Human Immunology 73 (2012) 207-213

Contents lists available at SciVerse ScienceDirect

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

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ARTICLE INFO

*Article history:*
Received 7 October 2011
Accepted 21 December 2011
Available online 14 January 2012

*Keywords:*
Cyclophosphamide
CD8$^+$
Treg Cancer Autoimmunity

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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0198-8859/$36.00 
doi:10.1016/j.humimm.2011.12.020

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

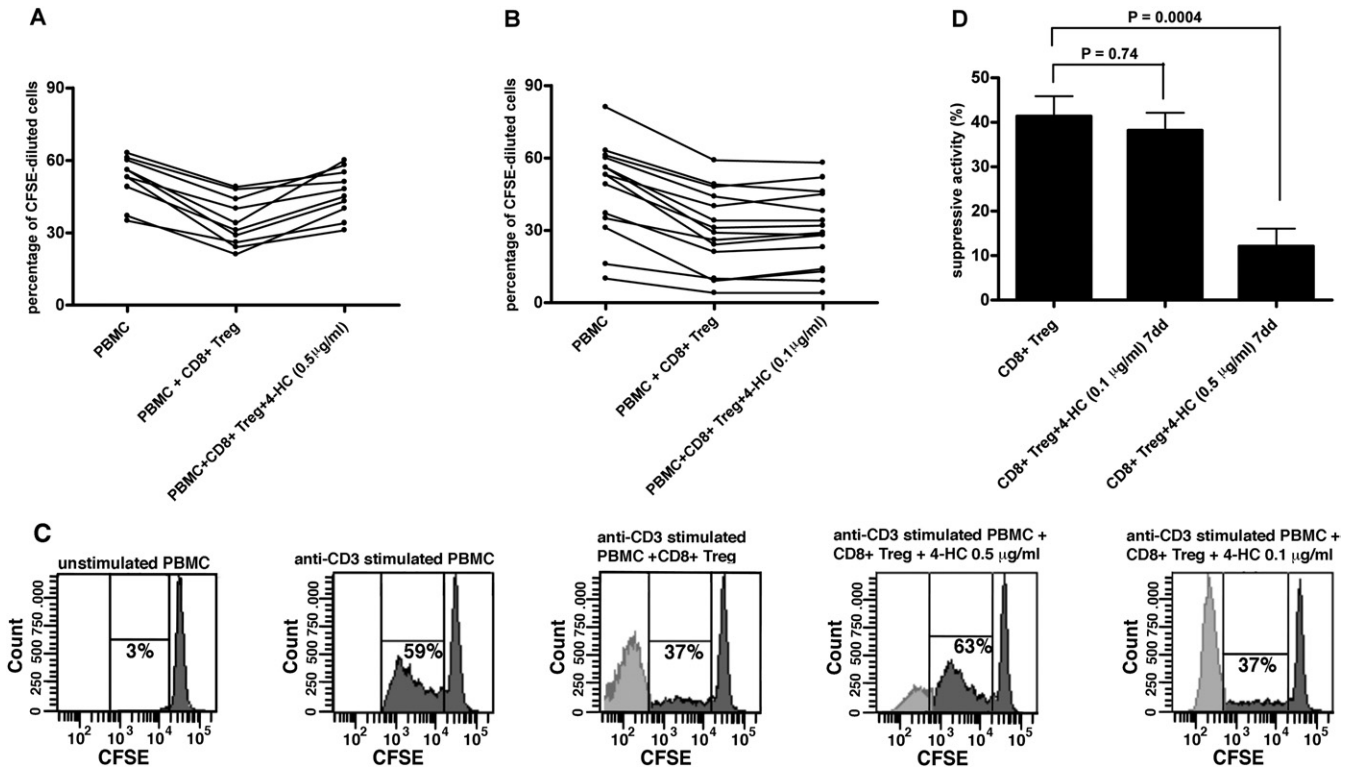


**Fig. 1.** Analysis of 4-hydroperoxycyclophosphamide (4-HC) effects on $CD8^+$ regulatory T cell (Treg) generation. (A and B) Anti-CD3 monoclonal antibody (mAb)-induced peripheral blood mononuclear cell (PBMC) proliferation (expressed as a percentage of carboxyfluorescein succinimidyl ester [CFSE]-diluted 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.

phosphate-buffered saline) with specific mAbs at 4°C for 30 minutes in the dark. The following mAbs were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD45RA, phycoerythrin (PE)-conjugated anti-CD127, allophycocyanin-conjugated anti-CD39, PE–cyanin 7–conjugated anti-CD8, and PerCP-conjugated anti-CD28. After staining procedures, the cells were acquired and analyzed by a FACSCanto flow cytometer using FACSDIVA software.

