immune escape mechanism.

In addition to the generation of an effective antitumor immune response, targeting these suppressive mechanisms is

essential for maximizing the efficacy of cancer immunotherapy. Many approaches are being investigated to achieve this end, some of which come from well-known old-school drugs that are being repurposed to modulate the immune system for a better antitumor outcome.

Cyclophosphamide

Cyclophosphamide is an alkylating chemotherapeutic agent with strong immunosuppressive activity. It was introduced in 1958 and for almost 60 years has been considered the backbone of chemotherapeutic regimens in lymphoproliferative diseases as well as a wide range of solid tumors. It is also an integral part of the conditioning regimen commonly applied in myelo-ablative allogeneic stem cell transplantation. It is no wonder that cyclophosphamide is still on the World Health Organization's "List of Essential Medicines."

Depending on the dose of administration, cyclophosphamide affects tumors either through direct cytotoxic activity or through immune-enhancing mechanisms. Administering cyclophosphamide in high doses is cytotoxic, due to its activity as an alkylating agent, which leads to inhibition of DNA replication and apoptosis of both tumor and lymphoid cells. This cytotoxic effect on lymphoid cells is observed in actively replicating as well as resting, nonreplicating lymphocytes (1). Therefore, apart from its role as a potent chemotherapeutic agent, cyclophosphamide has gained attraction as an immunosuppressive treatment in many autoimmune disorders, such as vasculitis, systemic lupus erythematosus, and chronic graft-versus-host disease in recipients of allogeneic

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stem cell transplants. Including cyclophosphamide in regimens for haploidentical allogeneic transplants that are performed to treat hematologic malignancies can overcome immune rejection barriers that have prevented many populations from undergoing allogeneic transplants.

Cyclophosphamide as an Immune Modulator

The dose, timing, and sequence of cyclophosphamide administration play major roles in its effect on the immune system (Fig. 1). As outlined above, high doses of cyclophosphamide lead to the nonspecific depletion of immune cells and are therefore used for inducing lymphodepletion before adoptive cell transfer, conditioning patients before allogeneic stem cell transplantation, as well as chemotherapeutic approaches in aggressive types of lymphoproliferative disorders. On the other hand, low doses of cyclophosphamide can have antitumor effects by enhancing the immune response. Low doses of cyclophosphamide are effective in treating tumors in immune competent, but not nude, mice, indicating that the effect of low-dose cyclophosphamide is immune mediated, whereas high-dose cyclophosphamide led to antitumor responses in both types of mice (2). High doses of cyclophosphamide have also been found to decrease lymphocyte infiltration into tumors, whereas low doses lead to higher lymphocyte infiltration (2). Additionally, low-dose cyclophosphamide enhances vaccine-induced immune responses, whereas higher doses impeded vaccination (3).

How Does Cyclophosphamide Enhance the Immune Response?

Low doses of cyclophosphamide enhance immune responses through affecting an array of immune cells within the tumor microenvironment. Its effects simultaneously improve the effector and inhibit the suppressive arm of the antitumor immune response.

The effect of cyclophosphamide on Tregs

Tregs are a fundamental component of immunosuppressive mechanisms of tumors; Treg depletion enhances antitumor immunity and promotes tumor regression (4, 5). Therefore, several approaches have been studied to target Tregs, including our group's use of PI3K-Akt pathway inhibitors to selectively and effectively target Tregs, which has translated into therapeutic efficacy in murine tumor models (5).

Low-dose cyclophosphamide [given in single or metronomic (repeated) doses], is associated with a temporary reduction in both the number and function of Tregs within the tumor microenvironment, its associated lymph nodes, and peripheral blood

