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# Cyclophosphamide inhibits the generation and function of CD8$^+$ regulatory T cells

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ABSTRACT

CD8$^+$ regulatory T cells (Treg) and CD4$^+$CD25$^+$ Treg infiltrate human cancers, thus favoring tumor immune escape. Therefore, in the setting of antitumor therapeutic protocols, it is important to associate antitumor treatment with agents that are able to inhibit Treg function. Cyclophosphamide (CY) has been demonstrated to be effective in counteracting CD4$^+$CD25$^+$ Treg activity. Hence, we tested its inhibitory efficacy on human CD8$^+$ Treg. Because CY is a prodrug, 4-hydroperoxycyclophosphamide (4-HC), a derivative of CY that in aqueous solution is converted to 4-hydroxycyclophosphamide, an active metabolite of CY, was used. 4-HC significantly inhibited CD8$^+$ Treg generation and function but only at the higher tested concentration (0.5 $\mu$g/mL), that is, in the therapeutic range of the drug. The lower 4-HC concentration tested (0.1 $\mu$g/mL) was almost ineffective. 4-HC inhibitory effects were related to apoptosis/necrosis induction. When CD8$^+$CD28$^+$ non-Treg were analyzed for comparative purposes, significantly lower cytotoxic rates among these cells were observed than among CD8$^+$ Treg, which were differentiated because they did not express the CD28 molecule. These data demonstrate that CD8$^+$ Treg are inhibited through cytotoxic phenomena by CY, thus supporting the use of this drug at adequate concentrations and schedules of administration as a Treg inhibitor in combinatorial chemo- or immunotherapeutic anticancer protocols.



## 1. Introduction

Regulatory T cells (Treg) play a pivotal role in the maintenance of immunologic self-tolerance, downregulating the activation/proliferation of self-reactive T cells and thus preventing the development of various autoimmune diseases [1,2]. However, Treg have been detected among tumor-infiltrating lymphocytes [3,4]. Treg infiltrating cancer lesions are thought to be responsible for the inhibition of antitumor immune responses, thus favoring tumor immune escape. Indeed, administration of ipilimumab [5], an agent blocking the CTLA4-CD80/CD86 interaction—one of the molecular circuits adopted by Treg for suppressing immune responses [6]—has therapeutic effects against cancer [7]. The importance of counteracting tumor-infiltrating Treg function to set antitumor protocols of chemo- and/or immunotherapy that are more efficient than those currently in use is therefore clear. However, Treg belong to different T cell lineages [8,9] and each Treg subtype uses different mechanisms to induce immune suppression [10,11]. Hence, the search for additional agents that can block tumor-infiltrating Treg is mandatory. The chemotherapeutic agent cyclophosphamide (CY) has been reported to be effective on Treg. CY is a nitrogen mustard alkylating agent that exhibits great cytotoxicity against cells actively replicating their DNA [12,13]. CY is an inactive prodrug that requires activation by the hepatic cytochrome P-450 enzyme system to form the active metabolite 4-hydroxycyclophosphamide, which is in equilibrium with its tautomer aldophosphamide. These 2 intermediate metabolites rapidly diffuse out of hepatic cells into the circulation and are subsequently taken up by other cells, including cancer cells. Within the cells, aldophosphamide decomposes to form the cytotoxic phosphoramide mustard, which produces the interstrand DNA cross-links responsible for the cytotoxic properties of the drug. The selective toxicity on tumor cells occurs because the concentration of the enzymes converting aldophosphamide into the cytotoxic metabolite is higher in tumor cells than in normal cells [14]. CY is one of the most successful and widely used drugs for the treatment of hematologic and solid malignancies [15,16], as well as for the treatment of different autoimmune disorders [17], and therefore is commonly considered an immunosuppressive drug. However, evidence exists that CY may have immunostimulatory effects (*i.e.*, enhancing the therapeutic activity of adoptive T cell immu-

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notherapy) [18]. Recent studies have linked the immunostimulating effect of CY to the selective inhibition/depletion of $CD4^+CD25^+$ Treg in both experimental [19–21] and human [22,23] tumors. However, the Treg repertoire also includes $CD8^+$ Treg; no information on the sensitivity of $CD8^+$ Treg to CY is currently available. This article reports data on the effects of 4-hydroperoxycyclophosphamide (4-HC), a derivative of CY that in aqueous solution is converted to 4-hydroxycyclophosphamide (the active metabolite of CY) [24], on $CD8^+$ Treg function and viability.