### 2.5. Analysis of apoptotic and necrotic cells

$CD8^+$ purified T cells, preexposed or not to 4-HC, were incubated with Pe–cyanin 7–conjugated anti-CD8 and allophycocyanin-conjugated anti-CD28 for 15 minutes at room temperature in the dark, following the manufacturer's instructions. The percentage of apoptotic cells was assessed by flow cytometry after cell incubation with FITC-conjugated annexin V (BD Pharmingen, San Diego, CA). To quantify necrotic cells, 7-aminoactinomycin D (BD Biosciences, Franklin Lakes, NJ) was added to the cells immediately before flow cytometric analysis. Cells stained only by FITC-conjugated annexin V were considered apoptotic, whereas cells stained by both FITC-conjugated annexin V and 7-aminoactinomycin D were enumerated as necrotic cells.

### 2.6. Statistical analysis

All results are expressed as means ± standard deviation. Statistically significant differences between mean percentage data (related to apoptosis, necrosis, and suppressive activity of different T cell subsets treated or not with 4-HC) were analyzed using the Mann–Whitney test for nonparametric values. Correlations between variables were analyzed by Spearman's correlation test. Differences were considered significant when $p < 0.05$. The statistical analyses were performed using GraphPad Prism 4.0 Software (GraphPad Software Inc., San Diego, CA).

## 3. Results

### 3.1. Analysis of the effects of 4-HC on $CD8^+$ Treg generation and function

To evaluate the effects of 4-HC on *in vitro* generation of $CD8^+$Treg, *ex vivo* purified $CD8^+$ T cells were incubated for 7 days with the drug used at 0.1 or 0.5 $\mu$g/mL concentrations. The suppressive activities of $CD8^+$Treg generated in the presence or absence of the drug were comparatively analyzed. Control $CD8^+$Treg exerted a suppressive activity ≥25% in all analyzed samples. $CD8^+$Treg preincubated with 0.5 $\mu$g/mL of 4-HC exhibited significantly lower suppressive activity than control $CD8^+$Treg (12% vs 41%, $p = 0.0004$; Fig. 1). To the contrary, the suppressive activity of $CD8^+$Treg pretreated with 4-HC at 0.1 $\mu$g/mL concentration was comparable to that of control $CD8^+$Treg (38% vs 41%, $p = 0.74$; Fig. 1).

To also assess the effects of 4-HC on the function of already generated $CD8^+$Treg, *in vitro*–generated $CD8^+$Treg were incubated for 24 hours with the drug used at the same concentrations as above. Again, the suppressive activities of treated or untreated $CD8^+$Treg treated were comparatively analyzed. $CD8^+$ Treg incubated with 0.5 $\mu$g/mL of 4-HC exhibited significantly reduced suppressive activity compared with control $CD8^+$Treg (20% vs 41% $p = 0.0004$; Fig. 2). Instead, the suppressive activity of $CD8^+$Treg incubated overnight with 4-HC at 0.1 $\mu$g/mL concentration was comparable to that of control $CD8^+$Treg (38% vs 41%, $p = 0.45$; Fig. 2).