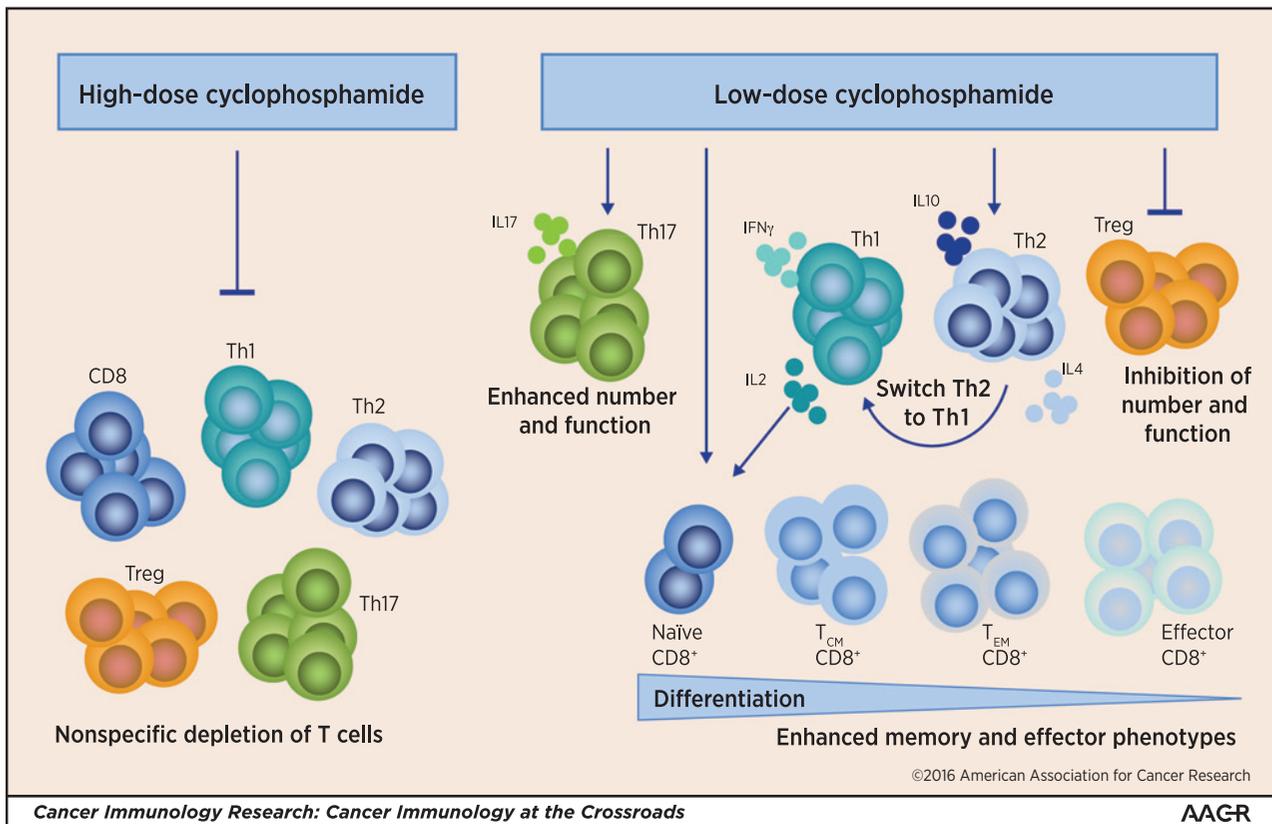


Figure 1. The immune-modulatory effect of cyclophosphamide on T cells. When given in high doses, cyclophosphamide leads to nonspecific depletion of T cells. However, at low doses, cyclophosphamide exerts a range of effects on different T-cell subsets: These include selective depletion of Tregs and inhibition of their suppressive function; switching the secretion of cytokines from Th2 to Th1; and enhancement of Th17, memory, and effector CD8 T-cell phenotypes.

(6–8). This results in enhancing antitumor immune responses when used alone or in combination with cancer immunotherapies (6, 9, 10). We and others have demonstrated in mice that single-dose cyclophosphamide induced apoptosis in Tregs starting 24 hours after the exposure, with a nadir at 3–4 days and full reconstitution reaching pretreatment numbers within 10 days (6).

In humans, the effect of administration of metronomic cyclophosphamide can selectively deplete Tregs and restore both T-cell and natural killer (NK)-cell function in patients in whom both the numbers and function of Tregs were inhibited (11). In patients with metastatic breast cancer, a transient but statistically significant decrease in Treg numbers accompanies an increase in tumor-specific T cells (12). Total reconstruction of Tregs follows the prolonged metronomic treatment with cyclophosphamide in humans, due to the enhanced proliferation of Tregs that maintains their suppressive ability (12).

Low-dose cyclophosphamide has been tested in animal models as part of combination immunotherapy with immune therapeutic agents, including different vaccines and immune checkpoint inhibitors. We found in a murine tumor model that adding a single dose of cyclophosphamide to a combination of HPV16-E6 peptide vaccine with PD-1 binding antibody leads to statistically significant enhancement of antitumor therapeutic efficacy and survival. Tumor-infiltrating Tregs decreased significantly, while infiltrating cytotoxic CD8⁺ T cells and effector CD4⁺Foxp3⁻ T cells increased significantly, yielding an enhanced antigen-specific