## 2. Subjects and methods

### 2.1. Generation of $CD8^+$ Treg from peripheral blood

$CD8^+$ Treg were generated as described previously [25]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by centrifugation on a Ficoll–Hypaque gradient (Biochrom AG, Berlin, Germany) for 30 minutes at 1800 rpm. PBMC were incubated in RPMI 1640 culture medium (Gibco by Life Technologies Ltd., Paisley, UK) with 10% fetal calf serum (Invitrogen) in culture flasks (Corning Life Sciences, Amsterdam, The Netherlands) at 37°C overnight. $CD8^+$ T lymphocytes were purified from nonadherent cells by magnetic bead separation using microbeads conjugated with monoclonal antibody (mAb) specific for the CD8 antigen (Dynal CD8 positive isolation kit, Invitrogen by Life Technologies Ltd., Paisley, UK). Purified $CD8^+$ T lymphocytes ($2 \times 10^5$ cells/well) resuspended in culture medium consisting of RPMI 1640 (Gibco by Life Technologies Ltd., Paisley, UK) with 10% fetal calf serum (Invitrogen by Life Technologies Ltd., Paisley, UK) were incubated with 20 U/mL of interleukin (IL)-2 (Proleukin, Eurocetus, Amsterdam, The Netherlands) and 10 ng/mL of IL-10 (PeproTech) in 96-well flat-bottom plates (Corning Life Sciences, Amsterdam, The Netherlands) at 37°C for 7 days.

At the end of the incubation, the cells were collected, washed, counted, and used as suppressors in a proliferation suppression assay.

### 2.2. Proliferation suppression assay

The suppressive activity of Treg was evaluated by monitoring the inhibition of dye dilution in PBMC stained with carboxyfluorescein succinimidyl ester (5 $\mu$M; Molecular Probes, Invitrogen by Life Technologies Ltd., Paisley, UK) before the test. Thereafter, the cells were pulsed with anti-CD3 UCHT1 mAb (5 $\mu$g/mL, BD Bioscience, Franklin Lakes, NJ) and cultured for 5 days in a 96-well round-bottom plate ($1 \times 10^5$ cells/well) in the presence (or absence) of *in vitro*–generated $CD8^+$ Treg ($1 \times 10^5$ cells/well). At the end of the incubation the samples were washed in phosphate-buffered saline and analyzed using a FACSCanto flow cytometer (BD Bioscience, Franklin Lakes, NJ) using FACSDiva software (BD Biosciences, Franklin Lakes, NJ).

### 2.3. 4-HC treatment

4-HC was purchased from Niomech–IIT GmbH (University of Bielefeld, Bielefeld, Germany). The drug was opportunely diluted to be used at a final concentration of 0.5 or 0.1 $\mu$g/mL, concentrations within the therapeutic range of 4-HC [26]. To evaluate the effects on $CD8^+$ Treg generation, the drug was added to cultures during the 7-day incubation with IL-2 and IL-10. To analyze 4-HC effects on already generated $CD8^+$ Treg, the drug was added to cultures on the 6th day of generation for 24 hours.