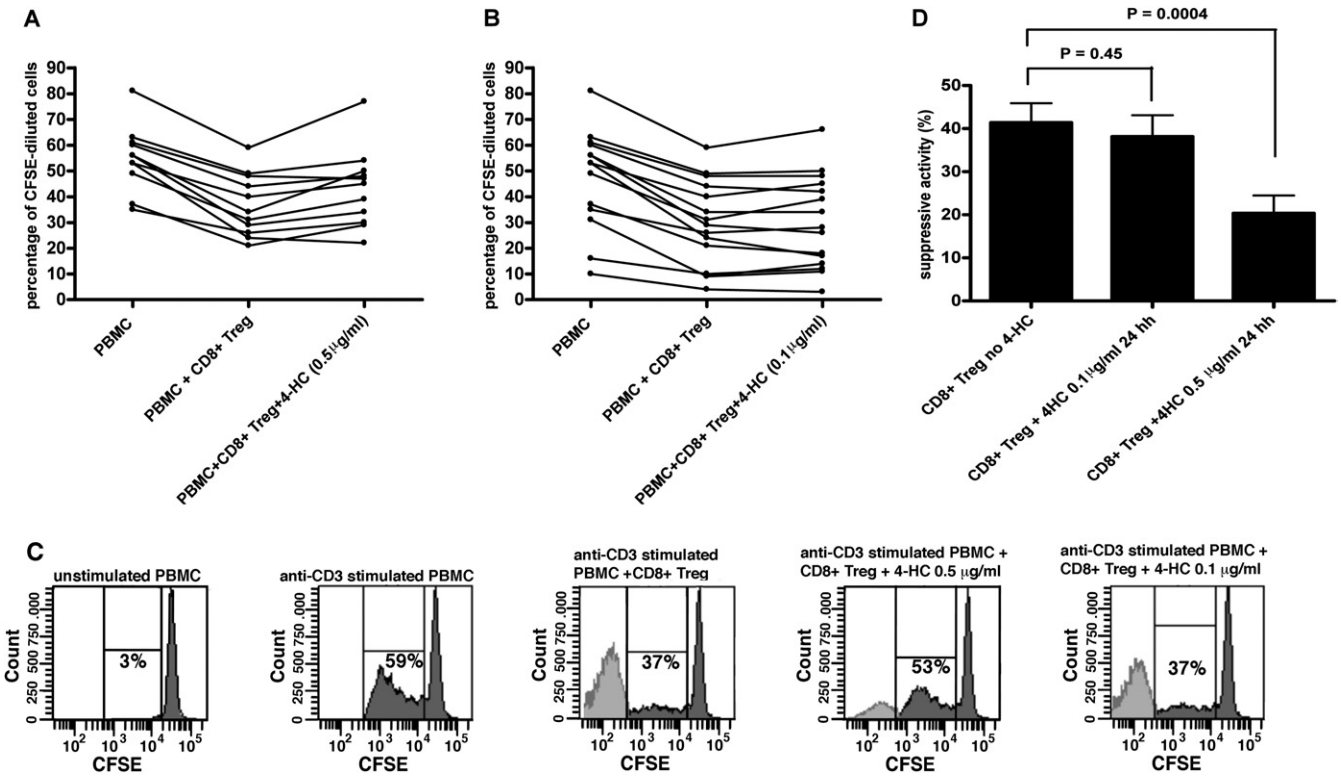


**Fig. 2.** Analysis of 4-hydroperoxycyclophosphamide (4-HC) effects on $CD8^+$ regulatory T cell (Treg) suppressive activity. (A and B) Anti-CD3 monoclonal antibody (mAb)-induced peripheral blood mononuclear cell (PBMC) proliferation (expressed as a percentage of carboxyfluorescein succinimidyl ester [CFSE]-diluted cells) in the presence or absence of *in vitro*–generated $CD8^+$ Treg incubated for 24 hours with or without 4-HC used at 0.5 (A) or 0.1 (B) $\mu$g/L (C) Representative results of proliferation analysis of anti-CD3 mAb-stimulated PBMC from donor 16 cultured in the presence or absence of $CD8^+$ Treg incubated or not for 24 hours in the presence 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 *in vitro*–generated $CD8^+$ Treg incubated for 24 hours with either 0.1 or 0.5 $\mu$g/mL 4-HC.

**Table 1**
Comparative analysis of antigen expression on basal $CD8^+$ T cells as well as on $CD8^+$ regulatory T cells (Treg) generated in the presence or absence of different concentrations of 4-hydroperoxycyclophosphamide (4-HC)

| Antigen | Basal $CD8^+$ T cells | A $CD8^+$ Treg | B $CD8^+$ Treg+0.1 $\mu$g/mL 4-HC | C $CD8^+$ Treg +0.5 $\mu$g/mL 4-HC | *p* values |
|---|---|---|---|---|---|
| CD127 | 76[a] ± 16 | 46 ± 19 | 39 ± 11 | 39 ± 22 | A vs B: $p = 0.5$; A vs C: $p = 0.5$ |
| CD39 | 2 ± 3 | 3 ± 2 | 4 ± 4 | 5 ± 4 | A vs B: $p = 0.8$; A vs C: $p = 0.4$ |
| CD45RA | 75 ± 11 | 72 ± 20 | 64 ± 27 | 62 ± 27 | A vs B: $p = 0.7$; A vs C: $p = 0.7$ |

[a]Data are expressed as percentages of positive cells.