immune response (9). These findings were further confirmed in a murine model of pancreatic ductal adenocarcinoma: Adding low-dose cyclophosphamide to the combination of PD-1 antibody with GVAX (granulocyte-macrophage colony-stimulating factor-secreting allogeneic pancreatic tumor cells) vaccine improved survival. This improvement was a result of enhancing tumor-specific CD8 T-cell numbers and activity with PD-1 blockade and suppressing Tregs through low-dose cyclophosphamide treatment (13). Similar results were obtained in a prostate cancer murine model in which animals were treated with a different checkpoint inhibitor, anti-CTLA-4 antibody, combined with GVAX. Although effector CD8 T cells were significantly enhanced with the dual combination, adding low-dose cyclophosphamide enhanced the therapeutic efficacy by a transient depletion of Tregs (14). A photodynamic therapy-generated cancer vaccine was tested in combination with low-dose cyclophosphamide in a murine tumor model, which showed that cyclophosphamide enhanced the vaccine's efficacy by reducing the number of Tregs (15).

Several mechanisms were proposed for cyclophosphamide-mediated Treg depletion and loss of function (Fig. 2A). The ability of Tregs to detoxify cyclophosphamide metabolites is impaired, rendering them more prone to the agent's cytotoxic effects than other T cells. Tregs express more CD39, an ectoenzyme that hydrolyzes extracellular ATP to ADP, causing the efflux and loss of intracellular ATP and resulting in less intracellular ATP in

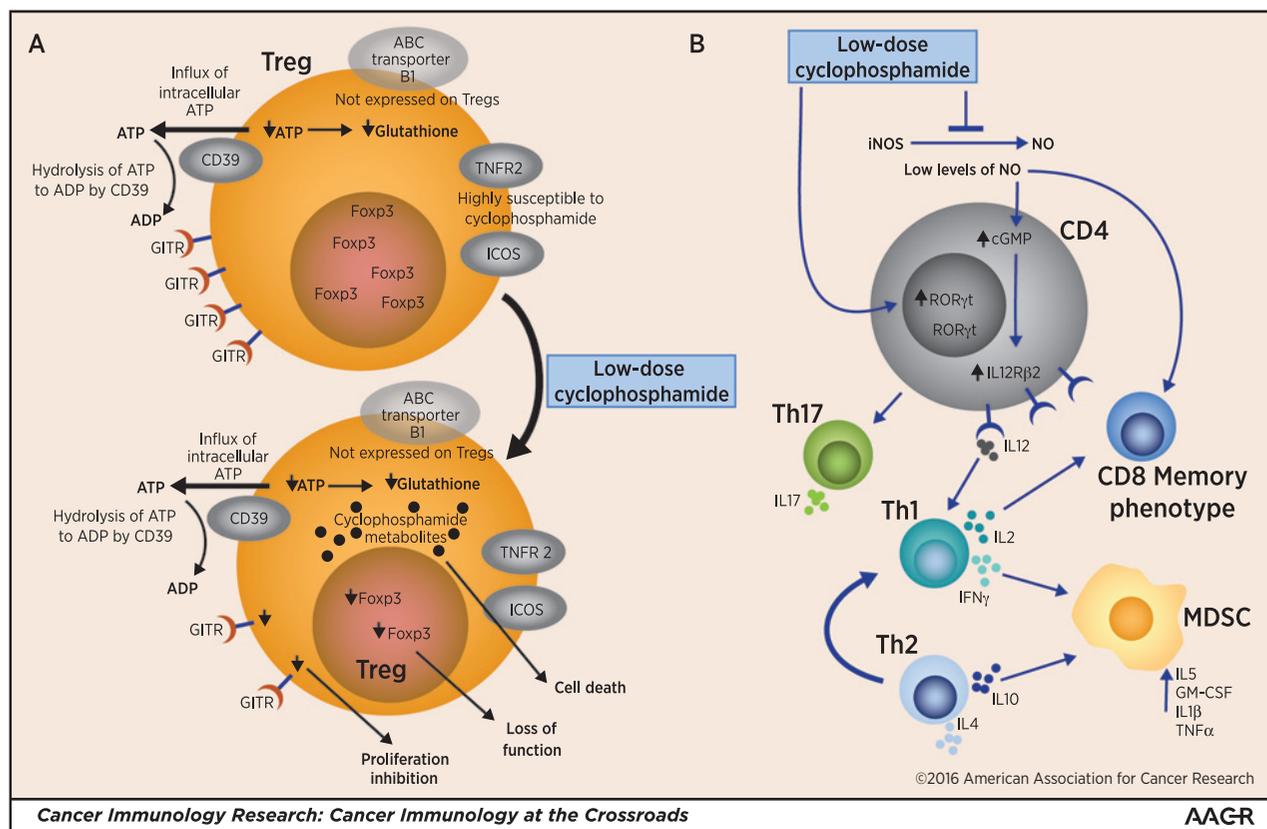


Figure 2. A, the mechanisms by which low-dose cyclophosphamide inhibits the number and function of Tregs. B, the mechanisms by which low-dose cyclophosphamide modulates different immune cells.