### 2.4. Immunofluorescence analyses

Cell expression of membrane antigens was analyzed by immunofluorescence by incubating the cells ($1 \times 10^5$ cells in 100 $\mu$L of

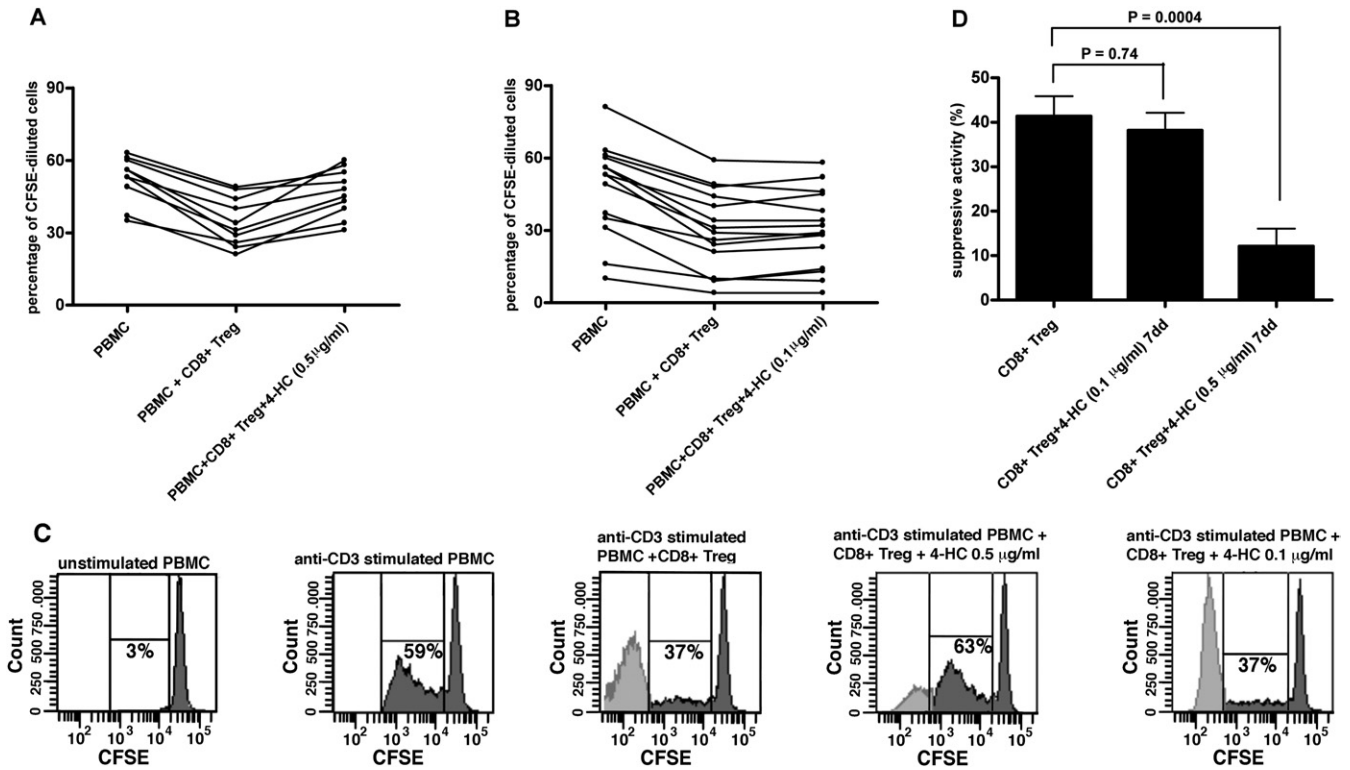


**Fig. 1.** Analysis of 4-hydroperoxycyclophosphamide (4-HC) effects on $CD8^+$ regulatory T cell (Treg) generation. (A and B) Anti-CD3 monoclonal antibody (mAb)-induced peripheral blood mononuclear cell (PBMC) proliferation (expressed as a percentage of carboxyfluorescein succinimidyl ester [CFSE]-diluted cells) in the presence or absence of $CD8^+$ Treg generated with or without 4-HC used at 0.5 (A) or 0.1 (B) $\mu$g/mL. (C) Representative results of proliferation analysis of anti-CD3 mAb-stimulated PBMC from donor 16 cultured in the presence or absence of $CD8^+$ Treg generated in the presence or absence of either 0.5 or 0.1 $\mu$g/mL of 4-HC. Percentages of proliferating cells are indicated. (D) Comparison between the mean suppressive activity of untreated $CD8^+$ Treg and that of $CD8^+$ Treg generated in the presence of either 0.1 or 0.5 $\mu$g/mL 4-HC.