### 3.2. 4-HC does not alter the phenotype of $CD8^+$ Treg

CD127, the IL-7 receptor, is downmodulated in Treg subpopulations [27,28] so it is commonly monitored for assessing T cell differentiation toward regulatory function. CD39 is a nucleosidase often expressed by Treg cells [29,30] and CD45RA is expressed on non-antigen-specific $CD8^+$ Treg [9,31]. To assess whether the inhibition of $CD8^+$ Treg suppressive activity was dependent on phenotypic changes eventually induced by 4-HC, the expression of CD127 (IL-7 R), CD39, and CD45RA antigens was analyzed. Table 1 demonstrates that 4-HC did not cause significant variations of surface antigen expression when added to $CD8^+$ T cell cultures during $CD8^+$ Treg generation. Comparable findings were achieved when *in vitro*–generated $CD8^+$ Treg were incubated for 24 hours with 4-HC (Table 2).

### 3.3. 4-HC induces $CD8^+$ Treg apoptosis and necrosis

4-HC is an antiblastic drug; hence, its pharmacologic activity could be related to cell death induction. Therefore, the rates of apoptotic and necrotic cells in $CD8^+$ Treg exposed to 4-HC were analyzed. Because peripheral blood–derived $CD8^+$ Treg do not express the CD28 antigen [25], the percentages of apoptotic and necrotic cells were calculated by gating $CD8^+CD28^-$ and $CD8^+CD28^+$ T cells separately. Interestingly, the percentage of apoptotic cells among $CD8^+CD28^-$ Treg generated in the presence of 0.5 $\mu$g/mL of 4-HC was significantly higher than that observed among untreated $CD8^+CD28^-$ Treg (Fig. 3A). Similarly, a significantly higher percentage of apoptotic cells was detected among *in vitro*–generated $CD8^+CD28^-$ Treg incubated for 24 hours with 0.5 $\mu$g/mL of 4-HC compared with untreated $CD8^+CD28^-$ Treg (Fig. 3A). To the contrary, comparable rates of apoptotic cells between treated and untreated cells were observed when $CD8^+CD28^-$ Treg were incubated with 0.1 $\mu$g/mL of 4-HC either during or after Treg generation (Fig. 3A).

The analysis of necrosis in $CD8^+CD28^-$ Treg demonstrated a significantly increased percentage of necrotic cells among $CD8^+CD28^-$ Treg generated in the presence of 4-HC, independent of drug concentration, compared with untreated $CD8^+CD28^-$ Treg (Fig. 3B). To the contrary, no significant differences in necrotic cell percentage were detected between $CD8^+CD28^-$ Treg incubated or not for 24 hours with 4-HC (Fig. 3B).

To achieve comparative information on sensitivity to 4-HC by Treg ($CD8^+CD28^-$) and non-Treg ($CD8^+CD28^+$) T cell populations, the percentages of apoptotic or necrotic cells were also analyzed within the $CD8^+CD28^+$ T cells. $CD8^+CD28^+$ T cells incubated for 7 days with 0.5 $\mu$g/mL of 4-HC exhibited higher apoptosis percentages compared with untreated cells (Fig. 3C). Similarly a significantly higher percentage of apoptotic cells was detected among $CD8^+CD28^+$ T cells after their incubation for 24 hours with 0.5 $\mu$g/mL of 4-HC compared with untreated cells. To the contrary, comparable rates of apoptotic cells between treated and untreated cells were detected when $CD8^+CD28^+$ T cells were incubated for 24 hours or 7 days with 0.1 $\mu$g/mL of 4-HC (Fig. 3C). Significantly increased percentages of necrotic T cells were only observed among $CD8^+CD28^+$ T cells incubated for 7 days with 0.1 or 0.5 $\mu$g/mL of 4-HC, but not among $CD8^+CD28^+$ T cells incubated for 24 hours with 4-HC, compared with untreated cells (Fig. 3D). Interestingly, the comparison of apoptotic cell percentages between $CD8^+CD28^-$ and $CD8^+CD28^+$ T cells subpopulations indicated higher apoptotic rates in the former than in the latter T cell subpopulation under all culture conditions (Fig. 4A). Similarly, comparison of necrotic cell percentages between $CD8^+CD28^-$ and $CD8^+CD28^+$ T cells indicated a higher level in the former than in the latter T cell subpopulation under all culture conditions, with the exception of cells incubated for 7 days with 0.5 $\mu$g/mL of 4-HC (Fig. 4B).