comparison with that of conventional T cells. Low ATP reduces the concentration of the antioxidant glutathione and the cells' ability to detoxify metabolites (16). Tregs also lack expression of the ATP-binding cassette (ABC) transporter B1, which extrudes drugs and metabolites from cells. This makes Tregs more susceptible to cyclophosphamide (17). CD39-expressing Tregs are associated with increased expression of tumour necrosis factor receptor (TNFR2; ref. 18), which is expressed in one of the maximally suppressive Treg phenotypes. Interestingly, Tregs expressing TNFR2 and Inducible T-cell costimulator (ICOS) were found to be highly susceptible to cyclophosphamide (19).

In addition to its cytotoxic effect, cyclophosphamide was also reported to inhibit the proliferation and the function of Tregs through the downregulation of glucocorticoid-induced tumor TNFR-related protein (GITR) and Foxp3 (6). Both GITR and Foxp3 are important molecules for the suppressive function of Tregs. GITR acts as a Treg costimulatory molecule that enhances Treg proliferation upon binding to its ligand, and Foxp3 regulates the transcription of many factors involved in Tregs' suppressive function. Hence, once downregulated by cyclophosphamide, Treg function and proliferation are impaired, leading to a reduction in their numbers within the tumor.

The effect of cyclophosphamide on effector T cells

Low doses of cyclophosphamide can enhance the number and cytotoxic function of effector T cells. The combination of metronomic cyclophosphamide with a DepoVax vaccine containing HPV16E7 peptide targeting an HPV16-induced murine tumor model led to a sustainable antitumor effect. This effect resulted from the selective lymphodepletion ability of cyclophosphamide, resulting in the preservation of vaccine-induced CD8 T cells and the subsequent enhancement of their cytotoxic ability (20). Additionally, low-dose cyclophosphamide can augment the memory phenotypes of CD4 and CD8 T cells, which are superior mediators of antitumor immunity. In a murine prostate cancer model, low-dose cyclophosphamide greatly enhanced the generation of memory and effector CD4 and CD8 T cells when combined with cell-based vaccines encoding hyper-IL6 and hyper-IL11 (21). Cyclophosphamide also has a considerable effect on the phenotype of T cells, especially switching the Treg-Th17 balance to a less suppressive Th17 phenotype. Pancreatic cancer-bearing mice were treated with mHSP65-TTL vaccine (mixed recombinant mycobacterial heat-shock protein 65 and pancreatic cancer tissue lysate) in combination with a low dose of cyclophosphamide. Cyclophosphamide alone or in combination with mHSP65-TTL upregulated mRNA expression of ROR γ t (22), suggesting an enhancement of the Th17 phenotype.

Although the effect that cyclophosphamide has on effector cells is an expected outcome, given cyclophosphamide's depletion of Tregs, several mechanisms were put forward to explain this enhancement of effector cells (Fig. 2B). Cyclophosphamide can directly enhance the proliferation of effector T cells (11, 12). Effector T cells released from Treg suppression significantly increase their numbers within the tumor microenvironment. Cyclophosphamide's effect is also due to the polarization of T cells into the more effective antitumor phenotypes (Th1, Th17, and memory CD8 T cells). Low doses of cyclophosphamide were associated with a switch to the secretion of Th1 cytokines [interferon gamma (IFN γ) and IL2] instead of Th2 associated cytokines (IL4 and IL10; refs 23 and 24), which favors a positive antitumor immune response. Evidence for Th17 polarization

upon cyclophosphamide administration is provided by the increase in IL17 secretion (25) and upregulation of ROR γ t (22). Cyclophosphamide inhibits nitric oxide synthase (iNOS), an enzyme that leads to the generation of nitric oxide (NO). Low NO helps convert cyclic guanosine-5-triphosphate into 3',5'-cyclic guanosine monophosphate (cGMP). Increased cGMP within CD4 cells leads to the induction of IL12 receptor β 2 (IL12R β 2), allowing induction of Th1 differentiation by IL12 (26).