To verify whether 4-HC-mediated inhibition of suppressive activity could be strictly related to its cytotoxic effects on $CD8^+$ Treg, the suppressive activity of $CD8^+$ Treg variably exposed to 4-HC was correlated with drug-induced mortality (considering the sum of percentages of apoptotic and necrotic cells): Fig. 4C illustrates a clear correlation between the 2 variables, thus supporting the concept that the 4-HC impact on $CD8^+$ Treg activity is mainly mediated by cytotoxic phenomena.

## 4. Discussion

The results of this study indicate the following: (1) 4-HC, at a dose of 0.5 $\mu$g/mL, inhibits both $CD8^+$ Treg generation and function; and (2) 4-HC effects on $CD8^+$ Treg correlate with apoptosis/necrosis induction.

Several previous studies have established that Treg cells are crucial for maintaining peripheral tolerance by suppressing immune responses against self-antigens and that numeric or functional defects of these cells have been linked to the development of autoimmunity [2,32,33]. By contrast, high concentrations of Treg cells were observed in the peripheral blood and in the tumor environment of patients affected with a wide range of human cancers, where they suppress, rather than enhance, immune responses, thus leading to immune tolerance toward cancer cells and promoting tumor growth [4,34,35]. Therefore, the depletion of Treg or inter-

**Table 2**
Comparative analysis of antigen expression on basal $CD8^+$ T cells as well as on $CD8^+$ regulatory T cells (Treg) incubated or not with different concentrations of 4-hydroperoxycyclophosphamide (4-HC) for 24 hours

| Antigen | Basal $CD8^+$ T cells | A $CD8^+$ Treg | B $CD8^+$ Treg+0.1 $\mu$g/mL 4-HC | C $CD8^+$ Treg+0.5 $\mu$g/mL 4-HC | *p* values |
|---|---|---|---|---|---|
| CD127 | 76[a] ± 16 | 46 ± 19 | 44 ± 13 | 40 ± 16 | A vs B: $p = 0.9$; A vs C: $p = 0.5$ |
| CD39 | 2 ± 3 | 3 ± 2 | 3 ± 2 | 4 ± 5 | A vs B: $p = 1$; A vs C: $p = 0.8$ |
| CD45RA | 75 ± 11 | 72 ± 20 | 70 ± 23 | 70 ± 25 | A vs B: $p = 0.9$; A vs C: $p = 0.9$ |

[a]Data are expressed as percentages of positive cells.

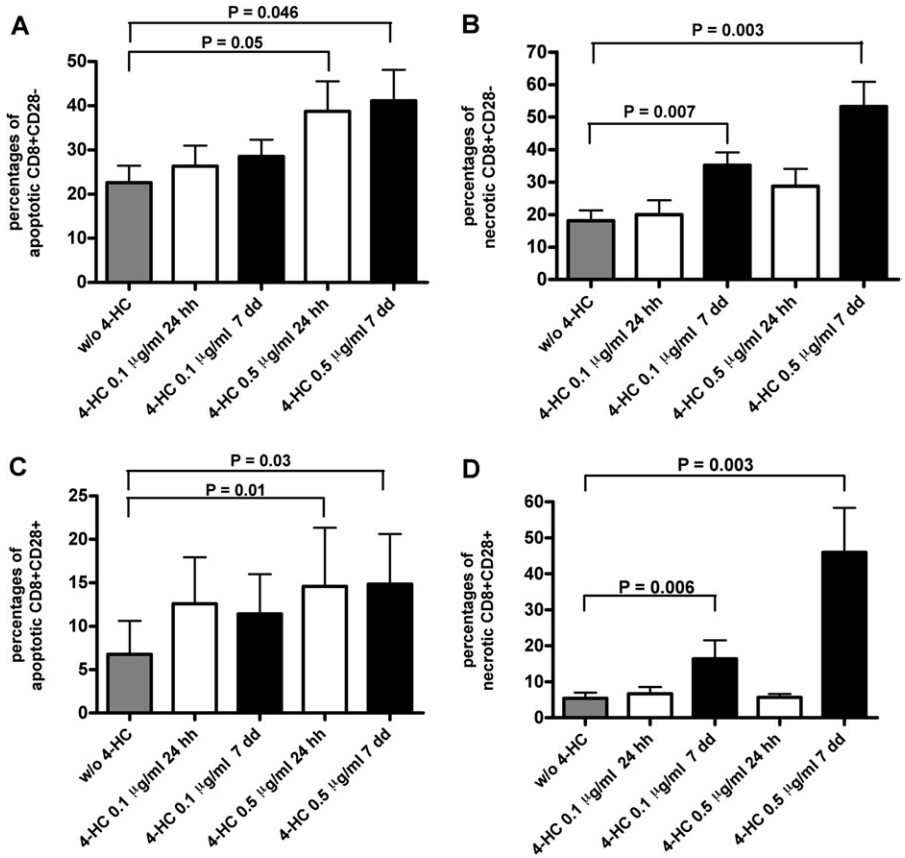


**Fig. 3.** Analysis of apoptosis and necrosis induction by 4-hydroperoxycyclophosphamide (4-HC) during or after $CD8^+$ Treg generation. Purified $CD8^+$ T cells were cultured with interleukin (IL)-2 and IL-10 for 7 days to generate $CD8^+$ Treg. 4-HC was added at the beginning of incubation (black columns) or on the 6th day of culture for 24 hours (white columns). Cultures performed without addition of 4-HC served as controls (gray columns). Immunofluorescence analyses for the evaluation of apoptotic (A and C) or necrotic (B and D) cells were performed separately at the end of incubation gating for CD8 + $CD28^-$ (A and B) or $CD8^+CD28^+$ (C and D) T cell subpopulations.

ference with their activity could represent an important strategy to prevent tumor immune escape. In the past, several approaches have been tried to target regulatory T cells [36]; among them, the use of cyclophosphamide was reported to be effective in inhibiting $CD4^+CD25^+$ Treg activity [19]. However, to be considered a valid Treg inhibitor, an agent must target not only $CD4^+CD25^+$ but also $CD8^+$ Treg, which have been observed to be highly represented within tumor-infiltrating lymphocytes [4] and able to strongly inhibit antitumor immune responses [37,31]. Here we demonstrate that 4-HC counteracts both $CD8^+$Treg generation and function. This finding is important because it supports the use of CY as an inhibitor of the regulatory immune response in combinatorial protocols in which this drug is associated with other cytotoxic drugs, as well as with antitumor immunotherapies. However, the effects on $CD8^+$ Treg were mainly evident when the drug was used at a concentration of 0.5 $\mu$g/mL, which corresponds to the serum concentration observed after intravenous administration of 10 to 20 mg/kg of CY [26]. To the contrary, low or null effects on $CD8^+$ Treg were observed at the lower drug concentration (0.1 $\mu$g/mL), which corresponds to a CY dose of about 2 mg/kg. Although relevant pharma-