The effect of cyclophosphamide extends to cytotoxic CD8 T cells. IL2 secretion, which is associated with cyclophosphamide administration, enhances the memory phenotype of cytotoxic CD8 T cells (23). Additionally, the decrease in NO, outlined above, leads to enhancement of memory CD8 T cells (27). Memory CD8 T cells are superior mediators of antitumor immune responses due to their enhanced proliferative and cytotoxic abilities (28). This explains, at least in part, the enhanced antitumor immune response associated with cyclophosphamide treatment.

The effect of cyclophosphamide on MDSCs

Despite the effect low-dose cyclophosphamide has in reducing the number and function of Tregs, it has been reported to increase the number of suppressive MDSCs (Gr1⁺CD11b⁺) within the tumor microenvironment. It is speculated that this phenomenon is due to the increased production of inflammatory mediators, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL1 β , IL5, IL10, IFN γ , and tumor necrosis factor alpha [TNF α ; (Fig. 2B)]. These chemokines induce MDSC expansion and activation that may eventuate their accumulation, which, when coupled with the increased NO production, could lead to the inhibition of effector T-cell proliferation (29). It is apparent, however, from the enhanced therapeutic efficacy reported in most studies with significant increases in the number of cytotoxic effector T cells, that the potential inhibition of effector T cells by MDSCs might not significantly affect antitumor cytotoxicity (9, 10).

Cyclophosphamide: An Immune Modulator in the Clinic

It has been over 30 years since North showed that cyclophosphamide enhances the effect of adoptively transferred T cells to control tumors in mice, but clinical translation of these results has been difficult (30). While reviewing all trials is outside the scope of this article, herein we summarize some of the current ongoing activities in this area. The best evidence for cyclophosphamide's beneficial enhancement of tumor immunity was observed in a trial of multi-peptide vaccines and GM-CSF in patients with advanced renal cell cancer. The addition of cyclophosphamide 3 days before vaccination reduced the number of Treg cells in the blood and improved overall survival. Progression-free survival was not affected, as has been observed in other vaccine trials (31).

Several vaccine platforms were tested in combination with cyclophosphamide. In patients with advanced melanoma, pre-treatment with metronomic low-dose cyclophosphamide enhanced antigen-specific immune responses when combined with NY-ESO-1 vaccine. Although a reduction in Treg numbers was not detected 2 weeks after cyclophosphamide treatment, an earlier transient drop in their number is speculated (32). Metronomic doses of cyclophosphamide were also tested in combination with a survivin HLA class I peptide vaccine using the DepoVax

platform (DPX-Survivac) in ovarian cancer patients. Antigen-specific immune responses were observed in the form of memory and effector CD4 and CD8 T cells. A transient drop in Treg numbers was observed, and a drop in Treg functionality is speculated to have aided in the enhancement of the antitumor immune response (33).

In patients with pancreatic adenocarcinoma, low-dose cyclophosphamide was used to target Treg cells in combination with two different vaccines: GVAX, or the *Listeria*-based vaccine CRS-207, which expresses mesothelin (34). In another study, pancreatic adenocarcinoma patients were given the GVAX vaccine in combination with low-dose cyclophosphamide. Intratumoral tertiary lymphoid aggregates were observed, and microarray analysis identified a suppressed Treg pathway and an enhanced Th17 pathway, which were associated with enhanced antitumor immune responses and better overall survival. In addition, an upregulation of the PD-1–PD-L1 pathway was observed after treatment, suggesting a potential for combination with immune checkpoint inhibitors (35).

Currently, multiple clinical trials using cyclophosphamide as an adjuvant treatment for cancer, and specifically as an immune modulator, are ongoing. The combinations being investigated include combinations with various vaccines, CAR-T cells, SMAC mimetic LCL161, and immune checkpoint inhibitors. The results of these studies are eagerly awaited. The critical parameter for evaluation of ways to maximize the benefit of cyclophosphamide

remains to be determined. Changes in Treg number or function, antigen-specific T-cell response, or changes in MDSCs are potential endpoints for analysis, but ultimately, improvement in patient survival is most important.

Final Note

Cyclophosphamide is an economically attractive chemotherapeutic drug that is approved by the FDA for clinical treatment of a wide range of cancers. This drug can be repurposed to augment novel immunotherapeutic agents. Preclinical and clinical studies have already shown great promise for the combination of this old-school chemotherapeutic agent with cancer vaccines and immune checkpoint inhibitors. The use of cyclophosphamide, a potent immune modulator, in combination with various immunotherapies harbors great potential for enhancing both adoptive cell transfer and vaccine-based cancer immunotherapies.

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No potential conflicts of interest were disclosed.

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