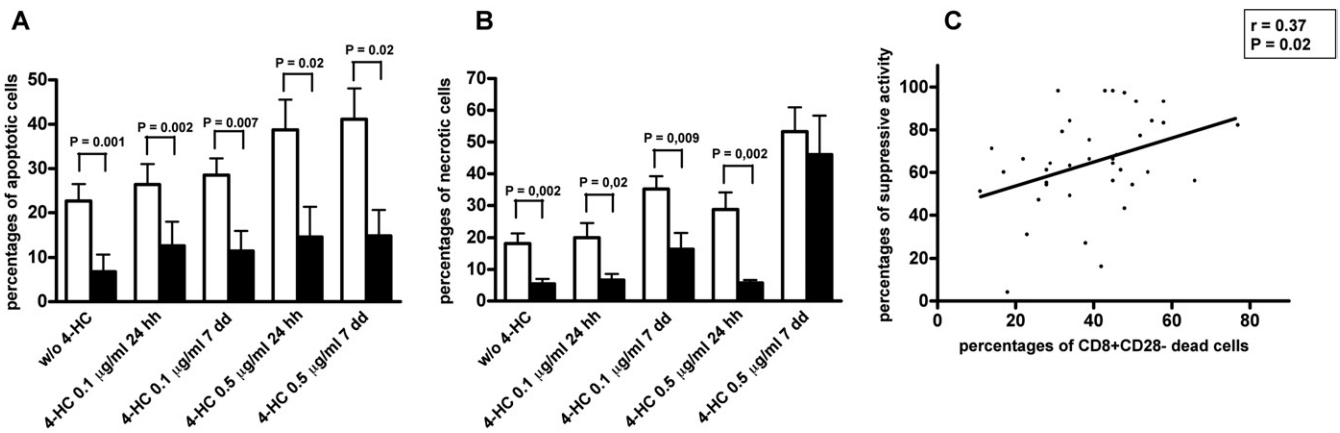


**Fig. 4.** Comparative analysis of 4-hydroperoxycyclophosphamide (4-HC) cytotoxic effects between $CD8^+CD28^-$ regulatory T cells (Treg; white columns) and $CD8^+CD28^+$ T cells (black columns) and correlation of $CD8^+CD28^-$ Treg mortality with their suppressive activity.

cokinetic changes may occur between different species, it is noteworthy that CY was still reported to be effective in inhibiting $CD4^+CD25^+$ Treg function when administered in mice at very low doses (2 mg/mouse corresponding to ~100 mg/kg) [20]. Moreover, a reduction in circulating CD4 + $CD25^+$ Treg numbers was achieved in humans with an administered dose of CY as low as ~1.5 mg/kg/day [22]. These observations likely suggest that the dosage at which 4-HC is effective on $CD8^+$ Treg (0.5 $\mu$g/mL) is higher than that targeting $CD4^+CD25^+$ Treg. Moreover, data relative to the administration of an intravenous CY dosage determining a drug serum concentration close to 0.5 $\mu$g/mL indicated only a moderate reduction in $CD4^+$ non-Treg percentage [38]. This finding agrees with our unpublished observation that 4-HC added at 0.5 $\mu$g/mL concentration to unselected PBMC or purified $CD4^+$ T cells does not significantly modify the percentage of $CD4^+CD25^-$ T cells (not shown). Subsequently, new schedules of CY treatment, tailored using timing and dosages of administration appropriate for each of the 2 different Treg subsets, should be set to efficiently target the whole Treg compartment.

Another consideration pertains to the field of treatment of autoimmune diseases. CY has been largely adopted as an immunosuppressive drug [17] administered at doses providing serum concentrations comparable to those demonstrated here to be able to inhibit $CD8^+$ Treg. Indeed, the prolonged use of CY at such doses may lead to the progressive eradication of the whole Treg compartment, thus exposing patients to the risk of becoming resistant to the drug and/or being affected with severe inflammatory rebounds. Experimental evidence of these events has been reported [39,40].

The mechanism by which 4-HC exerts its effects on $CD8^+$ Treg is cytotoxity. Indeed, 4-HC inhibition of $CD8^+$ Treg function was reported to be directly correlated with the amount of apoptotis/necrosis–dependent mortality of the target cells. Because peripheral blood $CD8^+$ Treg do not express the CD28 molecule, it was possible to differentiate a $CD8^+CD28^-$ Treg subset from a $CD8^+CD28^+$ non-Treg subpopulation. Interestingly, when the rates of apoptosis/necrosis induction was comparatively analyzed in $CD8^+CD28^-$ Treg and $CD8^+CD28^+$ non-Treg, a significantly higher sensitivity to 4-HC was observed in the former of the 2 T cell subpopulations, reminiscent of what was already demonstrated when sensitivity to CY of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets was comparatively analyzed [20]. Because increased sensitivity to CY seems to be a common feature of both $CD4^+CD25^+$ and $CD8^+$ Treg, it is possible to envisage the setting of newly articulated protocols for CY administration specifically aimed at deleting Treg sparing non-Treg subpopulations (*i.e.*, using shortened or pulsed timing of administration). Clinical trials specifically designed to investigate this issue could clarify whether CY can be really considered a valid inhibitor of Treg function to be used in anticancer treatment protocols.

## Acknowledgments

This work was supported in part by a grant from Compagnia di San Paolo entitled "Immunoterapia anti-tumorale: analisi d'efficacia dei principali protocolli tradizionali d'immunizzazione e validazione dell'efficacia terapeutica dell'inibizione dell'interleuchina 10 nel trattamento del melanoma" and in part by a PRIN grant from MIUR entitled "Immunoterapia anti-tumorale operata attraverso l'inibizione dei circuiti regolatori citochino-dipendenti."

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